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UNIT OF BACTERIAL GENETICS

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DOCTORAL DISSERTATION

Regulated assembly of lipopolysaccharide and sensing of its alterations in *Escherichia coli*

Supervisor

Professor Satish Raina

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Index of contents

List of figures

List of abbreviations

ABC	ATP-Binding Cassette
APS	ammonium persulfate
ATP	adenosine triphosphate
Bas	bacterial adaptative response
BLAST	Basic Local Alignment Search Tool
CRP	cAMP receptor protein
ECA	enterobacterial common antigen
ECA	enterobacterial common antigen
ECF	extracytoplasmic function
EHEC	enterohemeorrhagic *Escherichia coli*
EMSA	electrophoretic mobility shift assay
Gal	galactose
Glc	glucose
GlcN	glucosamine
GlcUA	glucuronic acid
Hep	L-*glycero*-D-*manno*-heptose
Hok	host killing factor
IM	inner membrane
IPTG	isopropyl *β*-D-1-thiogalactopyranoside
Kdo	3-deoxy-*α*-D-*manno*-oct-2-ulosonic acid
LA	lysogeny broth with agar
Lap	lipopolysaccharide assembly protein
LB	lysogeny broth
Lpp	Braun's lipoprotein
LPS	lipopolysaccharide
Lpt	lipopolysaccharide transporter
MALDI-TOF	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MgrR	Mg^{2+} responsive RNA
ncRNA	non-coding ribonucleic acid
nt	nuclotide
Ntr	nitrogen responsive

OM	outer membrane
OMP	outer membrane protein
ONPG	*ortho*-nitrophenyl-*β*-galactoside
PAGE	polyacrylamide gel electrophoresis
P-EtN	phosphoethanolamine
PMB	polymyxin B
ppGpp	guanosine 3′,5′-bis(diphosphate)
Qse	quorum sensing
RACE	rapid amplification of cDNA ends
Rcs	regulator of capsule synthesis
RNAP	RNA polymerase
RpoD	σ^D, σ^{70}, housekeeping sigma factor
RpoE	σ^E, σ^{24}, extracytoplasmic function sigma factor
RpoH	σ^H, σ^{32}, heat shock sigma factor
RpoN	σ^N, σ^{54}, nitrogen-limitation sigma factor
RpoS	σ^S, σ^{38}, stationary phase sigma factor
Rse	regulator of sigma E
RT	room temperature
SDS	sodium dodecyl sulfate
SlrA	suppressing *lap* deficiency RNA
sRNA	small ribonucleic acid
TCS	two-component systems
TDP	thymidine diphosphate
TEMED	*N,N,N′,N′*-tetramethylethane-1,2-diamine
TMP	thymidine monophosphate
TPR	tetratricopeptide repeats
TSS	transcription start site
TTP	thymidine triphosphate
UDP-GlcNAc	uridine diphosphate *N*-acetylglucosamine
UPEC	uropathogenic *Escherichia coli*
UTR	untranslated region
wt	wild type
X-Gal	5-bromo-4-chloro-3-indolyl-*β*-D-galactopyranoside

1. THE AIM OF THE RESEARCH

The aim of this research project was to study the mechanism of the transcription regulation of the *rpoE* gene. The *rpoE* gene encodes a RNA polymerase subunit, which is essential for the viability of *Escherichia coli*. RpoE belongs to the family of extracytoplasmic function sigma factors. Hence, in *E. coli* RpoE controls transcription initiation of genes whose products are involved in synthesis and transport of outer membrane (OM) components. RpoE also transcribes certain non-coding small RNAs, which act as translational repressors of expression of major OM components to provide a negative feed back control. As is often the case with alternative sigma factors, RpoE is tightly regulated at transcriptional as well as post-translational level.

The transcriptional regulation of the *rpoE* gene has not been fully understood. Initially, two promoters were identified, out of which the downstream promoter was known to be positively autoregulated by RpoE. However, transcription from upstream region is sustained under several stress and non-stress conditions including entry into the stationary phase, high osmolarity or cold shock and defects in lipopolysaccharide (LPS) assembly and biosynthesis.

Genetic approaches were employed, using screen of random transposon libraries and complete set of deletion derivatives of non-essential genes, to identify genes whose products could either negatively or positively regulate transcription from the distal promoter region. This analysis revealed lack of CRP, RpoS, RpoN to reduce the transcriptional activity. However, majority of transposon mutants that caused increase in transcription had mutations either in LPS biosynthesis or in the gene encoding the key regulator of LPS biosynthesis RfaH. In complementary approach genes, which when overexpressed caused an increase in transcription identified a novel non-coding sRNA RirA, the *qseG* gene and several genes encoding lipoproteins. The RirA sRNA was shown to regulate the activity of RfaH transcriptional factor and in the excess of RirA, LPS core biosynthesis and incorporation of *O*-antigen were severely compromised. Thus, such multiple divergent regulations of the upstream promoter suggested that there might be more than one promoter that respond to such diverse signals. Indeed, mapping of 5′ ends of the *rpoE* mRNA identified five new transcriptional initiation sites (P1 to P5) located distal to the promoter regulated by RpoE (now named as the *rpoE*P6 promoter). Thus, several single-copy chromosomal

*rpoE*P-*lacZ* promoter fusions were constructed and analyzed for their activity in order to distinguish their specific regulations. Experiments showed that three major promoters, P2, P3 and P4, are recognized by different sigma factors (RpoN, RpoD and RpoS) and these promoters are activated in response to unique signals. The *rpoE*P4 promoter exhibits the highest basal activity, which is regulated by modulators of RpoS or exposure to increased osmolarity in accordance to its positive regulation by the stationary phase sigma factor RpoS. The nitrogen-limitation responsive sigma factor RpoN-recognized *rpoE*P2 promoter was shown to be positively regulated by a QseE/F two-component system and a NtrC activator. Transcription from the housekeeping sigma factor RpoD-regulated P3 promoter was shown to be induced when the LPS composition and assembly have severe defects and by the overproduction of certain lipoproteins. The activation of the *rpoE*P3 promoter due to LPS defects and imbalance in lipoprotein amounts was shown to require the RcsB response regulator. The P3 promoter was also found to be positively regulated by the global regulator CRP. Thus, results described in this PhD thesis revealed a complex network in the regulation of the *rpoE* transcription by multiple sigma factors, activators and the mechanism of sensing of LPS alterations in *E. coli* was elucidated.

Lipopolysaccharide is essential for the viability of *E. coli*, because of that its synthesis and translocation to the call surface should be tightly regulated. Proper execution of LPS synthesis requires the presence at the inner membrane of fully active stoichiometric amounts of all LPS biosynthetic enzymes. Moreover, only completely synthesized LPS molecules should be subsequently translocated. This coupling of LPS synthesis and its translocation requires LapA and LapB proteins (lipopolysaccharide assembly proteins) and have also been a part of my research.

LapB ensures that LPS assembly is completed before its translocation by acting as a scaffold-like protein for LPS biosynthetic enzymes. Thus, LapB function is to enable that only the mature LPS is delivered to MsbA and in subsequent steps LapA and LapB could function together with lipopolysaccharide transporter, Lpt complex. The support for this comes from co-purification of Lap proteins with Lpt proteins. The pull down experiments revealed that LapA and LapB co-purified among others with protease FtsH, which tightly regulates LpxC, the enzyme that catalyzes the first committed step of lipid A biosynthesis. Such regulated proteolysis of LpxC is needed to maintain balance between phospholipids and LPS ensuring outer membrane integrity and hence the

bacterial viability. These data suggest that LapB contributes to turnover of LpxC, ensuring balanced biosynthesis of LPS and phospholipids, and couples LPS synthesis to translocation, thereby explaining its essentiality.

During the progression of my PhD research, another novel RpoE-regulated non-coding sRNA was identified, during suppressor analysis of deletion derivatives of a key LPS assembly factor (LapB). Thus, the aim of my research was to examine the molecular basis of the suppression of Δ(*lapA lapB*) mutants by the overexpression of novel non-coding RNA, SlrA. In order to do that, specific defects of Δ(*lapA lapB*) mutants were analyzed and verified if overexpression of the RpoE-regulated *slrA* sRNA could suppress some of these defects. Overexpression of the *slrA* sRNA, resulted in restoration of normal expression of *rpoE* regulon members, whose activity are otherwise hyperelevated in Δ(*lapA lapB*) mutants. Furthermore, the inability of growth in the presence of detergents, as well as the accumulation of LpxC, was examined. To fully understand the mechanism of the suppression of Δ(*lapA lapB*) mutants defect by *slrA* sRNA proteomic changes were analyzed. The whole cell extract from the Δ(*lapA lapB*) mutant was compared to that obtained from the wild type, both after overexpression of the *slrA* RNA. Proteins whose amounts differed significantly were identified by MALDI-TOF. This analysis revealed highly reduced amount of Lpp lipoprotein when the *slrA* sRNA was overexpressed, revaling that the main substrate for SlrA is the most abundant protein Lpp (Braun's lipoprotein). Thus, it was shown that SlrA functions in *trans* to repress Lpp synthesis and also dampens the RpoE-dependent extracytoplasmic stress response.

2. INTRODUCTION

Bacteria respond to changes in their environment by altering transcription. Transcription is the first step of gene expression, which is the synthesis of RNA from a DNA template mediated by RNA polymerase. Initiation of this process requires promoter sequences recognition by a specific subunit of RNA polymerase called sigma factor. Out of seven sigma factors present in *Escherichia coli*, only two are essential for the viability under all growth conditions. One of them is the σ^E (RpoE) sigma factor, which is a part of bacterial stress response regulon. It is involved in the maintaining of the cell envelope integrity during stress as well normal growth conditions. It plays role in the transcription initiation of the genes, whose products are involved in synthesis and transport of outer membrane components as well as proteins responsible for correct outer membrane and periplasmic protein folding and assembly (Strauch *et al*., 1989; Danese & Silhavy, 1997; Dartigalongue *et al*., 2001; Rezuchova *et al*., 2003; Rhodius *et al*., 2006; Martorana *et al*., 2011).

E. coli cells are surrounded by the cell wall-like component (cell envelope), which determinates cell shape, the mechanical strength and elasticity, and allows to face unpredictable and often hostile environment. Such Gram-negative bacteria cell envelope is composed of an inner and an outer membrane separated by an aqueous compartment called a periplasm (Beveridge & Davies, 1983; Ellen *et al*., 2010; Silhavy *et al*., 2010).

There are several structural and functional differences between two membranes of the cell envelope. The most striking is the fact that the outer membrane (OM) is asymmetric. Its periplasmic side is composed of phospholipids, which also build both leaflets of the inner membrane (IM). However, the layer facing the environment outside the cell is composed of glycolipid called lipopolysaccharide (LPS). It is believed that the presence of this glycolipid in the outer leaflet of the OM is responsible for generally higher resistance of Gram-negative bacteria, as compared to most Gram-positive bacteria, to many toxic chemicals such as antibiotics and detergents, allowing survival in hostile environments and host colonization or infection (Gan *et al*., 2008; Gunn, 2000; Nikaido, 2003; Vollmer & Holtje, 2004; Vollmer & Seligman, 2010).

LPS is an important signaling molecule for the innate immune system. It binds to a receptor constituted of TLR4 and MD2 on the plasma membrane of innate immune cells and induces a signaling cascade resulting in the activation of the production of

proinflammatory mediators, such as TNF-α and IL-6 and the adaptive immune response. The overstimulation of such response is dangerous to the host and can result in septic shock, and hence LPS is also known as endotoxin (Miyake, 2004; Maeshima & Fernandez, 2013).

The OM differs from the IM in case of the structure of integral membrane proteins. The integral proteins in the IM contain α-helices mostly composed of hydrophobic residues, while in the OM proteins have mostly amphipathic *β*-strands, which form a *β*-barrel structure (Fairman *et al*., 2011). Besides integral proteins, membranes also contain lipoproteins anchored via an N-terminal domain (Bos *et al*., 2007).

Such complex structure of the OM serves challenges for its biogenesis. Both lipid and protein components are synthesized in the cytoplasm or at the inner membrane and must be translocated across the IM bilayer, the aqueous periplasmic space and further assembled at the final destination. More problematically, the cell compartments external to the IM are devoid of ATP and other high-energy carriers, so the energy for the OM components translocation is either provided by substrates that have been energized before their translocation across the IM or complex protein machines connected to the IM, which can use the energy released by ATP hydrolysis in the cytoplasm or the proton motive force (Oliver, 1996).

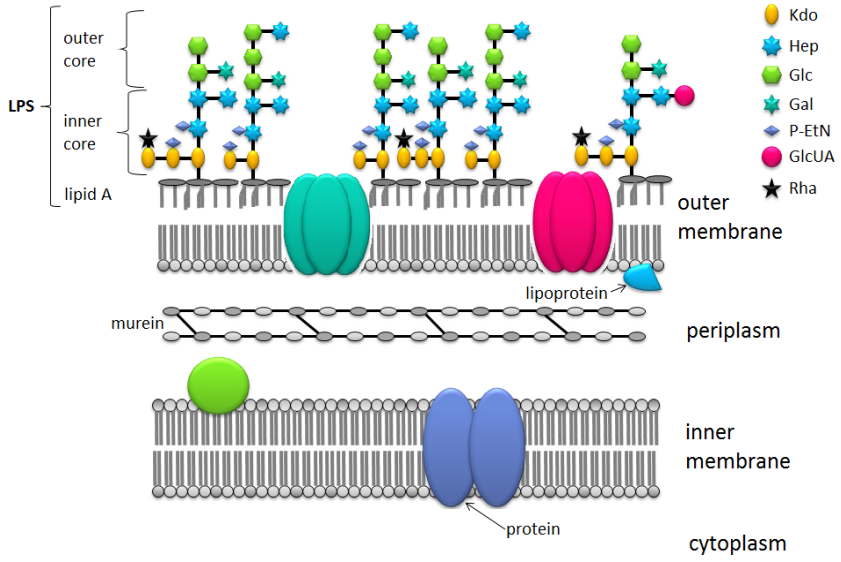

Fig. 1. Model of *E. coli* K-12 cell envelope (adapted from Raetz & Whitfield, 2002 and Klein *et al*., 2014).

2.1. Structure and biosynthesis of lipopolysaccharide

LPS has highly heterogeneous composition, made of the most conserved hydrophobic endotoxin part called lipid A, the inner and outer core oligosaccharide and in smooth-type bacteria, a distal polysaccharide called *O*-antigen.

Lipid A of *E. coli* is phosphorylated and acylated $\beta(1'\rightarrow6)$-linked glucosamine disaccharide, which is anchored in the outer membrane. To lipid A are attached a carbohydrate moiety of varying sizes. Firstly, there is the inner core, usually composed of residues of 3-deoxy-α-D-*manno*-oct-2-ulosonic acid (Kdo) and L-*glycero*-D-*manno*-heptose (Hep). Both lipid A and the inner core have generally conserved structure. However, they often carry non-stoichiometric modifications. The heterogeneity of LPS depends on growth conditions and is controlled by various regulatory factors (Klein *et al*., 2011).

2.1.1. Lipid A structure and biosynthesis

The lipid A structure is generally well conserved among Gram-negative bacteria, although there can be differences in the length of the fatty acyl chains and the number and positions of the secondary fatty acids. Moreover, there may also occur differences in lipid A structure within the same species caused by specific growth conditions. One such example is the replacement of lauroyl chain by palmitoleate upon growth of *E. coli* at low temperature, which could be a part of homeoviscous adaptation to cold temperature (Carty *et al*., 1999).

Other modifications within lipid A structure may also occur during or even after LPS transport to the OM. These are the removal or substitution of phosphate groups or of acyl chains, which often help to overcome host's defense systems (Bos *et al.,* 2004; Doerrler *et al.,* 2004; Needham & Trent, 2013; Raetz *et al.,* 2007; Tefsen *et al.,* 2005; Klein & Raina, 2015).

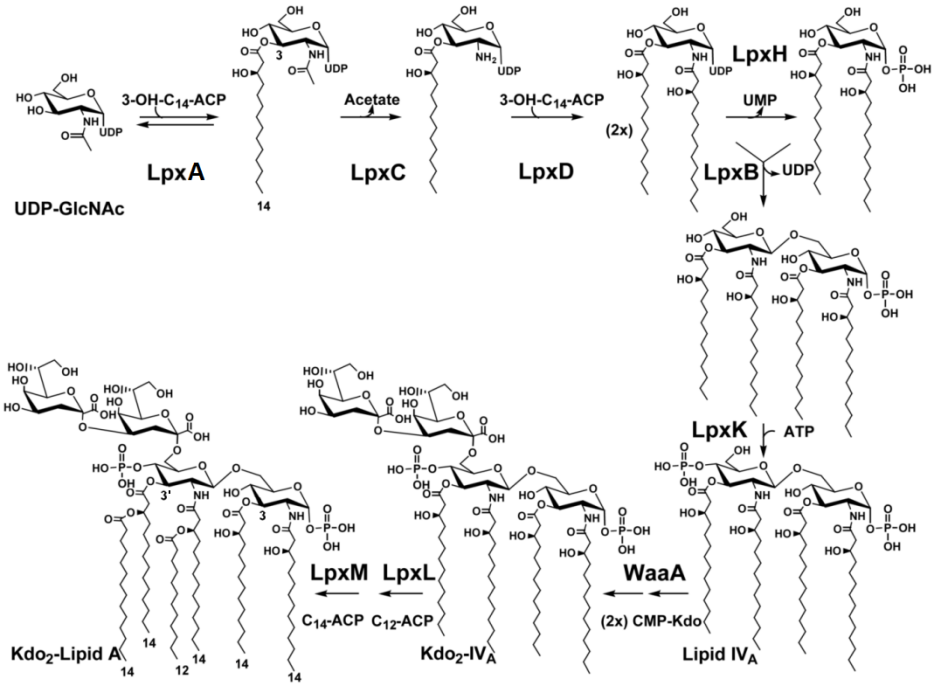

Fig. 2. Biosynthesis of the Kdo_2-lipid A in *E. coli* K-12.

Lipid A is synthesized from a sugar nucleotide uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc), which is firstly acylated with *β*-hydroxymyristate. This reaction is mediated by the LpxA *O*-acyltransferase (Fig. 2) and it has an unfavorable rate constant, being reversible in nature (Anderson *et al*., 1993). Hence, following it is the deacetylation, which constitutes the first committed reaction of the pathway (Anderson *et al*., 1988; Young *et al*., 1995; Sorensen *et al*., 1996; Jackman *et al*., 1999). This step is mediated by the zinc metalloenzyme LpxC encoded by a conserved, single-copy gene present in diverse Gram-negative bacteria, which does not display sequence similarity to other deacetylases or amidases, making it an excellent target for the novel antibiotics design (Onishi *et al*., 1996; Jackman *et al*., 2000).

After deacetylation step mediated by LpxC, a second *β*-hydroxymyristate moiety is added by LpxD *N*-acyltransferase. The generated UDP-2,3-diacylglucosamine is combined with the product of its hydrolysis by pyrophosphatase LpxH (that is 2,3-diacylglucosamine-1-phosphate called lipid X) to form a *β*(1′→6)-linked disaccharide. This reaction is mediated by synthase LpxB and its product is phosphorylated at the 4′ position by a specific kinase LpxK to form lipid IV_A.

2.1.2. Biosynthesis of the Kdo_2-lipid A in *E. coli* K-12

Next step in LPS biosynthesis is the incorporation of two Kdo residues by WaaA, where the second Kdo is incorporated much more rapidly, preventing an accumulation of the intermediate with the single Kdo. This addition of two Kdo units enables lauroyl and myristoyl residues addition by LpxL and LpxM respectively to the distal glucosamine unit. The (*R*)-3-hydroxyl groups of both acyl chains are added to the non-reducing GlcN residue of lipid IV_A, leading to the synthesis of hexaacylated lipid A. Under conditions of cold shock (12°C) instead of laurate, palmitoleate is inserted by the σ^E-dependent LpxP transferase, adjusting the fluidity of outer membrane (Carty *et al*., 1999).

From above described LPS biosynthetic enzymes, only LpxL, LpxM and LpxP are not essential for the *E. coli* viability. Δ(*lpxL lpxM*) mutants, as well as mutants lacking all the late acyltransferases Δ(*lpxL lpxM lpxP*), have a mild 30 to 40% increase in the σ^E activity (Klein *et al*., 2009). However, a triple deletion Δ(*lpxL lpxM lpxP*) derivative does not grow on rich media at tempertures above 30°C, indicating the importance of these late acyltransferases.

Lipid A can be also modified with palmitate by PagP transferase. Transcription of the *pagP* gene is positively regulated by the PhoP/Q two-component system (Raetz *et al*., 2007). Such PagP-dependent modification does not induce σ^E (Klein *et al*., 2009).

Under σ^E-inducing conditions and the activation of BasS/R two-component system also 4-amino-4-deoxy-L-arabinose (L-Ara4N) addition is observed. The biosynthesis and attachment of L-Ara4N involves genes organized in the *arnB-arnC-arnA-arnD-arnT-arnE-arnF* operon, whose transcription is under the positive control of the BasS/R two-component system (Hagiwara *et al*., 2004; Lee *et al*., 2005).

The LPS structure obtained during reactions described above (lipid A with two Kdo residues) is a minimal LPS required for the *E. coli* viability under optimal growth conditions (Klein *et al*., 2011). However, in slow-growth conditions, at lower temperatures even lipid IV_A can be sufficient for the viability, like it takes place in *waaA* mutants. Such mutants have the σ^E signal transduction pathway highly induced (Klein *et al*., 2009). On the other hand, due to adaptation to specific niches LPS is more expended by a non-repeating oligosaccharide core, serving as a barrier to many harmful conditions.

The inner core has common structure composed of Kdo, Hep and phosphate residues. However, the outer core is more diverse, which is probably caused by higher exposure to different environmental stresses. Because of these differences, the *E. coli* core was divided into different types: R1, R2, R3, R4, B and K-12 (Fig. 3). *E. coli* B synthesizes a truncated LPS and contains only two glucose residues in the outer core, lacking at the same time galactose (Klein *et al*., 2011; Klein *et al*., 2013). Strains that cause extraintestinal infections have mostly the R1 type core and the most verotoxigenic, enterohemorrhagic isolates have mostly the R3 type core (Amor *et al*., 2000; Raetz & Whitfield, 2002).

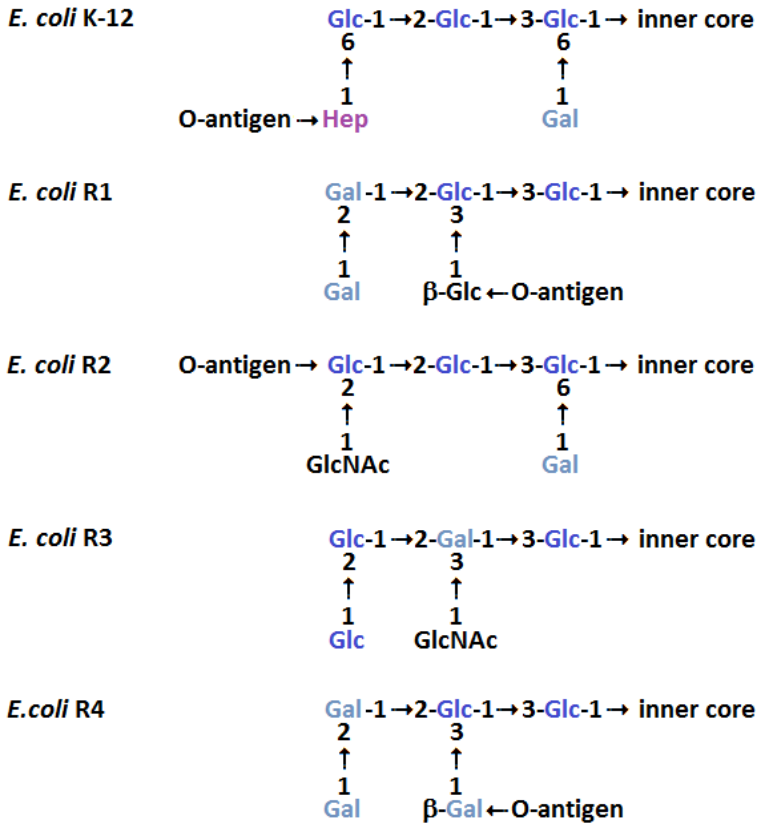

Fig. 3. Structures of the known outer core oligosaccharides types from *E. coli* (adapted from Raetz & Whitfield, 2002 and Klein *et al*., 2013).

2.1.3. The inner core synthesis

The oligosaccharide core is sequentially synthesized by glycosyltransferases located at the cytoplasmic surface of the inner membrane. After Kdo_2-lipid A is synthesized, the next step in the inner core synthesis is α(1→5) addition of the first heptose (HepI) to KdoI, which is directly transferred to lipid A by WaaC (RfaC) heptosyltransferase (Fig.

4). Subsequently, the second heptose is α(1→3)-linked to HepI by WaaF heptosyltransferase II.

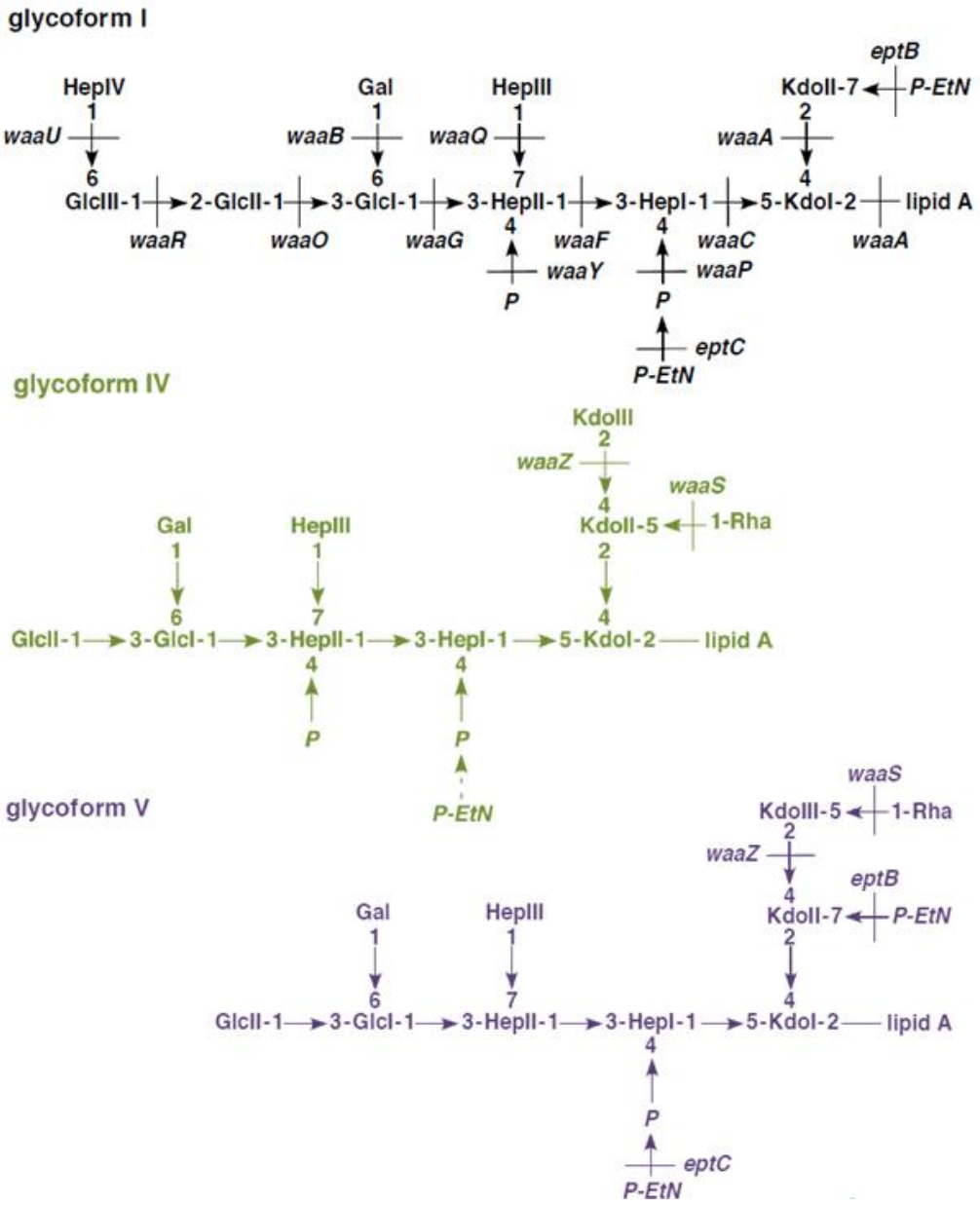

Fig. 4. Examples of LPS structures of *E. coli* K-12 (Klein *et al*., 2011 and Klein *et al*., 2013).

The mutants lacking *waaC* or *waaF* genes, similarly to *waaA* mutants, have altered outer membrane proteins (OMPs) and truncated LPS (Beher & Schnaitman, 1981), which leads to the elevated σ^E-dependent extracytoplasmic stress response (Klein *et al*., 2009). Those genes are transcribed as a *rfaD-waaF-waaC-waaL* operon. Mutants in the *rfaD* gene, which encodes ADP-L-*glycero*-D-*manno*-heptose-6-epimerase required for Hep biosynthesis have LPS lacking heptose and less amounts of OMPs (Missiakas *et*

al., 1996). Genes, whose products are required for either heptose biosynthesis (*rfaD*) or its incorporation (*waaC* and *waaF*) are essential for bacteria growth at elevated temperatures (Murata *et al*., 2011; Raina & Georgopoulos, 1991).

The next step in LPS biosynthesis is the phosphorylation of the first heptose by WaaP kinase, which is needed for the third heptose incorporation and complete phosphorylation of HepII by WaaY.

Even though *waaP* mutants do not have altered OMPs (Yethon *et al*., 2000), phosphorylation of HepI by WaaP plays a crucial role in the OM stability (Yethon *et al*., 1998) and *waaP* mutants have some characteristics of deep-rough phenotype[1] similar to *waaC* or *waaF* deletion mutants.

The third heptose addition is mediated by WaaQ heptosyltransferase. In its absence, the phosphorylation of the second Hep does not occur (Klein *et al*., 2013). Under phosphate-limiting growth conditions, the third Hep can be further modified by non-stoichiometric substitution by glucuronic acid (GlcUA) (Fig. 5). This modification is always associated with simultaneous loss of phosphate on the second Hep (Klein *et al*., 2013). This modification can also occur in strains with core type of R1, R4, *E. coli* B and *Salmonella* (Klein *et al*., 2013). In the case of *E. coli* B, the GlcUA residue incorporation can occur even in phosphate-rich medium, probably due to constitutive induction of the *waaH* gene whose product is required for the GlcUA incorporation (Klein *et al*., 2013).

[1] Deep-rough mutants have changed surface hydrophilicity causing increased sensitivity to hydrophobic compounds (some antibiotics, dyes, detergents, fatty acids, phenols, and polycyclic hydrocarbons). Such mutants exhibit a constitutive σ^E-dependent stress response (Missiakas *et al*., 1996, Klein *et al*., 2009), leading to changed outer membrane composition with reduced OMPs content. Deep-rough mutants also release significant amounts of periplasmic enzymes unless medium is supplemented with high concentration of Mg^{2+}. Those mutants have lost ability to express pili and flagella. They secrete a form of hemolysin with reduced hemolytic activity and overproduce capsular polysaccharide, what results as mucoid phenotype (Raetz & Whitfield, 2002; Noor *et al*., 2009; Murata *et al*., 2011).

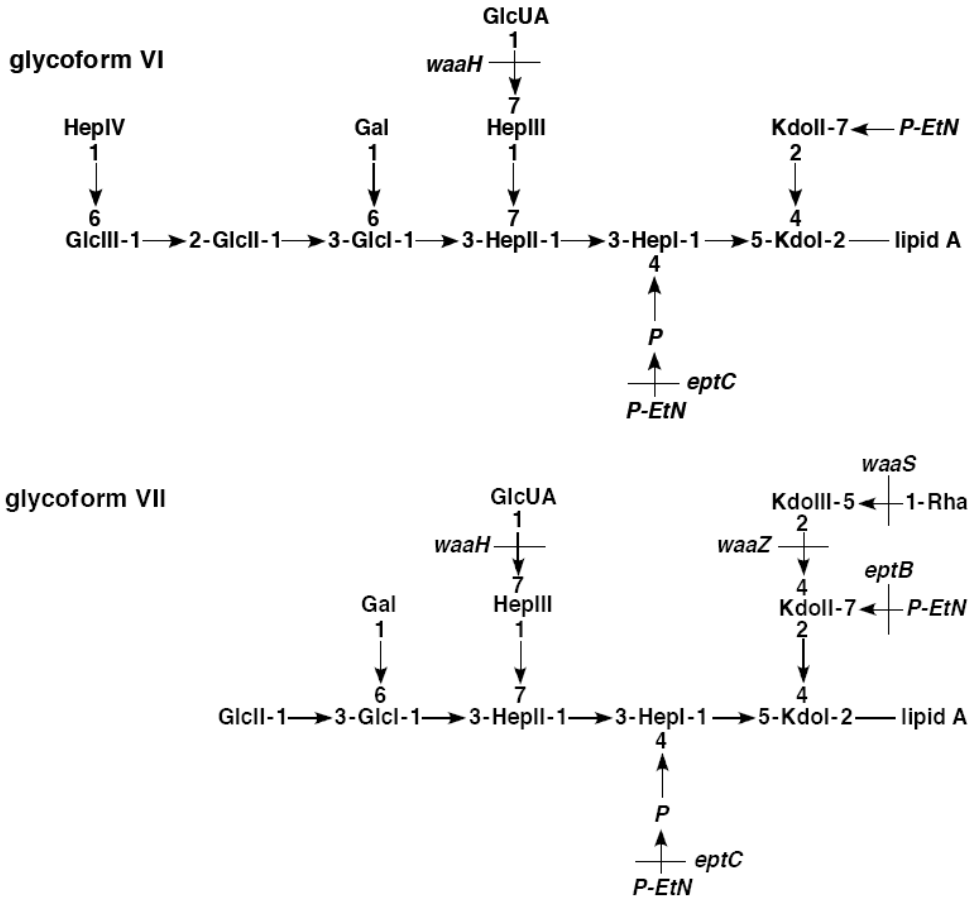

Fig. 5. LPS structures from *E. coli* K-12 with GlcUA substitutions on HepIII (Klein *et al*., 2013).

Moreover, the first Hep of the inner core can be modified by the addition of phosphoethanolamine (P-EtN) by EptC. This incorporation is positively regulated by the PhoB/R two-component system and it does not require the BasS/R two-component system induction. However, at the basal level it can occur without any regulatory inducible system (Klein *et al*., 2013).

2.1.4. The outer core synthesis and non-stoichiometric substitutions

The first sugar of the LPS outer core of *E. coli* K-12, glucose (GlcI), is α(1→3) added to HepII by WaaG glucosyltransferase (Fig. 4). To this glucose WaaB mediates transfer of galactose (Gal) and WaaO mediates the addition of the second glucose by the α(1→3)-linkage and to which the third glucose (GlcIII) can be added by WaaR by α(1→2)-linkage. Further to GlcIII WaaU can transfer the fourth heptose (HepIV), to which *O*-antigen can be ligated by WaaL at the periplasmic surface of the inner membrane.

It was shown that bacteria overexpressing the *waaL* gene have increased the σ^E activity due to LPS accumulation causing an altered ratio of outer membrane proteins (Raina *et al*., 1995).

waaR mutants have LPS truncated after the second glucose and, unlike mutants with LPS truncated in the inner core, *waaR* mutants do not show elevated σ^E levels and have normal OMPs content. The inner core of LPS in *waaR* mutants additionally contains non-stochiometric substitution by a third Kdo (mediated by WaaZ) and rhamnose (Rha) (by WaaS) linked to KdoII creating glycoform IV. Alternatively, depending upon P-EtN transfer by EptB transferase to KdoII, glycoform with a third Kdo is synthesized that have simultaneous addition of Rha by WaaS to the third Kdo, constituting the additional new glycoform V (Fig. 4) (Klein *et al.*, 2011).

It was shown that amounts of WaaR glycosyltranferase are reduced upon σ^E induction. It occurs due to the translational repression by non-coding RNA RybB. Mutants lacking anti-σ^E factor (RseA), which exhibit constitutive induction of σ^E, have increased abundance of glycoforms with the third Kdo accompanied by a truncation in the outer core (Klein *el al*., 2011). Such mutants preferentially accumulate glycoform V, because of constitutive transcriptional induction of the *eptB* gene, encoding phosphoethanolamine transferase, which adds P-EtN to the second Kdo. The *eptB* gene expression is negatively regulated by the Hfq-dependent RNA, MgrR (Mg^{2+} responsive RNA) (Moon & Gottesman, 2009). This is another small regulatory RNA that effects the LPS composition. The role of non-coding RNAs in the regulation of LPS composition is described in the following chapter.

2.1.5. The role of non-coding RNAs in regulation of LPS modifications and the envelope stress response

A non-coding RNA is a functional RNA molecule that generally is not translated into a protein. Most of bacterial regulatory RNAs, called small RNA (sRNA), act by base pairing with target mRNAs and regulate the gene expression. Such antisense sRNAs can be categorized as *cis*-encoded sRNAs, transcribed from the opposite DNA strand with respect to the gene they regulate, and *trans*-encoded sRNAs, where the antisense sRNA gene is located at a different locus than the target gene.

However, some sRNAs act in different ways than base pairing with target mRNAs. sRNAs like CsrB or 6S RNA, can bind to protein targets and modify their function. The

CsrB RNA binds CsrA protein and by that inhibits its activity. Another example of regulatory RNA with a protein target is 6S RNA, which, during the stationary phase, binds RNA polymerase and represses the expression from a subset of σ^{70}-dependent promoters and also activates the σ^{S}-dependent transcription at several promoters (Wassarman & Storz, 2000; Kim *et al.*, 2004; Trotochaud & Wassarman, 2004).

Most of the previously characterized bacterial sRNAs encoded on the chromosome act by base pairing with mRNA targets that are encoded in *trans* (at a chromosomal position different from their target). Correct coupling between small RNA and mRNA is achieved due to RNA base-pairing sequence and elements of the secondary structure. It results in alternations of the gene expression. The most common mode of action invoked by antisense RNA is the negative regulation. It can occur through degradation of newly-created RNA duplexes by RNases or the attenuation of translation, when sRNA binds to the 5′ untranslated region (UTR) and occludes the ribosome-binding site and, as a consequence, inhibits protein translation. Moreover, there are known regulatory antisense RNAs that act far upstream of the AUG codon of the regulated gene (Hammer & Bassler, 2007; Prevost *et al*., 2007). The rarest mechanism characteristic for antisense RNA regulators comprises activation of translation, where base pairing of the antisense RNA disrupts an inhibitory structure, which sequesters the ribosome-binding site and makes the translation possible (Urban & Vogel, 2008; Repoila & Darfeuille, 2009).

In contrast to *cis*-encoded sRNAs, the potential base-pairing between *trans*-encoded sRNAs and their targets is generally more limited and discontinuous. Antisense RNAs functioning in this manner often require the RNA chaperone Hfq, which promotes the pairing of complementary RNA molecules (Brennan, 2007). Hfq is an abundant RNA-binding protein, which is homologous to Sm and Lsm proteins that form the core of splicing and mRNA degradation complexes in eukaryotes (Aiba, 2007; Gottesman, 2005; Maki *et al*., 2008). Hfq facilitates association between the antisense RNA and its target, probably by increasing of a local concentration of both antisense RNA and mRNA. Hfq most likely also prevents sRNA degradation while the target mRNA is not available.

It was earlier revealed that all examined *trans*-encoded antisense RNAs co-immunoprecipitate with Hfq, but the recruitment for the RNA chaperone seems to be versatile (Zhang *et al*., 2003). Otherwise, the competition between antisense RNAs for

binding Hfq could be an important factor that regulates the antisense RNAs activity. However, recent discovery of some *trans*-acting sRNAs, like RaiZ, use ProQ as the RNA chaperone (Smirnov *et al*., 2017). ProQ also binds many *cis*-acting sRNAs, although function of various such sRNAs that bind ProQ is not fully known.

Due to limited and discontinuous base-pairing between *trans*-encoded sRNAs and their targets, one *trans*-encoded antisense RNA can influence multiple targets and could act as the global regulator of a particular physiological response (Masse *et al*., 2007; Bejerano-Sagie & Xavier, 2007; Valentin-Hansen *et al*., 2007). It has been revealed that a large number of *trans*-encoded antisense RNAs provide a negative feedback regulation, in a direct or an indirect manner, so that they act as important factors contributing to many regulatory circuits in the cell. *Trans*-encoded antisense RNA molecules mostly regulate genes associated with the bacterial response for environmental conditions. They influence the global envelope stress response, LPS modifications and expression of outer membrane proteins (Klein *et al*., 2011; Murata *et al*., 2011; Klein & Raina, 2017).

As mentioned above, MgrR is an example of small regulatory RNA that effects the LPS composition. The MgrR expression is positively regulated by the PhoP/Q two-component system in response to changes in the Mg^{2+} concentration and it is also significantly decreased upon the high Ca^{2+} concentration. The lower Mg^{2+} concentration, sensed by the PhoQ/PhoP two-component system, leads to th eexpression of MgrR, changing the LPS composition. The *mgrR* sRNA negatively controls the translation of the *eptB* gene, encoding phosphoethanolamine transferase, which adds P-EtN to the second Kdo. Therefore, the *mgrR* mutant is more resistant to polymyxin B than the wild type, likely due to the increased *eptB* expression (Klein *et al*., 2013; Moon & Gottesman, 2009; Reynolds *et al*., 2005; Moon *et al*., 2013).

Lipopolysaccharide modifications are also regulated by the *rybB* sRNA. This RpoE-transcribed sRNA represses the WaaR glycosyltranferase synthesis upon the RpoE activation. Mutants lacking RseA, the anti-sigma factor specific to RpoE, exhibit constitutive induction of RpoE with concomitant increased abundance of glycoforms with the third Kdo accompanied by a truncation in the outer core (Klein *el al*., 2011). As well, the *rybB* sRNA was initially identified as a translational repressor of genes encoding several OMPs, such as proteins OmpC and OmpW (Johansen *et al*., 2006).

The expression of genes encoding OMPs is also negatively regulated by another non-coding RNA, MicA, which is similarly transcribed by the essential RpoE sigma factor (Johansen *et al*., 2006; Udekwu & Wagner, 2007). Overexpression of either MicA or RybB sRNAs suppresses phenotypes of RpoE depletion. MicA negatively regulates the expression of a number of genes. Its first identified target was an outer membrane protein, OmpA. MicA interestingly represses the translation of the *phoP* mRNA and enhances its degradation. Thus, MicA, an RpoE-regulated sRNA, links the LPS regulatory two-component system PhoP/Q with RpoE (Klein & Raina, 2017; Coornaert *et al*., 2010).

MicA upon association with Hfq binds to the translation initiation region of the *ompA* mRNA and blocks the ribosome-binding site, thereby preventing translation (Udekwu *et al*., 2005; Rasmussen *et al*., 2005). This sRNA also leads to the destabilization of the transcript encoding different outer membrane protein, OmpX. In this negative regulation of the *ompX* expression an additional non-coding RNA, CyaR, also participates. Transcription of the *cyaR* is directly activated by the cAMP receptor protein (CRP), hence the name of CyaR (cyclic AMP-activated RNA). It was shown that the *cyaR* expression is rapidly up-regulated in the response to the RpoE activation, however the mechanism of this activation is unknown (Johansen *et al*., 2008).

As highlighted above, heterogeneous LPS composition due to several non-stoichiometric substitutions is observed mainly in the response to changes in growth conditions and induction of envelope stress response. Hence, glycoforms IV and V were also found in LPS of *E. coli* K-12 grown in phosphate-limiting medium, where BasS/R and PhoB/R two-component systems are induced (Klein *et al*., 2011). Thus, overlapping control of LPS alterations by the RpoE sigma factor, BasS/R and PhoB/R two-component systems was described. It was also shown that the third Kdo incorporation is controlled by an alarmone ppGpp, guanosine 3′,5′-bis(diphosphate) (Klein *et al*., 2011), a global regulator of the gene expression, which among others modulates competition between sigma factors (Magnusson *et al*., 2005). In summary, LPS modifications are regulated by σ^E, as well as two-component systems and non-coding sRNAs.

2.2. Transport of lipopolysaccharide

Three enzymes involved in initial steps of LPS synthesis (LpxA, LpxC and LpxD) are located in the cytoplasm. The next two steps are mediated by enzymes (LpxB and LpxH), which are associated with the inner membrane and the remaining enzymes involved in this pathway are integral IM proteins (Raetz & Whitfield, 2002). Therefore, synthesized LPS has to be transported from the inner leaflet of the inner membrane to its final destination, the cell surface. Unlike to a well-elucidated LPS biosynthesis, the mechanism by which such large amphipathic molecule is transported across the periplasm is less understood. At the beginning, LPS is flipped from the cytoplasmic to the periplasmic face of the inner membrane by the essential ATP-binding cassette (ABC) transporter MsbA (Doerrler & Raetz, 2002). It was recently reported that MsbA might use the energy not only from ATP hydrolysis but also by coupling LPS transport to a transmembrane electrochemical proton gradient. Thus, a functional integration of these both forms of metabolic energy was shown (Singh *et al*., 2016). The ATP hydrolysis by MsbA is induced by lipid A binding (Doshi & van Veem, 2013). MsbA uses the energy to switch between conformations facing either the cytoplasm or the periplasm by a twisting of the transmembrane domains of its dimer and by that inserting LPS into the outer leaflet of the IM. However, details of this mechanism remain unclear (Ward *et al*., 2007; Zoghbi *et al*., 2016).

MsbA shows specificity to the level of LPS acylation. MsbA has higher affinity to the hexa- and the pentaacylated lipid A as compared to tetraacylated derivatives (Doerrler & Raetz, 2002; Vorachek-Warren *et al*., 2002; Tran *et al*., 2008; Tran *et al*., 2010).

Once LPS is flipped out by MsbA, it is translocated across the periplasmic space and assembled in the cell surface by the transenvelope complex of seven essential proteins, named Lpt (from LPS transport). This LPS transporter system is structurally and functionally well conserved among Gram-negative bacteria (Bos *et al*., 2007; Malojčić *et al*., 2014). As demonstrated by the example of hybrid Lpt machines obtained from *E. coli* and *Pseudmonas aeruginosa* proteins are functional. As well, *P. aeruginosa* LptA homologue complements *E. coli* Δ*lptA* mutants (Bollati *et al*., 2015).

Firstly, LPS must be extracted from the inner membrane. The energy needed for this step of LPS transport is most likely obtained by ATP hydrolysis mediated by a cytoplasmic protein LptB (Ruiz *et al*., 2008; Sperandeo *et al*., 2008), whose dimers

form an ABC transporter with inner membrane proteins LptF and LptG that interacts with LptC (Narita & Tokuda, 2009; Chng *et al*., 2010; Sherman *et al*., 2014).

The first protein of the Lpt subsequent transporter shown to bind LPS is LptC (Okuda *et al*., 2012; Tran *et al*., 2010). However, LptC cannot extract LPS on its own (Okuda *et al*., 2012). Two possible mechanisms of LPS extraction were proposed (Simpson *et al*., 2015). In the first model, both LptF and LptG or just one of them may interact directly with LPS and then pass it to LptC. This model is supported by the genetic evidence in *Burkholderia cenocepacia*, suggesting that LptG recognizes L-Ara4N-modified LPS (Hamad *et al*., 2012). The second model proposes that LptFG somehow stimulate LptC to extract LPS from the inner membrane. Moreover, a more recent study reported the isolation of mutants lacking LptC (Benedet *et al*., 2016). This suggests that LptFG is able to directly extract LPS or indirectly promote LPS extraction by controlling LptA when LptC is absent (Okuda *et al*., 2012; Tran *et al*., 2010; Benedet *et al*., 2016). In either model, LptFG must use physical interactions with LptB to connect ATP hydrolysis in the cytoplasm with LPS extraction from the IM. In fact, Simpson *et al*. (2016), identified a specific binding site in LptB for the coupling helices of LptFG that is responsible for coupling of ATP hydrolysis with LptFG function to extract LPS from the IM.

LptC is composed of a single N-terminal transmembrane and a large periplasmic domain (Sperandeo *et al*., 2007; Tran *et al*., 2010). LptC lacking its membrane-spanning region can still bind the $LptB_2FG$ complex (Villa *et al*., 2013). The periplasmic domain is structurally homologous to LptA as well as to the periplasmic domain of LptD (Suits *et al*., 2008; Tran *et al*., 2010). This domain interacts with the N-terminal of the periplasmic component of Lpt transporter, LptA. Further, the C-terminal of LptA interacts with the N-terminal of LptD (Freinkman *et al*., 2012). In this way, LptA bridges the IM and the OM. However, it is still unknown how many LptA monomers comprise this bridge (Okuda *et al*., 2016).

Subsequent transfer of LPS from LptC to LptA is dependent on ATP hydrolysis (Okuda *et al*., 2012). It was shown that LptA can displace LPS from the purified periplasmic domain of LptC *in vitro* but it will not happen inversely (Freinkman *et al*., 2012; Sperandeo *et al*., 2011; Tran *et al*., 2010). These differences in binding affinity may be a part of the driving force for the transport of the amphipathic LPS across the aqueous

periplasm to the complex of LptD and LptE at the OM (Chng *et al*., 2010; Sperandeo *et al*., 2007; Tran *et al*., 2008; Wu *et al*., 2006).

LptD is an OM protein consisting of a N-terminal periplasmic domain and a C-terminal transmembrane *β*-barrel domain, which is the largest monomeric *β*-barrel protein identified up to now in the Gram-negative bacteria OM. Its size is sufficient to permit LPS translocation across the OM bilayer. LptE is a lipoprotein anchored in the inner leaflet of the OM (Chng *et al*., 2010; Freinkman *et al.*, 2011; Takase *et al.*, 1987). The structure of the LptDE complex was resolved by Huang and Dong laboratories (Dong *et al.*, 2014; Qiao *et al*., 2014). These structures provide several clues to the mechanism of LPS transport across the OM. LptE is positioned almost entirely inside the LptD *β*-barrel and the remainder part is located on the periplasmic face of the barrel. The interior groove of the periplasmic N-terminal domain of LptD is very hydrophobic (similarly to LptA and LptC) and it may receive the hydrophobic moiety of LPS from LptA (Qiao *et al.*, 2014), while sugar moieties are probably oriented by the LptE protein inside the hydrophilic *β*-barrel (Malojčić *et al.*, 2014; Dong *et al*., 2014; Qiao *et al.*, 2014). In this way, the lipid A without entering the lumen of the LptD *β*-barrel can be directly inserted into the outer leaflet of the OM by the lateral gate made by the opening of the LptD *β*-barrel between strands *β*1 and *β*26, whereas the polysaccharide core passes through the barrel (Gu *et al*., 2015; Li *et al*., 2015; Okuda *et al*., 2012; Qiao *et al.*, 2014; Tran *et al*., 2010).

For this step of LPS translocation, two highly conserved proline residues in *β*1 and *β*2 strands of LptD (P231 and P246) are critical, which was shown in studies with LptDE from Gram-negative bacteria (*Yersinia pestis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*) (Botos *et al*., 2016). These prolines are needed for perturbing the secondary structure of strands and generating a local gap that enables the lateral migration of LPS molecules (Botos *et al*., 2016; Sperandeo & Polissi, 2016).

Recently, the ‘PEZ’ model of LPS transport was proposed, where the Lpt transporter is compared to a PEZ dispenser with candies being pushed by a spring located at the bottom (Okuda *et al*., 2016). In this case, LPS molecules similarly to candies are pushed from the outer leaflet of the IM towards LptC by the $LptB_2FG$ complex driven by ATP hydrolysis by LptB in the cytoplasm. Then, LPS is further pushed from LptC to LptA and to the LptDE complex, in a process also relating on ATP hydrolysis mediated by $LptB_2FGC$. The stream of LPS from the IM to the cell surface seems to be continuous

and the energy needed for this transport is sequentially provided by the cytoplasmic ATP hydrolysis. The model of such continuous stream of LPS is supported by the observation that both LptC and LptA are constantly occupied by LPS and the appearance of LPS-binding sites at the N-terminal domain of LptD (Okuda *et al*., 2016). LptE ability to disrupt LPS aggregates also may play the important role in this mechanism (Malojčić *et al.*, 2014).

2.3. The σ^E regulon

The *rpoE* gene encodes an extracytoplasmic function sigma factor called σ^E or σ^{24}. This RNA polymerase subunit was identified as the sigma factor necessary for the recognition of the P3 promoter of the *rpoH* gene (gene encoding a heat-shock sigma factor), which is the only efficiently transcribed *rpoH* promoter at very high temperature (Erickson & Gross, 1989; Wang & Kaguni, 1989; Raina *et al*., 1995). σ^E is also involved in the transcription initiation of the *rpoN* gene encoding the nitrogen-limitation responsive sigma factor, σ^{54} and the *rpoD* gene encoding primary sigma factor, σ^{70} (Dartigalongue *et al*., 2001). σ^{70} and σ^E are the only two of seven different sigma factors located in *E. coli* that are essential for the viability at all temperatures (De Las Peñas *et al*., 1997; Hiratsu *et al*., 1995).

σ^E is the main regulator of bacterial envelope stress response. Thus, $E\sigma^E$ controls the transcription initiation of genes, whose products are involved in synthesis and transport of outer membrane components (Table 1). These include genes like *plsB*, whose product catalyses phospholipid biosynthesis, or *lpxA*, *lpxB*, *lpxD*, and *lpxP* encoding enzymes involved in lipid A biosynthesis, or *rfaD*, *waaC*, *waaF*, *waaL*, *waaU* encoding products responsible for LPS core biosynthesis. σ^E regulates also transcription of genes encoding products involved in LPS translocation to the OM. These are *lptA*, *lptB* and *lptD* encoding components of the LPS transporter (Dartigalongue *et al*., 2001; Martorana *et al*., 2011).

The RpoE sigma factor is also required for the transcription initiation of genes encoding products responsible for the correct outer membrane and periplasmic protein folding and assembly, like genes encoding a periplasmic protein chaperone, *skp*, a cytoplasmic protein chaperone *clpX*, proteases like *htrA* (*degP*), *lon* and *ecfE* (*rseP*) and a metalloprotease *yfgC*, periplasmic peptidyl-prolyl *cis/trans* isomerases *fkpA* and *surA*, a disulfide oxido-reductase *dsbC*, as well as the OMP assembly complex including *bamA*, *bamB*, *bamC* and *bamD*. σ^E is also involved in transcription of genes encoding outer membrane proteins such as *ecfI* (*yidQ*), lipoproteins like *ecfH* (*yraP*), *yeaY*, a peptide antibiotic transporter *sbmA*, as well as inner membrane proteins *ecfG* (*ygiM*), *ecfL* (*yqjA*), and periplasmic proteins, as *efcF* (*yggN*), *ecfJ* (*ytfJ*) (Dartigalongue *et al*., 2001).

Another member of the σ^E regulon is the *bacA* gene, which encodes enzyme important for the synthesis of various bacterial cell wall components such as peptidoglycan, lipopolysaccharides, and teichoic acids. σ^E also initiates transcription of *wzb* and *wzc* genes, whose products are involved in the bacterial capsular polysaccharide production (Danese & Silhavy, 1997; Dartigalongue *et al*., 2001; Rezuchova *et al*., 2003; Rhodius *et al*., 2006).

σ^E is responsible for the transcription initiation of genes encoding products involved in the transcription process as previously mentioned *rpoD*, *rpoE*, *rpoH*, *rpoN*, as well as *greA* encoding a transcription elongation factor. σ^E also controls transcription of genes whose product are involved in the translation process and its regulation: *rsmA* (*ksgA*) encoding a ribosomal RNA small subunit methyltransferase A, *fusA* encoding an elongation factor G, *prfB* (*supK*) encoding a peptide chain release factor RF2 and *hpf* (*yhbH*) encoding a hibernation promoting factor (HPF) (Rhodius *et al*., 2006).

Table 1. Examples of genes belonging to the σ^E regulon.

Genes, whose products are involved in cell envelope components biosynthesis and transport	
Phospholipid biosynthesis	
plsB	glycerol-3-phosphate *O*-acyltransferase
LPS biosynthesis and transport	
lpxA	UDP-*N*-acetylglucosamine acetyltransferase
lpxB	tetraacyldisaccharide-1-P synthase
lpxD	UDP-3-*O*-(3-hydroxymyristoyl)-glucosamine *N*-acyltransferase
lpxP	palmitoleoyl-acyl carrier protein (ACP)-dependent acyltransferase
rfaD (htrM, waaD)	ADP-L-glycero-D-*manno*heptose-6-epimerase
waaC	ADP-heptose:LPS heptosyltransferase I
waaF	ADP-heptose:LPS heptosyltransferase II
waaL	*O*-antigen ligase
waaU	heptosyltransferase IV
lptA	periplasmic LPS-binding protein
lptB	ATP-binding protein of the Lpt transporter
lptD (imp)	LPS assembly OM complex subunit
bacA	undecaprenyl pyrophosphate phosphatase
Genes, encoding products involved in capsular polysaccharide synthesis	
wzb	protein-tyrosine phosphatase
wzc	protein-tyrosine kinase

Genes, whose products are involved in the protein folding and assembly	
bamA (ecfK)	subunits of the OMP assembly complex BamABCDE
bamB (yeaT, yfgL)	
bamC (nlpB)	
bamD (ecfD, yfiO)	
skp	periplasmic protein chaperone
clpX	subunit of ClpX-ClpP ATP-dependent serine protease
htrA (degP)	serine endoprotease
lon	DNA-binding ATP-dependent protease
ecfE (rseP)	inner membrane zinc RIP metalloprotease
yfgC	periplasmic predicted metalloprotease
fkpA	FKBP-type peptidyl-prolyl *cis*/*trans* isomerase
surA	peptidyl-prolyl *cis*/*trans* isomerase
dsbC	protein disulfide isomerase II
Genes, encoding other membrane components	
sbmA	peptide antibiotic transporter
ecfI (yidQ)	outer membrane proteins
ecfH (yraP)	lipoprotein
yeaY	lipoprotein
ecfL (yqjA)	inner membrane protein
ecfG (ygiM)	predicted signal transduction protein
Genes, whose products are involved in the transcription	
rpoD	σ^{D}, σ^{70} primary sigma factor
rpoE	σ^{E}, σ^{24} extracytoplasmic function sigma factor
rpoH	σ^{H}, σ^{32} heat shock sigma factor
rpoN	σ^{N}, σ^{54} nitrogen-limitation responsive sigma factor
greA	transcription elongation factor
ecfJ (ytfJ)	putative transcriptional regulator
Genes, whose products are involved in the translation	
rsmA (ksgA)	16S rRNA methyltransferase
fusA	elongation factor G
prfB (supK)	peptide chain release factor RF2
hpf (yhbH)	hibernation promoting factor (HPF)

2.4. Transcriptional regulation of the *rpoE* gene

As is the case with regulation of other alternative sigma factors, σ^E activity and amounts are tightly regulated at the transcriptional, as well as the post-translational level. The gene encoding σ^E, *rpoE*, is a part of a four-gene operon encoding anti-sigma factor and other two post-translational regulators of σ^E and organized as *rpoE-rseA-rseB-rseC*.

It is known that at very high temperatures the *rpoE* transcription occurs mostly by the recognition of the promoter positively regulated by σ^E (Raina *et al*., 1995) located at -75 position. It was also shown that σ^E binding to this promoter is repressed by the phosphorylated response regulator CpxR (Fig. 6) (De Wulf *et al*., 2002).

Fig. 6. Promoter of the *rpoE* gene regulated by RpoE. The -35 and -10 binding sites for σ^E factor are bold and underlined and potential CpxR-P recognition site is shown in the box (adapted from Raina *et al*., 1995 and De Wulf *et al*., 2002)

Two-component systems are a widespread form of signal transduction pathway of the bacterial response to environmental signals (reviewed by Parkinson, 1993). Quite like other typical two-component systems, the Cpx system consists of an inner membrane sensor kinase CpxA (Albin *et al*., 1986; Weber *et al*., 1988) and CpxR, a cytoplasmic response regulator (Dong *et al*., 1993). The sensor kinase, CpxA, responds to particular stimuli by autophosphorylation of conserved histidine residue. Then, phosphorylated CpxA transfers the phosphoryl group to a conserved aspartate residue of the cognate response regulator, CpxR. Phosphorylated CpxR binds to specific promoter sequences and by that regulates transcription (Hoch & Silhavy, 1995; Missiakas & Raina, 1997; Raivio & Silhavy, 1997).

The Cpx response is negatively regulated by the periplasmic protein CpxP. Transcription of the gene encoding CpxP is increased upon activation of the Cpx pathway (Danese & Silhavy, 1998). CpxP directly interacts with the sensor kinase CpxA and down-regulates the autophosphorylation activity of CpxA (Fleischer *et al*., 2007). However, in the presence of misfolded proteins in the periplasm, CpxP dissociates from CpxA and binds misfolded proteins, and along with them is degraded by HtrA (DegP) protease, which also is a part of the Cpx regulon as well as the

σ^E regulon (Raina *et al*., 1995; Danese *et al*., 1995; Isaac *et al*., 2005; Buelow & Raivio, 2005; Missiakas & Raina, 1997; Dartigalongue *et al*., 2001). It means that functions of the Cpx two-component system and σ^E are overlapping. They both sense environmental stresses and accumulation of misfolded extracytoplasmic proteins (Raivio & Silhavy, 1999; Dartigalongue *et al*., 2001; Klein *et al*., 2009).

2.5. Post-translational regulation of σ^E

Under optimal growth conditions without any extracytoplasmic stress, proteins in the periplasm or in the outer membrane are properly folded and when LPS is not truncated, most of σ^E molecules directly interacts with the N-terminal cytoplasmic domain of the anti-sigma factor, RseA (Missiakas *et al*., 1997; De Las Peñas *et al*., 1997; Collinet *et al*., 2000). RseA binds region 4.2 of sigma factor, which recognizes -35 element of promoters (Tam *et al*., 2002). Sequestering of σ^E by RseA to the inner membrane prevents binding of σ^E with RNA polymerase, thereby inhibits transcription of σ^E regulon genes. The affinity of RseA for σ^E seems to be increased by phosphorylation of the N-terminal domain of RseA by Etk kinase (Klein *et al*., 2003). This regulation seems to be enhanced by a periplasmic protein, that interacts with the C-terminal domain of RseA, RseB, the product of the third gene of the *rpoE-rseA-rseB-rseC* operon (Missiakas *et al*., 1997). The last gene of this operon encodes an inner membrane protein that for a change modulates the RpoE activity positively.

During extracytoplasmic stress, RseB dissociates from RseA, which enables RseA cleavage. Firstly, the periplasmic domain of RseA is cleaved by protease DegS activated by the interaction of its PDZ domain with OMPs C-terminal domain, which most likely should be localised inside the native proteins (Karshikoff *et al*., 1994; Cowan *et al*., 1995; Walsh *et al*., 2003; Wilken *et al*., 2004; Kanehara *et al*., 2002; Young & Hartl, 2003). Then, another inner membrane protease, EcfE (RseP), cleaves RseA, allowing proteolysis by cytoplasmic proteases, like ClpXP, releasing active σ^E in the cytoplasm, where it can initiate transcription from its operon and also a number of genes encoding products, which ensure proper execution of processes like folding as well as biosynthesis and transport of membrane components (Dartigalongue *et al*., 2001).

2.6. Mutants with the increased σ^E-dependent response

The RpoE sigma factor controls and responds to major outer membrane defects (Klein *et al*., 2011). The σ^E activity increases upon mutations in many genes of its regulon, like *ecfE*, *dsbC*, *surA*, *fkpA*, *skp* encoding enzymes involved in protein folding, as well as *sbmA* encoding the peptide antibiotic transporter. Also mutations in the gene encoding a component of the other transport system, *tolC*, which does not belong to the σ^E regulon, show the elevated σ^E activity, probably due to a reduction in the synthesis of OmpF and an increase in the level of OmpC porin synthesis (Misra & Reeves, 1987). The σ^E activity is increased during overproduction of various outer membrane proteins including OmpF and OmpC (Mecsas *et al*., 1993; Grigorova *et al*., 2004).

Mutants exhibiting constitutively the elevated σ^E activity have reduced amounts of OMPs, due to repression of OMPs synthesis by products of genes *rybB* and *micA*, σ^E inducible non-coding RNAs. *hfq* (encoding non-coding RNA-binding chaperone) mutants also show up-regulated σ^E activity (Thompson *et al*., 2007; Valentin-Hansen *et al*., 2007; Guisbert *et al*., 2007).

Additionally, the expression of the *rpoE* gene increases in early stationary phase under general growth conditions (Nitta *et al*., 2000). Increase in the σ^E activity is quite pronounced in strains containg mutations in genes encoding products involved in LPS biosynthesis described in chapter number 2.1. entitled "Structure and biosynthesis of lipopolysaccharide".

3. RESULTS AND DISCUSSION

3.1. Transcriptional regulation of the *rpoE* gene

My research project objective was to study the mechanism of the transcriptional regulation of the *rpoE* gene. At the beginning of my research, it was known that this gene encoding σ^E is a part of a four-genes operon *rpoE-rseA-rseB-rseC* and its transcription is regulated from two promoters *rpoE*P1 and *rpoE*P2 (Raina *et al*., 1995). The *rpoE* transcription at very high temperatures occurs mostly by the initiation from the downstream promoter, which is recognized by σ^E itself. The aim of my PhD research was to investigate the regulation of the upstream promoter. Firstly, I performed experiments with the strain carrying a single-copy *lacZ* promoter fusion (referred as the *rpoE*P promoter; strain number SR7917 and GK3115). This *rpoE*P-*lacZ* promoter fusion contains 529 bp region upstream of the promoter regulated by RpoE. Analysis of the activity of this single-copy promoter fusion revealed that transcription from the upstream region is sustained under several stress and non-stress conditions. Detailed examination of activity of *rpoE*P-*lacZ* promoter activity under different growth conditions revealed enhanced activity upon entry into the stationary phase, shift to either high osmolarity or cold shock or change of carbon source for growth from glucose to glycerol. Furthermore, the activity of *rpoE*P-*lacZ* fusion was also found to increase by factors that can induce LPS structural alterations. Thus, response to different environmental cues and growth phase-dependent induction of its activity suggested potential involvement of multiple divergent regulatory systems that control the activity of upstream promoter region. These initial results hinted that there might be more than one promoter with multiple transcription start sites (TSS) located within this region. Indeed, mapping of 5′ ends of *rpoE* mRNA identified five major new transcriptional initiation sites located distal to the promoter regulated by RpoE itself. The previously mentioned promoter fusion covered all these five promoter regions. Hence later, in order to verify which promoter responds to which of different known conditions that regulate the *rpoE* gene transcription, several single-copy chromosomal promoter fusions with the *lacZ* gene were constructed and analyzed for their activity. Experiments showed that individually these promoters are activated in response to unique and specific signals.

3.1.1. Insertional mutagenesis

Firstly, in order to identify genes, whose disruption affects the *rpoE* gene transcription, an insertional mutagenesis with use of Tn*10* transposon was performed in the wild-type (SR7917) and its mutant derivative with deletions of *basR*, *phoB* and *rcsB* genes and the *lacZ* gene fusion to the 529 bp region upstream of the promoter regulated by RpoE (strain number GK3115).

basR and *phoB* genes encode transcriptional regulator proteins that are a part of two-component systems and the *rcsB* gene encodes a DNA-binding transcriptional co-regulator of the Rcs complex signal transduction system. This strain was obtained during earlier research conducted in our laboratory and taking into account that ammonium metavanadate induces a non-stechiometric substitution in the LPS structure (our unpublished work and Tam & Missiakas, 2005). Such alterations are, among others, under control of σ^E, BasS/R and PhoB/R two-component systems (Klein *et al*., 2011). During the screening in Δ(*basR phoB*) mutant, the Rcs transcriptional regulator was identified as a possible activator of the *rpoE* transcription.

As it was mention before (paragraph number 2.1.2), the induction of the BasS/R two-component system leads to the substitutions by P-EtN and L-Ara4N in LPS. Such substitution and induction of the BasS/R two-component system is further enhanced in the phosphate-limiting conditions (Klein *et al*., 2009). It is also known that the BasS/R system is induced by elevated levels of metals, such iron and zinc ions, as well as ammonium metavanadate (Wösten *et al*., 2000; Lee *et al*., 2005) causing bacterial adaptive response (BAS) (Nagasawa *et al*., 1993) by controlling genes that are involved in the metal-response membrane modification, and response to acidic as well as anaerobic growth conditions (Hagiwara *et al*., 2004; Lee *et al*., 2005).

The second two-component system, PhoB/R, is highly induced by phosphate limitation (Makino *et al*., 1985). It regulates genes involved in phosphorus assimilation (Wanner, 1987) and similarly to BasS/R, PhoB/R controls genes involved in LPS modification for example *waaH* and *ugd* genes (Klein *et al*., 2011; Klein *et al*., 2013; Baek & Lee, 2006).

The Rcs signal transduction pathway consists of a histidine kinase sensor RcsC, a principal response regulator RcsB, an auxiliary activator RcsA, a phospho-transfer protein RcsD and a signal transductor RcsF (Majdalani & Gottesman, 2005; Clarke,

2010). As name indicates, Rcs (regulator of capsule synthesis) controls the expression of genes encoding products responsible for the biosynthesis of the capsular polysaccharide called colanic acid. The Rcs system responds to a variety of signals, including changes of temperature, osmolarity and membrane proteins (Sledjeski & Gottesman, 1996; Mouslim *et al*., 2003). RcsB can act as homodimers or heterodimers with RcsA, which results in enhanced transcription of regulated genes. However, under normal conditions, the RcsA synthesis is low and it is rapidly degraded by a Lon protease, but yet, at low temperatures synthesis of RcsA increases, leading to colanic acid production and a mucoid phenotype (Majdalani & Gottesman, 2005).

During my research, firstly, the insertional mutagenesis with use of Tn*10*::tet transposon was performed (as described in 4.2.19) and more than 10,000 candidates were streaked on MacConkey agar, and LA plates containing the indicator dye 5-bromo-4-chloro-3-indolyl-*β*-D-galactopyranoside (X-Gal) and tetracycline. Secondly, the insertional mutagenesis with use of Tn*10*::kan transposon was performed and around 20,000 candidates were streaked on LA containing X-Gal and kanamycin, as well as LA containing X-Gal along with ammonium metavanadate, which induced the *rpoE* transcription. Furthermore, additionally around 4,000 Tn*10* insertion mutants were streaked on LA containing X-Gal along with 0.4 M NaCl (which was also found to induced the *rpoE* transcription), as well as on MacConkey agar.

Some of candidates which seems to have reduced the *rpoE* transcription from upstream promoter region in Δ(*basR phoB rcsB*) were isolated. Bacteriophage P1 lysates were prepared on those mutants, and transductions were performed using two strains as recipients: GK3115 strain and strain SR7917 carrying the same *rpoE*P-*lacZ* fusion as GK3115, but without deletions of *basR*, *phoB*, *rcsB*.

The chromosomal DNA was isolated from mutants that seemed to have changed the *rpoE* fusion activity. The sequences flanking the Tn*10* insertion were synthesized using inverse PCR. Products of PCR were purified and obtained DNA products were sequenced (listed in Table 2 below).

Table 2. Genes in which Tn*10* insertion(s) mutations caused alterations in *rpoE*P activity.

Gene	Product encoded by the gene
waaC	ADP-heptose:LPS heptosyltransferase I
waaF	ADP-heptose:LPS heptosyltransferase II
waaQ	LPS core heptosyltransferase III
rssB	regulator of RpoS
rfaH	transcription antiterminator
glnA	glutamine synthetase
ecfL	putative inner membrane protein
cysE	serine acetyltransferase
carB	carbamoyl-phosphate synthase large subunit
cyaA	adenyl cyclase
ycjO	membrane component of a predicted ATP-dependent sugar transporter
atpI	ATP synthase, membrane-bound accesory subunit
pyrL	operon encoding aspartate carbamoyltransferase leader peptide

Among these, the isolation of Tn*10* insertions that conferred a Lac down phenotype mapping to *cyaA* and *glnA* genes were of specific interest. The *cyaA* gene encodes adenyl cyclase and activates the global transcriptional regulator CRP (Brickman *et al*., 1973; Danchin *et al*., 1984). CRP-cAMP positively regulates the expression of several genes and a decrease in the transcriptional activity of *rpoE*P-*lacZ* fusion suggested a positive regulation of transcription and hence was vigorously followed.

The second interesting result of the insertional mutagenesis experiment was that the deceased activity of the fusion of promoter region covering the 529 bp region upstream of the promoter regulated by RpoE was found due to a Tn*10* insertion in the *glnA* gene located at position 169 nucleotides in the coding region. This gene is the first gene of the *glnA*-*glnL*(*ntrB*)-*glnG*(*ntrC*) operon (Miranda-Rios, *et al*., 1987). The transposon insertion mutation found in the *glnA* gene was found to be polar on other genes of its operon. This operon encodes the two-component system comprised of the sensor kinase NtrB and the DNA-binding transcriptional dual regulator NtrC (Guterman *et al*., 1982). NtrC is known to act as an enhancer-like protein for many RpoN-regulated promoters (Syed & Gralla, 1997; Reichenbach *et al*., 2009). Both recognition sites for NtrC as well as RpoN consensus -12 and -24 regions can be indeed found upstream of the *rpoE* gene (Fig. 9). The regulation of the *rpoE* transcription by the NtrB/C two-component system

and RpoN was checked firstly by the comparison of *β*-galactosidase activity of the *rpoN* deletion mutants carrying different *rpoE* promoter fusions with the *lacZ* gene in media with nitrogen-limiting salts supplemented with NH_4Cl at concentrations of 3 or 10 mM. Direct regulation of one of *rpoE* promoters by RpoN was established in the further experiments, described in section 3.4.

Some of remaining genes sequenced during this approach are not potential transcription factors and observed reduction in the *rpoE* promoter activity was probably caused by the effect of their mutation on the metabolism. However, some potentially interesting results were obtained. One of these insertions was found in the *cysE* gene encoding serine acetyltransferase. It has been shown that loss of the *cysE* gene enhances biofilm formation in *E. coli* (Sturgill *et al*., 2004). On the other hand, significantly reduced biofilm formation was found in the Δ*rpoE* derivative of the uropathogenic *Escherichia coli* (UPEC) isolate UTI89 (Kulesus *et al*., 2008). RpoE is associated with the biofilm formation of *Salmonella pullorum* (Huang *et al*., 2015). Such results may suggest the role of RpoE in the biofilm formation.

In parallel, Tn*10* insertion mutants were screened for candidates that conferred an increasein the *rpoE* transcriptional activity. Such screens were a part of the same screen that identified mutants causing a decrease in the activity. However, for rescreening of Lac up Tn*10* insertion mutants, LA plates were supplemented by a lower amount of X-Gal (20 μg/ml) and colonies were streaked in parallel on MacConkey agar plates. Interestingly, numerous Lac up mutants did not grow on MacConkey agar, suggesting permeability defects with a concomitant increase in the *rpoE* transcriptional activity of such mutants. Several such mutants also exhibited a mucoid colony phenotype, indicating an increase in the production of colanic acid. Inability of some of these Tn*10* insertion mutants to grow on MacConkey agar plates and the mucoid colony phenotype is reminiscent of a deep-rough colony phenotype (Raina & Georgopoulos, 1991; Klein *et al*., 2009). Thus, all Tn*10* mutations that conferred a Lac up phenotype were first transduced back using bacteriophage P1- or T4-mediated transductions into parental strain SR7917 to confirm that this phenotype is due to a single Tn*10* insertion.

As vast majority of Lac up Tn*10* insertion mutants exhibited the deep-rough phenotype suggested defects in LPS biosynthesis. To group such mutants in a complementation group, linkage with a tightly linked marker to the LPS biosynthetic *waa* locus was carried out. A bacteriophage T4 lysate was prepared on the strain carrying Δ*waaH*

mutation with a substitution by the *cat* cassette (Klein *et al*., 2013). The *waaH* gene is located about 4.5 kb away from the *gmhD* (*rfaD*) gene and thus should give linkage between 85% to 95% with Tn*10* insertion mutation in genes within the large *waa* locus. Thus, those mutations that showed expected linkage were further characterized for the LPS structural analysis.

In subsequent experiments, the chromosomal DNA was isolated from all Lac up mutant strains and used to amplify by PCR flanking sequences to the Tn*10* insertion site to identify the corresponding gene in which insertion has occurred. Thus, mapping and sequencing of Tn*10* mutations conferring a Lac up phenotype revealed that the majority of them had insertion in LPS biosynthetic/LPS regulatory genes and a few in other loci. Among these, twelve Tn*10* insertions mapped to the *waaC* gene, three to the *waaF* gene and one each was located in *rfaH* and *waaQ* genes. Among strains that did not confer LPS related defects, two Tn*10* insertions were mapped to the *rssB* gene (both with Tn*10* insertion at nt position 240), one Tn*10* insertion in the *arcA* gene (nt position 72 within the coding region) and one at nt position -78 upstream of the initiation codon of the *ecfL* gene.

As the Tn*10* insertion mutation can be polar, likely the phenotype can be a consequence of lack of expression in downstream gene. Furthermore, if the gene of interest is transcribed as a part of an operon and located upstream of an essential gene, in such a case it is not possible to get a Tn*10* insertion mutation. Thus, to circumvent such potential missing of gene of our interest in transposon approach, total gene disruption library of the Keio collection (Baba *et al*., 2006) covering individually all non-essential genes with a non-polar deletion was used. For these experiments, pools of bacteriophage P1 and T4 lysates were made on all available 3909 strains and transduced into the strain SR7917 carrying a single-copy promoter fusion (with 529 bp upstream of the RpoE-regulated promoter). Lac up or down candidates were screened as was used for search of transposon mutants. The deletion mutation was mapped using linked markers. Specific non-polar deletions of genes, identified using transposon mutagenesis, were also reinvestigated to confirm previous results. In addition to the confirmation of results from transposon mutagenesis, this analysis identified additional genes that either down-regulate or induce transcription of the *rpoE*P-*lacZ*. Thus, a Δ*arcB* mutation led to an increase in transcription, while Δ*rpoS* and Δ*crp* caused a decrease in the activity of *rpoE*P-*lacZ* fusion. All such deletion derivatives carrying the *rpoE*P-*lacZ* fusion in

SR7917 were used for measurement of the *β*-galactosidase activity revealing involvement of several regulatory pathways.

All such genes, in which the Tn*10* insertion or a non-polar deletion conferred increase in the *rpoE*P activity, were thoroughly examined to explore in detail how transcription of the distal promoter region is regulated. Interestingly, the *ecfL* gene obtained in these approaches was identified as a part of σ^E regulon (Dartigalongue *et al*., 2001). It encodes a putative inner membrane protein belonging to a DedA family.

The DedA protein family is a highly conserved family of proteins present in bacteria, archaea, and eukaryota. However, functions of DedA family proteins are still not fully understood. They may be membrane transporters required for proton motive force maintenance, which may be needed for the normal function of several families of drug efflux pumps (Kumar & Doerrler, 2014). There are eight DedA family members present in *E. coli* (DedA, EcfL, YabI, YdjX, YdjZ, YghB, YohD and YqaA). Each of these DedA homologs is individually nonessential. However, deletion of all members of this family is lethal, meaning that the DedA family is collectively essential in *E. coli* (Boughner & Doerrler, 2012). Bacterial DedA family mutants display temperature sensitivity, cell division defects, altered membrane lipid composition, elevated envelope-related stress responses, and loss of proton motive force (Thompkins *et al*., 2008; Doerrler *et al*., 2013; Sikdar *et al*., 2013).

The chromosomal deletion of either the *ecfL* gene or of the next gene in the operon, *ecfM*, did not influence the *rpoE* transcription from the upstream promoter region. Hence, the double deletion Δ(*ecfL ecfM*) was constructed in the strain carrying the *rpoE* promoter fusion and examined for the *β*-galactosidase activity (Fig. 7). These results are consistent with observations that simultaneous deletions of *ecfL* and *ecfM* genes confer a membrane and cell division defects at low temperatures and also exhibit osmo-remedial growth defect at elevated temperatures (Thompkins *et al*., 2008).

Thus, EcfL effects the *rpoE* transcription by working together with EcfM as one signal transduction system. Obtained results show that DedA protein family has an impact on the RpoE transcription.

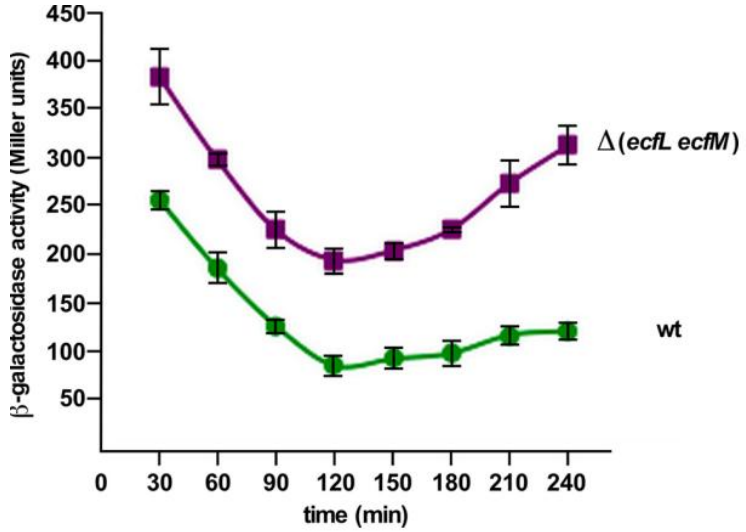

Fig. 7. *β*-Galactosidase assay showing increased activity of the *rpoE* promoter in the Δ(*ecfL ecfM*) mutant. Isogenic cultures of the wild-type and its Δ(*ecfL ecfM*) derivative carrying the *rpoE* promoter fusion were grown in LB medium at 37°C and analyzed for the *β*-galactosidase activity.

3.1.2. Multicopy expression that alters the *rpoE* transcription

In order to identify potential regulators of the *rpoE* transcription, I undertook another approach using strains carrying the single-copy chromosomal promoter fusion of upstream promoter region to the *lacZ* gene. Thus, to identify genes whose products when overexpressed, can either activate or repress transcription of the *rpoE* upstream promoter region, a multicopy library was transformed into competent cells of the strain SR7917 or its derivative SR16299 and plated on LA plates containing 0.3% glucose and appropriate antibiotic, chloramphenicol (20 μg/ml). Obtained transformants were streaked in parallel on MacConkey agar plates with two concentrations of the inducer [50 μM and 25 μM isopropyl 1-thio-*β*-D-galactopyranoside (IPTG)] and appropriate antibiotic. From candidates with increased and decreased reporter gene activity plasmids were isolated and re-transformed in the competent cells of SR16299 strain to confirm effect caused by the overexpression from the plasmids encoded genes and avoid misleading results. Such transformants were checked on MacConkey agar with 50 μM and 25 μM IPTG, as well on LA containing 50 μM IPTG, chloramphenicol and X-Gal (40 μg/ml), which enables approximation of expression level of *β*-galactosidase encoded by the *lacZ* gene.

From those, the best candidates (with the highest and lowest *rpoE*P-*lacZ* fusion activity comparing to a control strain of SR16299 transformed with a vector plasmid without insert) were chosen and plasmids obtained from these strains were transformed into strains carrying different fusions in order to disqualify non-specific changes in transcription. To confirm if changed *β*-galactosidase activity by the overexpression of genes encoded on these plasmids is specific to the *rpoE* promoter, additional control experiments were performed. Firstly, one control was SR10243 carrying the *htrP-lacZ* fusion. Thus, competent cells of SR10243 were prepared and used to transform with plasmids obtained from the strain SR16299 carrying the chromosomal *rpoE*P-*lacZ* fusion. Transformants that had changed the *β*-galactosidase activity comparing to the control (SR10243 transformed with the vector alone) were not taken for further investigations. Next, candidate plasmids were transformed into strains with promoter fusions with *lacZ* with the RpoE-regulated promoter of the *rpoE* gene and another promoter under RpoE regulation, that is *rpoH*P3-*lacZ*, as well as *lap*P2$_{hs}$, which is the RpoH-regulated heat shock promoter.

Fig. 8. The streaking of candidates with the reduced *rpoE*P promoter activity on MacConkey agar with 0.15 mM IPTG and chloramphenicol (20 μg/ml) and LA medium with X-Gal (40 μg/ml), 0.15 mM IPTG and chloramphenicol (20 μg/ml). As a control, SR16299 was transformed with empty plasmid.

The plasmids carrying promising genes, whose products are potentially more directly involved in the regulation of the transcription initiation by recognition of a *rpoE* promoter, were digested using restriction enzymes (BamHI or EcoRI), and agarose gel

electrophoresis was performed. Basing on these results, plasmids with different restriction pattern were sequenced. The genes identified in this approach are listed in Table 3 and Table 4 below.

Table 3. The list of genes which, when overexpressed, can reduce transcription of the *rpoE*P promoter.

Genes whose overexpression repress transcription of the *rpoE*P-*lacZ* fusion	
Gene	Product encoded by the gene
yghT	predicted protein with nucleoside triphosphate hydrolase domain
phoP	response regulator
fruR	Cra DNA-binding transcriptional dual regulator
fliZ	DNA-binding transcriptional regulator
rph	encoding RNase PH
rnpA	ribonuclease P protein component
ylbH	hypothetical protein
ycfP	hypothetical protein
ygbE	inner membrane protein
yiaN	component of L-dehydroascorbate transporter
yliB	peptide transporter subunit
rsmC	ribosomal RNA small subunit methyltransferase C
ldcC	lysine decarboxylase 2
rhaB	L-rhamnulose kinase
yliE	putative membrane-anchored cyclic-di-GMP phosphodiesterase
clsB	cardiolipin synthase 2
hyaF	hydrogenase-1 protein nickel incorporation factor
yabI	DedA family inner membrane protein
mltD	putative membrane-bound lytic murein transglycosylase D
rrmA	ribosomal RNA large subunit methyltransferase A
rffH	glucose-1-phosphate thymidylyltransferase
rfaD	ADP-L-*glycero*-D-*manno*-heptose-6-epimerase
rsmA	ribosomal RNA small subunit methyltransferase A

Table 4. The list of genes which, when overexpressed, can increase transcription of the *rpoE*P promoter.

Genes whose overexpression seemed to activate transcription of the *rpoE*P-*lacZ* fusion	
Gene	Product encoded by the gene
qseG	conserved OM α-helical protein, a part of the *qseE/F* operon
rirA	RfaH interacting RNA
csgG	lipoprotein
pgaB	lipoprotein
spr	lipoprotein
yhdV	lipoprotein
yceB	lipoprotein
yddW	lipoprotein
yghB	lipoprotein
rpoA	α subunit of RNA polymerase
pcnB	poly(A) polymerase I
hok	small toxin polypeptide
ompX	outer membrane protein X
ybhQ	predicted inner membrane protein
ugpA	putative membrane subunit of glycerol-2-phosphate ABC transporter
yhbS	predicted acyltransferase with acyl-CoA *N*-acyltransferase domain
sbmC	DNA gyrase inhibitor
yaeO	modulator of Rho-dependent transcription termination
eptA	phosphoethanolamine transferase
mog	molybdochelatase incorporating molybdenum into molybdopterin

Among various genes, whose expression caused a significant increase in transcription of the *rpoE*P-*lacZ* activity, the maximal induction was observed when the *qseG* gene was present in the multicopy. In this approach also several genes encoding lipoproteins (*csgG, pgaB, spr*, *yhdV*, *yceB*, *yddW* and *yghB*), which, when overexpressed, increase the *rpoE*P-*lacZ* activity, suggesting that such proteins are a part of signal transduction controlling *rpoE* promoters activity. Additionally, a set of plasmids was found to contain DNA sequences carrying part of the *waaQ* gene and divergently transcribed the *waaA* gene. These plasmids were obtained using multicopy library constructed using the chromosomal DNA cloned in a p15-based vector as previously described (Missiakas *et al*., 1993). Further characterization following DNA sub-cloning, revealed that this induction of the *rpoE*P activity is due to the presence of a novel sRNA located between *waaQ* and *waaA* ORFs. Detailed analysis revealed that this sRNA is generated by the

processing of 5′UTR of the *waaQ* mRNA (see below). Additionally, the overexpression of the *pcnB* gene encoding poly(A) polymerase I (PAP I) (Santos *et al*., 2006) also increases the *rpoE*P-*lacZ* activity. Among genes, whose induction repressed the *rpoE*P-*lacZ* activity, the *fliZ* gene was identified whose product could act as a negative regulator. As is evident, genes identified that could encode either positive or negative regulator based on analysis of gene deletions or multicopy dosage-dependent regulators fall in different functional groups. Some of them that can alter the *rpoE*P promoter activity are related to LPS biogenesis, the RpoS sigma factor activity or its amount, regulatory factor like CRP and the presence of higher amounts of lipoproteins. The function of QseG had not been elucidated and its overproduction caused maximal induction of the *rpoE*P promoter activity. However, further analysis revealed that all of these factors contribute to the *rpoE*P promoter activity and constitute an elaborate system of signal transduction to incorporate different signals and were analyzed in more detail. However, a direct participation of many other genes identified in multicopy approach could not be addressed and some of them are discussed below.

The PhoP response regulator of the PhoP/Q two-component system was already identify as a negative regulator of the *rpoE* expression during previous research in Professor Raina laboratory. It seems to be a part of negative feed-back regulation via PhoP/Q transcribed small non-coding RNA and is followed as a part of separate investigation.

Increased activity of the *rpoE* promoters located upstream from autoregulated promoter was found in strains overexpressing the *rpoA* gene. The *rpoA* gene encodes the α subunit of RNA polymerase that interacts with transcription factors at a number of promoters (Ishihama, 1992). It was shown that theoverexpression of α subunit of RNA polymerase inhibits the expression of *ompF* and *ompC* genes (Bowrin *et al*., 1994). Due to changes in the outer membrane proteins distribution, σ^E may be induced.

The gene encoding one of the outer membrane proteins (*ompX*) was also cloned during screen for genes that seemed to activate the *rpoE* transcription. It was already shown that the overexpression of OmpX increases σ^E activity (Mecsas *et al*., 1993). However, such changes should be rather mostly sensed by the promoter, which is autoregulated by σ^E after its release from the anti-sigma factor, RseA, caused by sensing of misfolded proteins by RseB and DegS.

Among genes whose overexpression altered transcription of the *rpoE* gene, also those genes were identified, whose products may have an effect on the mRNA instability: *rnpA* encoding ribonuclease P protein component, *rph* encoding RNase PH and *pcnB* that encodes poly(A) polymerase I [it was shown that increased polyadenylation of mRNAs results in the decreasing of mRNA half-life (Mohanty & Kushner, 1999)].

Some of genes cloned in this approach also belong to the σ^E regulon. These are *hok*, *yiaN* and *yghT*. The first one was selected as an activator of the σ^E response, however it encodes the host killing factor, and *β*-galactosidase released to medium with X-Gal during cell lysis could be misleading. *yiaN* and *yghT* genes were selected as down-regulators of σ^E response. The *yiaN* gene encodes a membrane component of L-dehydroascorbate transporter, and *yghT* encodes predicted protein with nucleoside triphosphate hydrolase domain. Analysis of the amino acids sequence with the use of Basic Local Alignment Search Tool (BLAST) revealed similarity of YghT to thymidylate kinase, which catalyses the phosphorylation of thymidine monophosphate (TMP) to thymidine diphosphate (TDP) utilizing ATP during biosynthesis of thymidine triphosphate (TTP).

The reduced transcription of the *rpoE* gene was found in the strain overexpressing the *yliE* gene, which encodes a putative membrane-anchored cyclic-di-GMP phosphodiesterase degrading cyclic-di-GMP (Jenal & Malone, 2006). Cyclic-di-GMP is the second messenger molecule involved in the control of a variety of cellular functions such as virulence and motility, although its principal role is the post-transcriptional regulation of the biofilm formation. Biofilm is a community of aggregated, surface-attached cells, which bacteria like *E. coli* can form in response to various stress conditions (Branda *et al*., 2005). Enhanced cells aggregation and surface adhesion along with synthesis of exopolysaccharides is promoted by the elevated intracellular level of cyclic-di-GMP (Simm *et al*., 2004). In contrast, the reduced cyclic-di-GMP level is associated with decreased biofilm formation, hence the overexpression of the *yliE* gene reduces biofilm formation (Boehm *et al*., 2009).

It was also shown that cyclic-di-GMP reduces Lon protease activity *in vitro* (Osbourne *et al*., 2014). Consequently, reduced cyclic-di-GMP levels, upon the *yliE* overexpression, may enhance Lon activity leading to among others RcsA degradation (Majdalani & Gottesman, 2005), which was identified as possible activator of the *rpoE*

gene transcription, which may explain the reduced *rpoE* promoter activity, when the *yliE* gene is overexpressed.

During the screen for genes that seemed to repress transcription of the *rpoE* upstream promoters fusion, the *clsB* gene encoding the second cardiolipin synthase was cloned (Guo & Tropp, 2000). There are three cardiolipin synthases in *E. coli*, ClsA, ClsB and ClsC. ClsA is responsible for synthesis of cardiolipin during exponential growth, while in the stationary phase all three cardiolipin synthases contribute (Tan *et al*., 2012). Cardiolipin activates Mqo malate dehydrogenase, which is a part of the RpoE regulon (Narindrasorasak *et al*., 1979; Huerta *et al*., 2003) and LpxK kinase that is involved in lipid A biosynthesis (Ray & Raetz, 1987). The overexpression of the *clsB* gene may cause increased cardiolipin synthesis followed by enhanced LpxK activity, which could cause LPS changes, possibly sensed by a pathway that controls one of the *rpoE* promoters.

Another gene, whose overexpression caused decreased transcription from the *rpoE* upstream promoters region, is the *yabI* gene encoding an inner membrane protein with five predicted transmembrane domains, and its C-terminus is located in the periplasm, being a member of the DedA family (Daley *et al*., 2005; Thompkins *et al*., 2008). Previously described insertional mutagenesis showed the impact on the *rpoE* transcription of another member of the DedA protein family, EcfL, which seemed to effect the *rpoE* transcription by working together with EcfM as one signal transduction system.

Transcription from the upstream promoter region of the *rpoE* gene was also reduced upon the *rffH* gene overexpression. This gene encodes glucose-1-phosphate thymidylyltransferase subunit of dTDP-glucose pyrophosphorylase involved in the synthesis of enterobacterial common antigen (ECA) (Marolda & Valvano, 1995). ECA is a family-specific surface antigen shared by all members of the Enterobacteriaceae and is restricted to this family. Biosynthesis of ECA shares the same sugar precursors and the same carrier lipid with LPS biosynthesis (Kuhn *et al*., 1988). It may mean that changed ECA synthesis could influence LPS synthesis and due to which the *rpoE*P activity or that the *rpoE* gene senses also changes in ECA. It was previously shown that mutations in the *rffH* gene caused activation of the RpoE-regulated *rpoH*P3 promoter, similarly to mutations in other genes, *wecE* and *wecF*, whose products are involved the synthesis of lipid III, the final intermediate in the ECA pathway. Strains carrying

mutation in *rffH*, as well as *wecE* and *wecF* accumulate ECA biosynthetic intermediate, lipid II and in the addition to activation of RpoE they activate Cpx signal transduction pathway (Danese *et al*., 1998).

Similarly to ECA, also murein synthesis shares precursor with LPS (that is the UDP-GlcNAc) (Mengin-Lecreulx *et al*., 1983). One of the genes encoding enzymes that may play a role in the recycling of muropeptides was found to reduce transcription of the *rpoE* upstream promoter when it was overexpressed. This is the *mltD* gene encoding putative membrane-bound lytic murein transglycosylase D, which degrades murein. Increased degradation of murein by the overexpression of the *mltD* gene encoded on the plasmid may increase demand for its synthesis from its precursor, which could influence the *rpoE* promoter via some signaling pathway.

The gene encoding one of the enzymes involved in LPS biosynthesis was also cloned during screen for genes that decreased transcription of the *rpoE*P-*lacZ* fusion. The *rfaD* gene (also known as *gmhD*, *htrM* or *waaD*), that encodes ADP-L-*glycero*-D-*manno*-heptose-6-epimerase required for Hep biosynthesis was cloned in this process. The *rfaD* gene is essential for bacterial growth at elevated temperatures. LPS of *rfaD* mutants lack Hep and have less amounts of OMPs and are supersensitive to hydrophobic drugs (Coleman & Leive, 1979; Coleman, 1983; Pegues *et al*., 1990; Raina & Georgopoulos, 1991; Ding *et al*., 1994; Missiakas & Raina, 1997; Murata *et al*., 2011). The *rfaD* gene has promoter that is recognized by RNA polymerase with an associated RpoE sigma factor (Dartigalongue *et al.*, 2001). As the overexpression of the *rfaD* gene reduced transcription from upstream promoter region of the *rpoE* gene suggests that perturbations in the LPS biosynthesis pathway influence transcription of the *rpoE* gene.

Another gene (*eptA*) encoding one of the enzymes involved in LPS modifications (Raetz & Whitfield, 2002) was also cloned during these experiments. EptA catalyses the addition of P-EtN to the lipid A part. Such modification of *E. coli* LPS is induced in cells grown under mild acidic conditions (Gibbons *et al*., 2005) and in LB supplemented with ammonium metavanadate (NH_4VO_3) (Zhou *et al*., 1999), which is consistent with parallel studies showing that an addition of ammonium metavanadate, induces *rpoE* upstream promoters.

Among other genes, which when overexpressed also lead to decrease in the *rpoE* transcription, included genes that encode methyltransferases of ribosomal RNA of small

as well as large subunit, *rsmA* (*ksgA*) and *rrmA*, respectively. RsmA also may play a role in protection of DNA against oxidative stress. It also suppresses a cold-sensitive phenotype of the *era* gene mutant, which encodes an essential GTP-binding protein (Lu & Inouye, 1998; Inoue *et al*., 2007; Zhang-Akiyama *et al.*, 2009). On the other hand, it has been shown that a *rrmA* mutant exhibit the growth defect, which is not caused by the lack of rRNA methylation, and thus RrmA seems to have another, as yet unidentified, function (Liu *et al*., 2004), which may contribute to the changed *rpoE* promoter activity.

During these experiments the *sbmC* gene, whose product also could act as a potential regulator was also identified. The *sbmC* gene encodes an inhibitor of DNA gyrase, enzyme introducing negative supercoiling during DNA replication (Fairweather *et al*., 1980; Nakanishi *et al*., 1998). The overproduction of SbmC causes growth defects and filamentous cell morphology (Nakanishi *et al*., 1998). SbmC also protects the cell from some of the DNA-damage- and SOS-response-inducing toxins, because of its interference with DNA binding by gyrase (Baquero *et al*., 1995; Wei *et al*., 2001; Chatterji & Nagaraja, 2002; Chatterji *et al*., 2003). It has been shown that the inhibition of the DNA gyrase activity causes increased synthesis of RpoH and its stabilization, resulting in the induction of heat shock proteins (Mizushima *et al*., 1996). Mutations in RpoH-dependent heat shock genes, like *groEL* and *grpE*, can be suppressed by overexpression of the *yaeO* gene, which was also obtained as a possible activator of the *rpoE* transcription during above-mentioned experiments. The *yaeO* gene encodes modulator of Rho-dependent transcription termination and it is also a multicopy suppressor of genes encoding products involved in cell division, such as *ftsA*, *ftsQ* and *rpoB* encoding β subunit of RNA polymerase (Chuang & Blattner, 1993; Pichoff *et al*., 1998; Kawamura *et al*., 2005).

3.2. Identification of new additional promoters by RACE analysis

The transcriptional regulation of the *rpoE* gene has not been fully understood. Initially, two promoters were identified, out of which the downstream promoter is positively regulated by RpoE. However, as described in above sections, transcription from the upstream region is sustained under several stress and non-stress conditions and induced upon several divergent stress conditions or upon overexpression of genes encoding proteins involved in different regulatory pathways. Based on our several previous observation and data presented in above sections, it is clear that transcription emanating from upstream promoter element(s) is induced upon some LPS structural alterations, entry into the stationary phase, challenge with high osmolarity and cold shock. These results were obtained with the use of a single-copy *lacZ* promoter fusion (referred as the *rpoE*P promoter) that contains the 529 bp region upstream of the promoter regulated by RpoE. Based on obtained mutational spectrum, analysis of multicopy inducers/repressors and changes in different growth conditions that alter the *rpoE*P promoter activity suggest existence of multiple divergent and interconnected modes of regulation of the upstream promoter region. Thus, it is likely that there may be more than one transcription start site (TSS) located within this region. Indeed, the RACE analysis (rapid amplification of 5′ and 3′ cDNA ends described in 4.2.2) with use of The GeneRacer from Invitrogen was performed. For the RACE analysis, total RNA was extracted from different growth conditions from the wild-type *E. coli* K-12 strains or its isogenic derivatives with defects in LPS Δ*waaC* and strains overexpressing the most prominent inducer the *qseG* gene. This mapping of 5′ ends of mRNA identified five major new transcriptional initiation sites located distal to the promoter regulated by RpoE itself (shown in Fig. 9). They were designated as P1 (located at position -381), P2 (-327), P3 (-327/-326), P4 (-218) and P5 (-153). Results of transcriptional activity presented in earlier sections were based on strains carrying *rpoE*P-*lacZ* fusion. This fusion covered all these 5 promoter regions. Hence, in order to verify which promoter responds to which of different conditions that regulate the *rpoE* gene transcription, several single-copy chromosomal *rpoE*P-*lacZ* promoter fusions were constructed and analyzed for their activity (as described in 4.2.3 and 4.2.15). Experiments showed that these promoters are activated in response to unique signals.

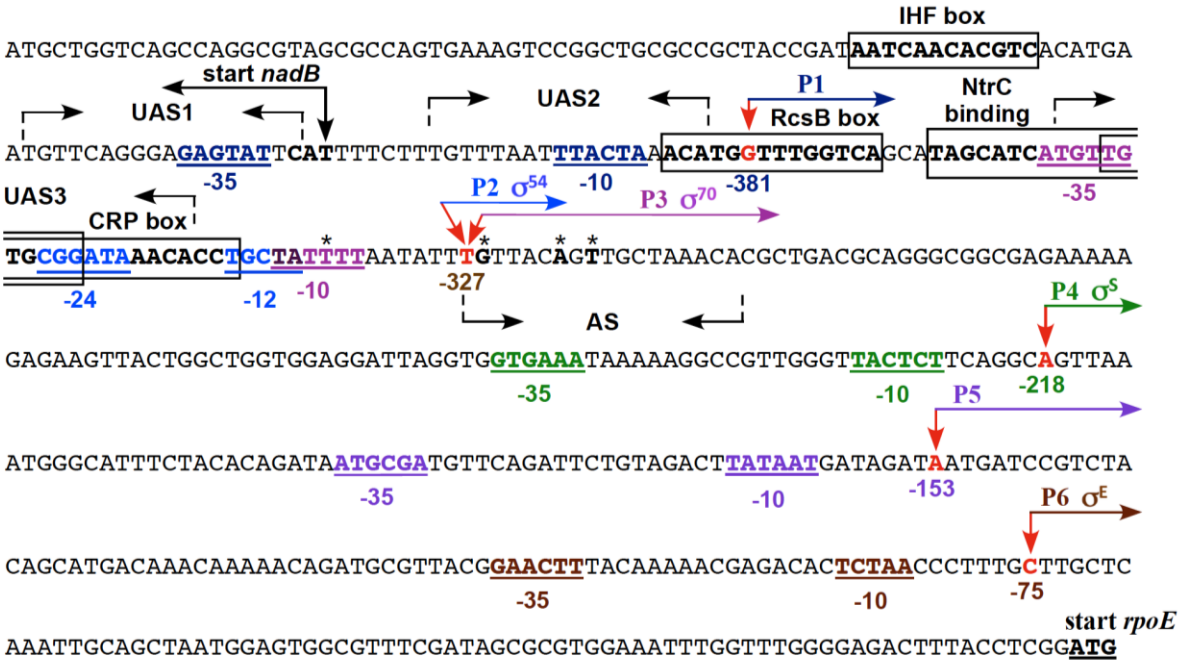

Fig. 9. The promoter region sequence of the *rpoE* gene. The red arrows indicate the position of transcription start sites. Symbol * indicates either minor start sites or processing sites.

Among these five promoters, *rpoE*P1 and *rpoE*P5 exhibit low basal activity in a single-copy and no specific growth conditions or *trans*-acting mutations were identified that could alter their activity. Even the frequency of plasmids containing the 5′ end of the P5 promoter obtained during the RACE analysis was the lowest of all. Thus, for further studies these two promoters were not followed.

Examination of DNA sequence showed that region located upstream of the P2 promoter contains a DNA sequence motif characteristic of the nitrogen-limitation responsive sigma factor, RpoN, recognition sites at -12 (GC) and -24 (GG) (marked in blue in Fig. 9). A very good match was observed with the promoter region of the RpoN-regulated genes (Fig. 10).

Fig. 10. The alignment of -24 and -12 promoter elements of the *rpoE*P2 promoter with different conserved RpoN-regulated promoters

RNA polymerase with RpoN cannot transcribe on its own, because it requires ATP-dependent activators like NtrC or PspF or QseF (Syed & Gralla, 1997; Reichenbach *et al*., 2009). NtrC is known to act as an enhancer-like protein for many RpoN-regulated promoters (Syed & Gralla, 1997; Reichenbach *et al*., 2009) and its recognition site is also present upstream of the *rpoE*P2 TSS as shown in Fig. 9. Examination of DNA sequences upstream of the *rpoE*P2 promoter also identified three potential QseF-binding sites marked UAS1, UAS2 and UAS3 (Fig. 9). These results are consistent with repeated cloning of the *qseG* gene as multicopy inducer of the *rpoE*P-*lacZ* activity. The *qseG* gene is co-transcribed as an operon with *qseF* (*glrR*) and *qseE* (*glrK*) genes. As described below, QseE and QseF constitute a two-component system and QseF has features resembling AAA activators belonging to the NtrC family. In the RACE analysis the usage of the P2 promoter was maximal when total RNA was extracted from bacteria overexpressing the *qseG* gene from a plasmid. Potential regulation of transcription from the *rpoE*P2 promoter by RpoN in concert with activators like QseF and NtrC is also supported by results of insertional mutagenesis, where Tn*10* insertion was found in the *glnA-ntrB*(*glnL*)*-ntrC*(*glnG*) operon. Such a Tn*10* insertion mutant exhibited deceased activity of the *lacZ* fusion of promoter region covering the 529 bp region upstream of the promoter regulated by RpoE (the promoter regulated by RpoE that is now named as *rpoE*P6).

Upstream of the position -327 the consensus sequences of -10 and -35 elements of housekeeping promoters can also be found, thus the same TSS could define the P3 promoter. The key step during the initiation of transcription of RpoD-recognized promoters is recognition of the -10 promoter element by the RpoD sigma factor. Base-

specific interactions occur primarily with thymine located at position -7 and adenine at position -11, which are flipped out of the single-stranded DNA and buried deep in RpoD protein pockets (Feklistov & Darst, 2011). In the Fig. 9, the presence of conserved -7T and -11A nucleotide residues can be seen. Hence, the TSS at -327 may be shared by RpoN and RpoD sigma factors. Examination of DNA sequence upstream of -10 element consensus regions for binding of CRP, and upstream of -35 element, consensus regions for binding of RcsB were predicted (Fig. 9).

3.3. How to distinguish between *rpoE*P2 and *rpoE*P3 promoters?

Predicted RpoN-regulated P2 and RpoD-regulated P3 promoters share the same transcription start site. Hence, the transcription initiation from this TSS could be mediated by RNA polymerase containing either RpoN or RpoD, or subjected to a dual usage. In order to distinguish which of these two promoters respond to particular known signals that regulate the *rpoE* gene transcription, mutations in consensus boxes recognized by sigma factors were introduced. To investigate the *rpoE*P2 activity, the -10 element corresponding to the P3 promoter containing conserved thymine residues at position -7 and adenine at position -11 was mutated to cytosine and guanine, respectively. In order to observe signals and the transcriptional activity only from the P3 promoter, the RpoN-recognition sites within P2 promoter region were mutated. The -12 GC was mutated into AT and -24 GG into GA. Such mutated DNA fragments were cloned in the promoter probe vectors (pRS550 and pRS551) and transferred in a single-copy to the chromosome with use of bacteriophage λRS45 to construct *lacZ* promoter fusions. The strain showing mainly the activity of the P3 promoter was named SR18987 (with intact -10 and -35 elements for RpoD recognition) and the construct exhibiting the RpoN-dependent P2 promoter activity was named SR19089.

3.4. Characterization of the *rpoE*P2 promoter

DNA sequence examination described in paragraph 3.2, showed the presence of nitrogen-limitation sigma factor (RpoN) recognition sites at -12 (GC) and -24 (GG) in the region located upstream of the P2 promoter. Since RNA polymerase with RpoN cannot initiate transcription on its own, potential NtrC or QseF binding boxes were also found within the sequence located upstream of TSS corresponding to the P2 promoter.

Thus, to demonstrate the RpoN-dependent transcriptional initiation from the *rpoE*P2 promoter *in vitro*, RpoN and its activators were cloned in expression vectors. All such clones carry a Hexa-His tag appended in-frame to the C-terminal end of the cognate gene. After the verification of such cloned genes, the expression was induced and proteins purified as described in 4.2.16. The results of SDS-PAGE are showed in Fig. 11 and Fig. 12 below.

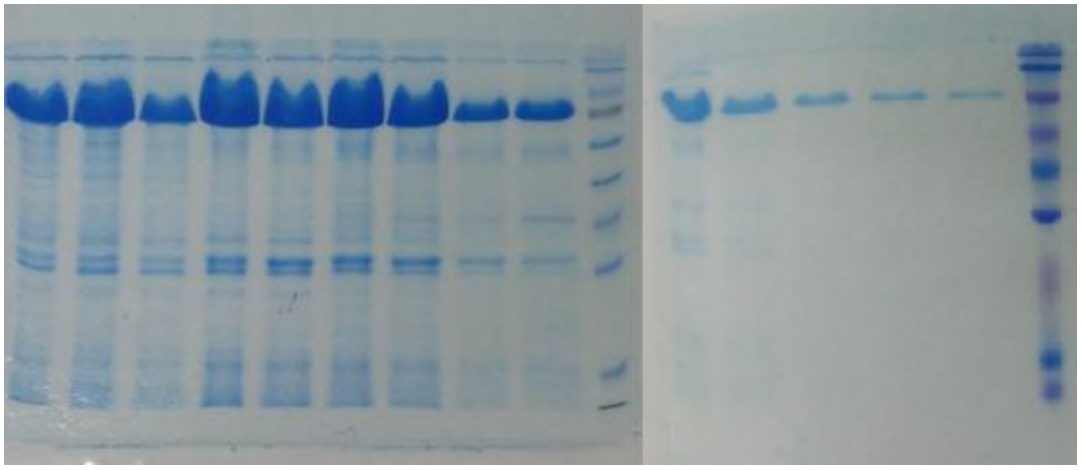

Fig. 11. RpoN purification. Purification was performed as described in paragraph 4.2.16. Protein was purified from 800 ml culture induced with 0.5 mM IPTG. Samples after elution of RpoN with indicated concentrations of imidazole, were resolved on 12.5% SDS-PAGE. Lines 1-3 - fraction eluted with 50 mM imidazole; lines 4 & 5 - elution with 100 mM imidazole; lines 6-8 - elution with 250 mM imidazole; line 9 - elution with 500 mM imidazole; line 10 - Prestained Protein Ladder from Fermentas (migration pattern with apparent molecular weights 15, 25, 35, 40, 55, 70, 100, 130, 170 kDa). Fractions with further elution with 500 mM imidazole are shown in lines 11-15; line 16 - HyperPAGE Prestained Protein Marker from Bioline (migration pattern with apparent molecular weights of 10, 15, 20, 25, 40, 50, 80, 125, 190 kDa).

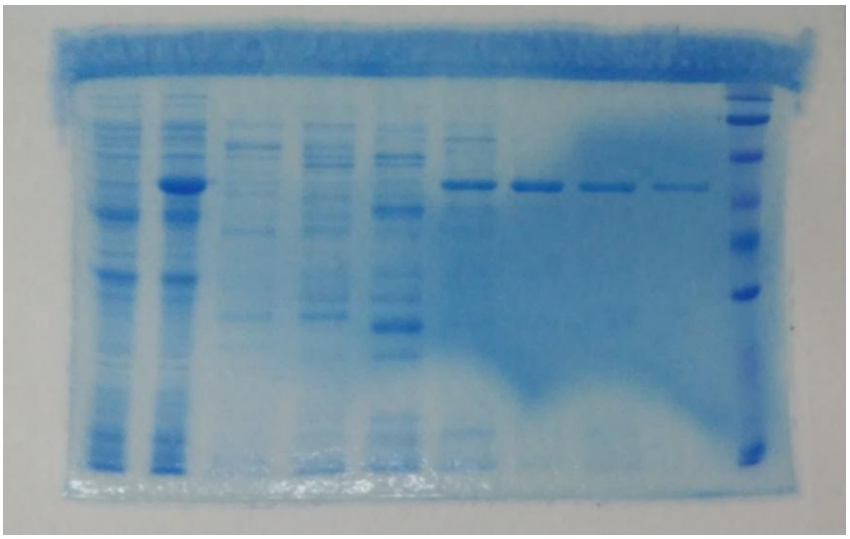

Fig. 12. NtrC purification. Purification was performed as described in paragraph 4.2.16. Protein was purified from 300 ml culture induced with 0.5 mM IPTG. Samples after elution of NtrC with indicated concentrations of imidazole were resolved on 12.5% SDS-PAGE. Line 1 - whole cell lysate without induction; line 2 - whole cell lysate after induction; line 3 - fraction eluted with 50 mM imidazole; line 4 -fraction eluted with 100 mM imidazole; line 5 - elution with 250 mM imidazole; line 6-9 - elution with 500 mM imidazole; line 10 - HyperPAGE Prestained Protein Marker from Bioline (migration pattern with apparent molecular weights 10, 15, 20, 25, 40, 50, 80, 125, 190 kDa).

Further, *in vitro* run-off assays, with reconstituted RNA polymerase containing RpoN and either NtrC or QseF as activators ,were performed in the presence of 5 mM ATP. During these experiments, the wild-type DNA template that covered the *rpoE*P2 promoter region and 85 bp downstream of TSS at -327 was used. From this template, the expected size of the transcript was observed when RpoN was supplemented with either NtrC or QseF (lanes number 2 and 4 in the Fig. 13). Additionally, the second DNA fragment was in parallel run-off experiments used as a template in order to validate recognition of this promoter by RpoN. This template DNA fragment covers the same region as was used with the first template but contained mutations at -12 and at -24 sites recognized by RpoN (GC to AT and GG to GA, respectively). As can be seen in Fig. 13, the initiation of transcription was significantly decreased after introduction of such mutations, especially with reaction carried in the presence of QseF as an activator (line number 5).

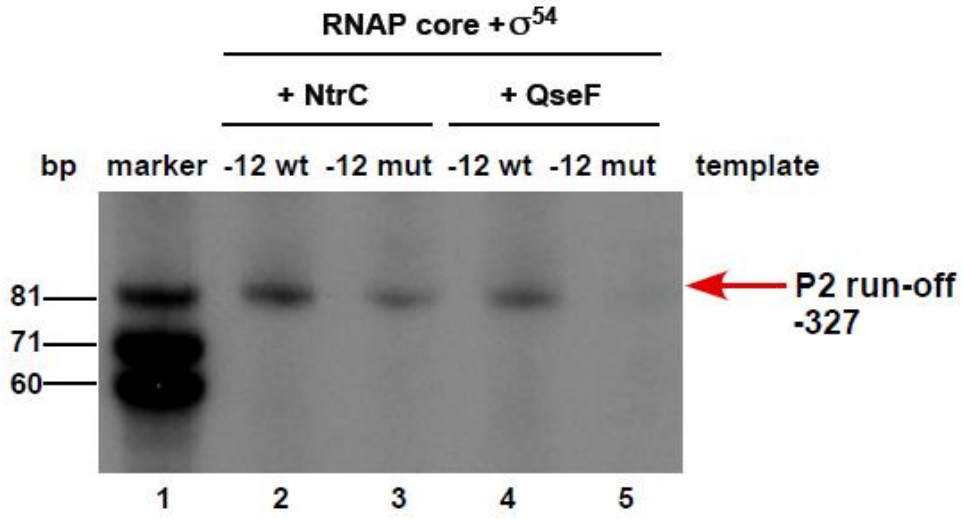

Fig. 13. *In vitro* transcription run-off assay showing recruitment of RNA polymerase complexed with RpoN for the P2 promoter of the *rpoE* gene. Lane 1 corresponds to the size standard. Lanes 2 and 4 corresponds to reactions with the wild-type DNA template in the presence of either NtrC or QseF. Lanes 3 and 5 correspond to the RNA transcript produced with DNA template mutated at -12 into AT and at -24 to GA. As it can be seen in the presence of either NtrC or QseF, RpoN complexed with the core RNA polymerase (RNAP) can initiate transcription from the *rpoE*P2 promoter using the 327 TSS.

Above results showed that the *rpoE*P2 promoter is indeed recognized by RNA polymerase containing RpoN and that QseF acts as the main activator/enhancer. Therefore, *in vivo* experiments were also performed. QseF is a part of two-component system (TCS) along with the sensor kinase QseE, which positively regulates transcription of the *glmY* sRNA (Reichenbach *et al*., 2009).

The *qseEF* genes are organized in an unusual manner for genes encoding components of TCS operon, because there is one more gene located between them. It is the *qseG* gene encoding an outer membrane protein, which in enterohemeorrhagic *E. coli* (EHEC) is necessary for translocation of type III secretion system effectors into epithelial cells and along with QseE and QseF is required for pedestal formation and they are involved in responding to phosphate and sulfate sources (Reading *et al*., 2009; Reading *et al*., 2010).

As mentioned in the section describing genes whose products, when overexpressed caused a significant induction of the *rpoE*P-*lacZ* fusion, the most prominent gene identified was the *qseG* gene. Since the *rpoE*P-*lacZ* fusion contained all five newly

identified promoters, the plasmid containing the cloned *qseG* gene was introduced systematically into different strains carrying individual single copy *rpoE-lacZ* promoter fusion. Consistent with *in vitro* results of run-off assays, overexpression of the *qseG* gene was found to induce the *rpoE*P2 promoter activity. This induction did not occur in the strain carrying the single-copy promoter fusion with the *lacZ* gene with mutations at -12 and -24 recognition sites (marked as *rpoE*P2*-*lacZ*), as well as when the *qseF* gene was deleted (Fig. 14). These results demonstrate that induction of this RpoN-regulated promoter by the *qseG* overexpression *in vivo* requires QseF response regulator as an activator. QseG in this process could cause activation of the QseE/F two-component system.

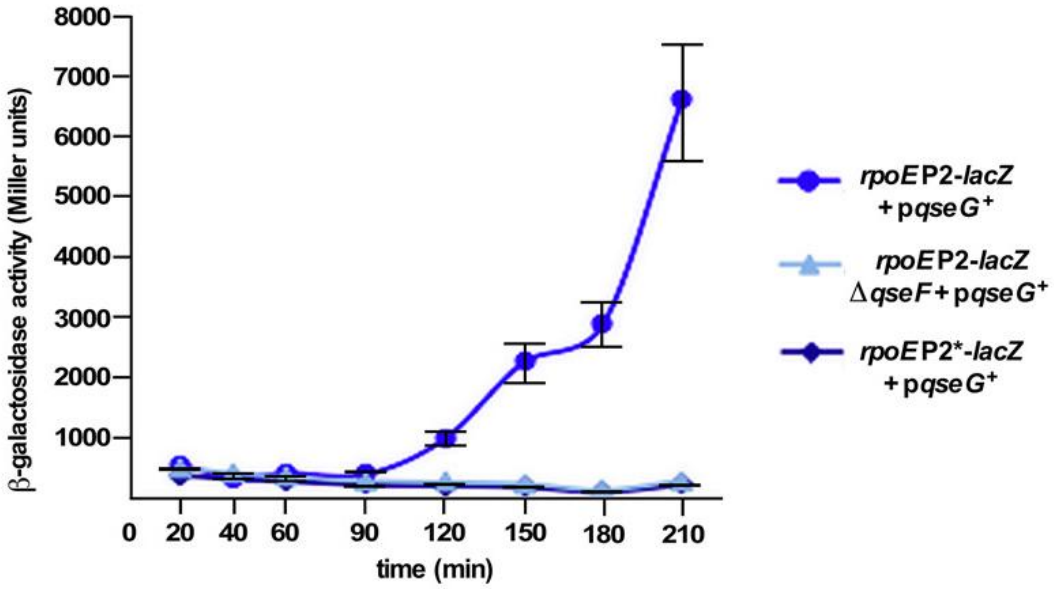

Fig. 14. *β*-Galactosidase assay showing the induction of the *rpoE*P2-*lacZ* promoter fusion upon overexpression of the *qseG* gene from the plasmid. Four independent cultures carrying the single copy chromosomal wild-type *rpoE*P2-*lacZ* fusion with or without the deletion of *qseF* gene, and four independent cultures with mutated (at -12 and -24 RpoN-recognition sites) *rpoE*P2*-*lacZ* fusion, all expressing the cloned *qseG* gene were analyzed for the *β*-galactosidase activity after different growth intervals in the presence of 50 μM IPTG.

3.5. Characterization of the *rpoE*P3 promoter

During examination of the sequence of promoter region of the *rpoE* gene, the consensus sequences of -10 and -35 elements of housekeeping promoters was found located upstream of the position -327. In order to validate if transcription form this TSS can be initiated by RpoD, *in vitro* transcription run-off assays were performed with the help of Dr. Klein. In this experiment, RNA transcripts were synthesized from two DNA template of 170 bp. One of these DNA fragments was mutated at the -10 element containing conserved thymine residues at position -7 and adenine at position -11, which were substituted by cytosine and guanine residues, respectively. An expected size of 85-nt RNA product was observed when RNA polymerase core with RpoD was incubated with the wild-type DNA template (lane 2 in the Fig. 15), whereas only a weak signal was visible when the mutated template was used in such assays (lane 3). Thus, it is concluded that RpoD complexed with the RNAP core can initiate transcription from the *rpoE*P3 promoter without any requirement of additional factor *in vitro*.

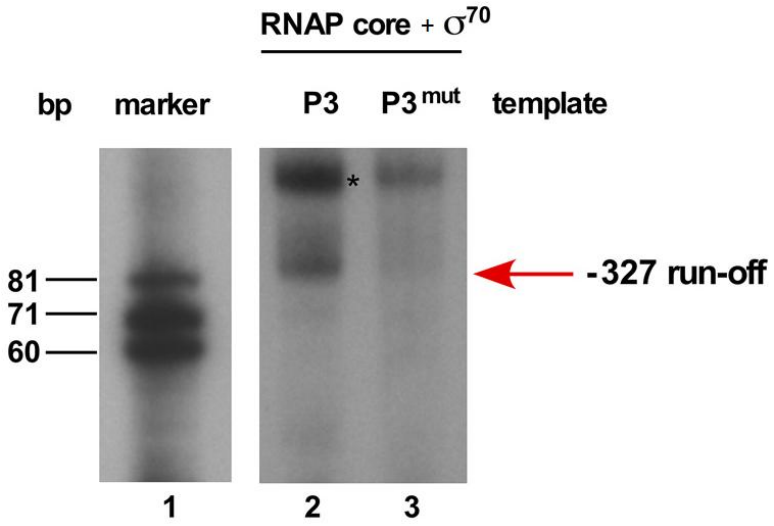

Fig. 15. *In vitro* transcription run-off assay showing recruitment of RNA polymerase complexed with RpoD (σ^{70}) for the P3 promoter of the *rpoE* gene. Lane 1 corresponds to the size standard. Lane 2 corresponds to the RNA transcript produced from the wild-type DNA template. Lane 3 corresponds to reaction with the DNA template mutated at -7 into and at -11. Band marked with an asterisk (*) symbol indicate nonspecific end-to-end transcription reaction products. As it can be seen, RpoD complexed with the RNAP core can initiate transcription from TSS at -327.

During the screen for deletion derivatives of non-essential genes, Δ*crp* mutants where found to have reduced *rpoE*P-*lacZ* activity (section 3.1.1). Furthermore, during the screen of Lac down Tn*10* mutants, a *cya*::Tn*10* insertion was identified. Thus, these results were further followed by transduction of Δ*crp* mutation into strains with different *rpoE-lacZ* fusions. Quantification of the *β*-galactosidase activity showed that CRP positively regulates the *rpoE*P3 promoter activity. As mentioned earlier in paragraph 3.2, upstream of the -327 TSS, consensus regions for binding to cAMP receptor protein (CRP) can be found. It is known that expression of promoters, which require CRP binding, is significantly reduced upon the addition of glucose to medium, because of low levels of cAMP (Browning & Busby, 2004). Therefore firstly, the activity of promoter fusions covering this region was checked on LA medium plates with addition of X-Gal, which enables approximation of expression level of *β*-galactosidase enzyme encoded by the *lacZ* gene. As can be seen in the Fig. 16, the *rpoE*P3-*lacZ* promoter activity was severely repressed when medium was supplemented with 0.5% glucose. It suggests CRP-mediated positive regulation of the *rpoE*P3 promoter.

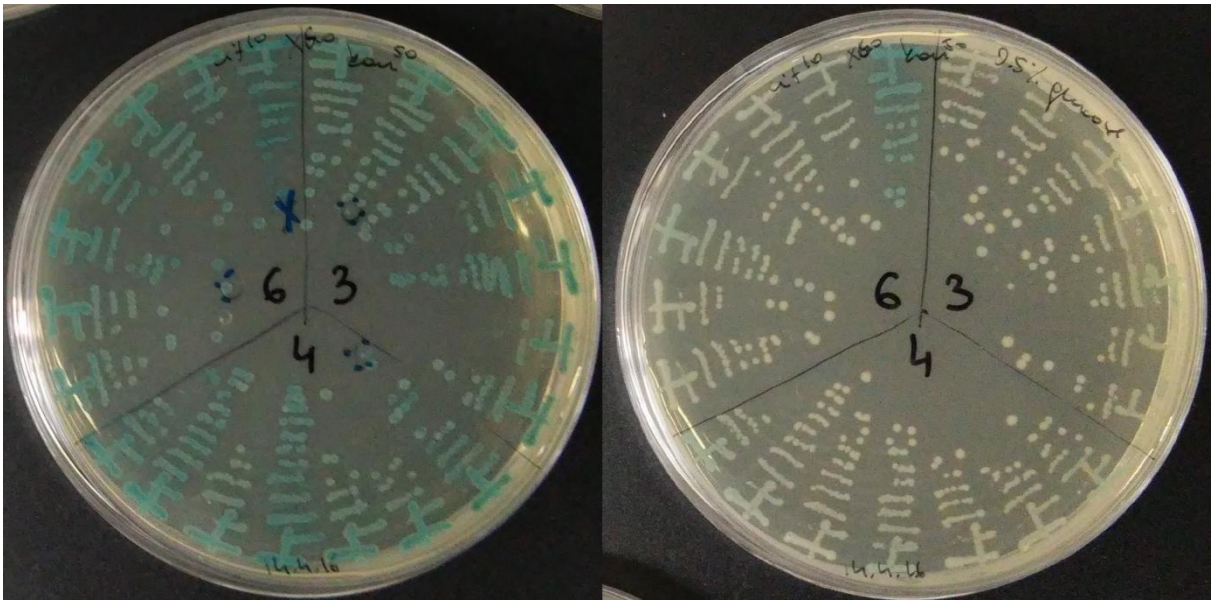

Fig. 16. Catabolite repression of the *rpoE*P3 promoter. Multiple colonies carrying *rpoE*P3-*lacZ* fusions were streaked simultaneously on LA medium with (plate on the right) or without addition of 0.5% glucose (plate on the left) containing also 10 mM sodium citrate, proper antibiotic and X-Gal, which in the presence of *β*-galactosidase encoded by the *lacZ* gene gives blue-colored product. The addition of 0.5% glucose significantly reduced the P3 promoter activity.

The regulation by CRP was further examined by qunatitaive measurement of the *β*-galactosidase activity with strains carrying nonmutated promoter region corresponding to -327 TSS fused to the *lacZ* gene and the *rpoE*P2-*lacZ* or *rpoE*P3-*lacZ* fusions in medium supplemented with either glucose or glycerol as the carbon source. Furthermore, *β*-galactosidase assays were carried out using isogenic wild-type and its derivative with deleted *crp* gene carrying proper promoter fusions. The decreased *β*-galactosidase activity was observed in the strain carrying the single copy chromosomal fusion of the *rpoE*P3-*lacZ* when the *crp* gene was deleted (Fig. 17).

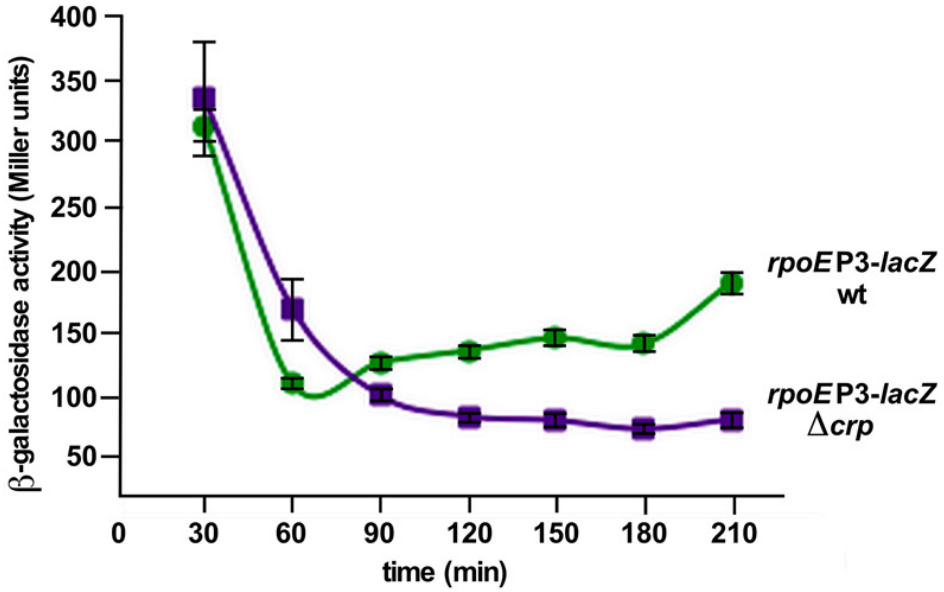

Fig. 17. *β*-Galactosidase assay showing the reduction of the *rpoE*P3-*lacZ* promoter fusion activity upon the *crp* gene deletion. The curved showed average from four independent wild-type and cultures with deletion of the *crp* gene carrying the single copy chromosomal fusion of the *rpoE*P3-*lacZ*. Cultures were analyzed for the *β*-galactosidase activity after different growth intervals in LB medium at 30°C.

In order to further examine the role of CRP in regulation of the *rpoE*P3 promoter activity, the minimal coding region of the *crp* gene was cloned in two expression vectors, pCA24N and pET22b. The cloned *crp* gene was tested for *in vivo* complementation of a *bona fide* Δ*crp* mutation. Verified plasmid constructs were then tested for induction of CRP protein. The high induction of CRP was obtained in the strain carrying pET22b plasmid with cloned the *crp* gene with a C-terminal hexa-His-tagged (Fig. 18). Such plasmid was transformed into BL21 strain derivative (strain with T7 polymerase cloned in the *lacZ* gene). This strain was further used for induction of

expression of the *crp* gene with 0.5 mM IPTG at 30°C. Pellet obtained from such liquid culture was used for CRP purification as described in 4.2.164.2.16 and samples were resolved on 12.5% SDS-PAGE (described in 4.2.17). Results of this experiment are shown below (Fig. 19).

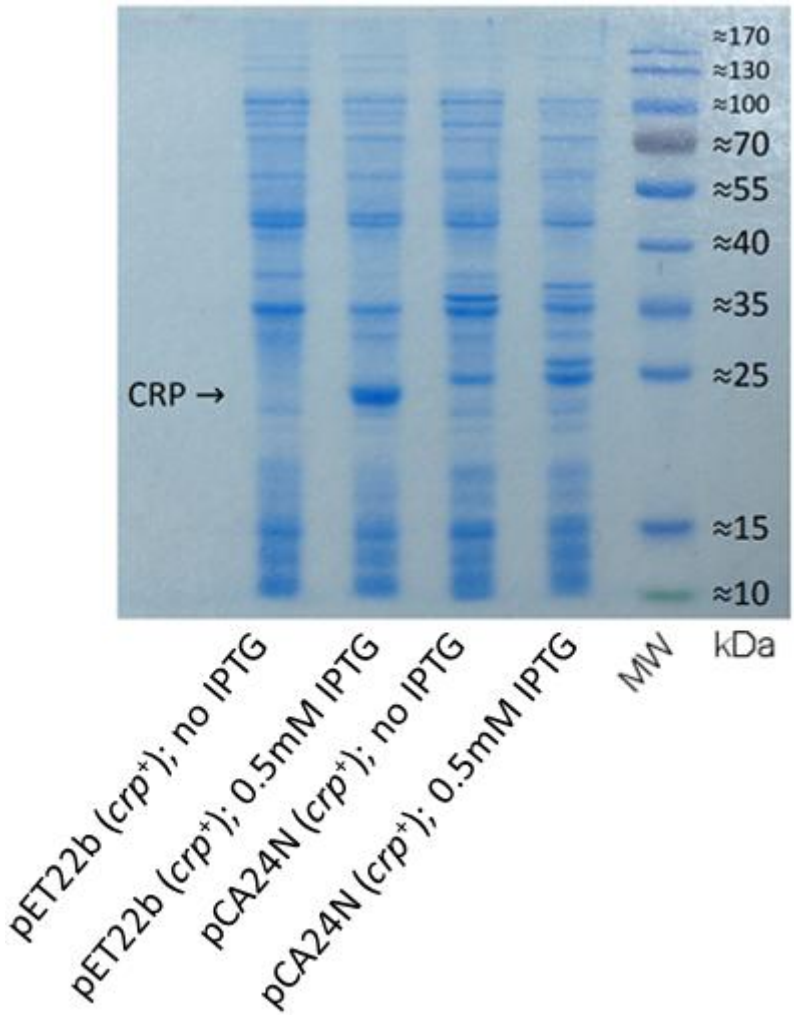

Fig. 18. CRP induction. An equivalent amounts of proteins were resolved on 12.5% SDS-PAGE. Line 1 - SR8576 (*E. coli* B strain BL21 derivative) + crp^+ in pET22b without induction; line 2 - induction of CRP in SR8576 + crp^+ in pET22b; line 3 - SR8294 + crp^+ in pCA24N without induction; line 4 - induction of CRP in SR8294 (*E. coli* K-12 wild-type) + crp^+ in pCA24N; line 5 - Prestained Protein Ladder from Fermentas (migration pattern with apparent molecular weights 15, 25, 35, 40, 55, 70, 100, 130, 170 kDa).

As it can be seen in Fig. 19, CRP was eluted with the lower concentration of imidazole. The fractions eluted with use of 500 mM imidazole solution contained only trace amounts of this protein.

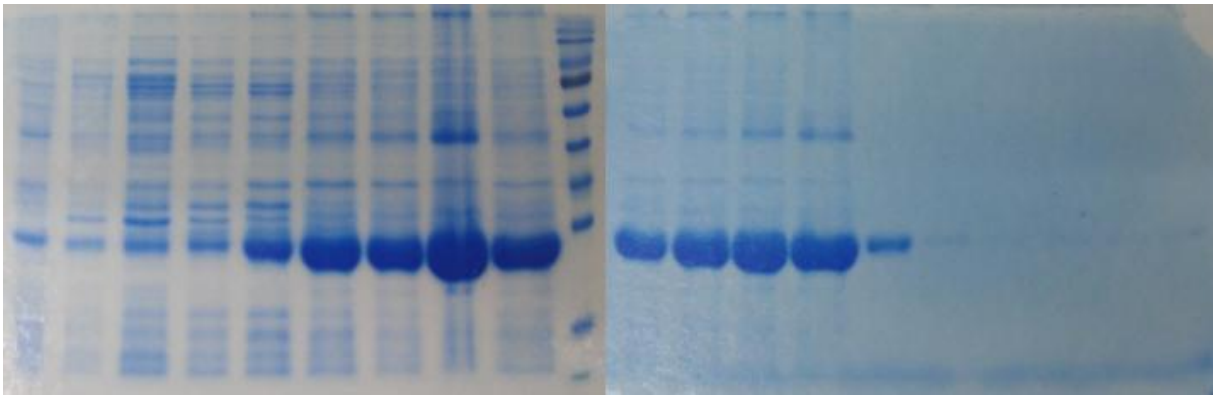

Fig. 19. CRP purification. Purification was performed as described in paragraph 4.2.16. Samples after the induction of CRP and after elution with indicated concentrations of imidazole, were resolved on 12.5% SDS-PAGE. Line 1 - induction of CRP in SR8576 + *crp*$^{+}$ in pET22b; line 2 - fraction after washing of the column; lines 3-4 - fractions eluted with 50 mM imidazole; lines 5-7 - fractions eluted with 100 mM imidazole; lines 8-9 - elution with 250 mM imidazole; line 10 - Prestained Protein Ladder from Fermentas (migration pattern with apparent molecular weights 15, 25, 35, 40, 55, 70, 100, 130, 170 kDa); line 11 - fraction eluted with 100 mM imidazole; lines 12-17 - elution with 250 mM imidazole; lines 18-20 - elution with 500 mM imidazole

Additional extended staining of the gel (Fig. 19) revealed the presence of different proteins in the samples, therefore additional step of chromatography followed by dialysis was necessary before using such samples in *in vitro* run-off assays.

Next, the direct binding of the CRP protein was examined by electrophoretic mobility shift assay (EMSA). For this experiment DNA fragment, covering 118-bp upstream of the *rpoE*P3 TSS was used. Such DNA was efficiently shifted in the presence of activated CRP (Fig. 20), which is consistent with the presence of CRP consensus box (Fig. 9). Overall, these results established that CRP positively regulates transcription of the *rpoE*P3 promoter and a CRP binding site is located upstream of the *rpoE*P3 promoter.

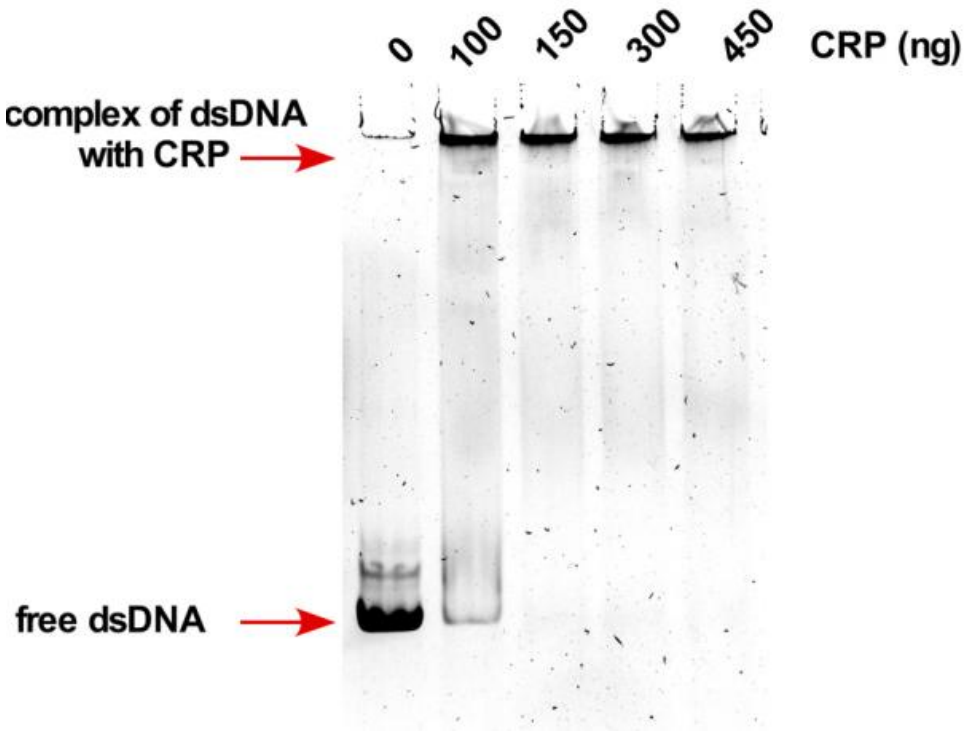

Fig. 20. The electrophoretic mobility shift assay showing binding of CRP to the DNA fragment covering the *rpoE*P3 promoter region. 118-bp DNA fragment was incubated with increasing concentrations of CRP activated by cAMP (lanes 2–5). Lane 1 is a negative control with DNA alone. Samples were resolved on a 6% native gel. Arrows show the position of CRP-DNA complex and free dsDNA.

3.6. Characterization of the *rpoE*P4 promoter

During *β*-galactosidase assays with strains carrying a single-copy chromosomal promoter fusion with the *lacZ* gene and the 529-bp region upstream of promoter regulated by RpoE, we observed increased expression upon entry to the stationary phase. It suggested that stationary phase sigma factor, RpoS, may be involved in the initiation of transcription within this region. Consistent with these results, the Δ*rpoS* mutation was identified in the initial screen that caused decrease in the *rpoE*P-*lacZ* activity. Similarly, two Tn*10* insertion mutations were also identified in the *rssB* gene that increased the *rpoE*P activity and more specifically of the *rpoE*P4 promoter. RssB acts as an adapter protein for the ClpXP-mediated degradation of RpoS and hence *rssB* mutants exhibit elevated RpoS levels. Similarly, during multicopy approach to identify various regulators, the *fliZ* gene was cloned. FliZ behaves as a repressor of RpoS by acting as its antagonist due to overlapping DNA binding specificity (Pesavento & Hengge, 2012). Therefore, its role is consistent with the obtained result that the *fliZ* overexpression decreased transcription of the *rpoE* gene, which was specific to the *rpoE*P4 promoter.

As mentioned above, RpoS is a substrate for the RssB-dependent proteolysis, which is enhanced in the absence of poly(A) polymerase I encoded by the *pcnB* gene (Santos *et al*., 2006). Consistent with other *in vivo* results, the *pcnB* gene was identified using multicopy approach. Thus, when the *pcnB* gene was overexpressed an increase in the transcriptional activity of the *rpoE*P-*lacZ* activity was observed.Thus, these results were further quantified, specifically to address regulation of the *rpoE*P4 promoter.

First of all a Δ*rpoS* mutation was introduced in strains carrying individual promoter fusions. Approximately 60% reduction, as compared to the wild-type, was observed for the strain with the *rpoE*P4-*lacZ* fusion (Fig. 21). The overexpression of the *pcnB* gene caused an approximately 40% increase in the *rpoE*P4 activity, since poly(A) polymerase I stabilizes RpoS (Fig. 21). Moreover, deletion of the *rssB* gene caused more than 35% increase in the *rpoE*P4 promoter activity, supporting results of insertional mutagenesis. Such results are overall consistent with regulation of the *rpoE*P4 promoter by the RpoS sigma factor.

It is known that RpoS activity is also influenced by changes in the osmolarity. A high osmolarity increases the RprA-dependent RpoS translation (Majdalani *et al.*, 2001;

Madhugiri *et al.*, 2010). For this reason, the activity of the *rpoE*P4-*lacZ* fusion was investigated after the addition of 0.5 M NaCl or 0.25 M sucrose. *β*-galactosidase assay revealed that these conditions caused about a 2- to 3-fold induction in the *rpoE*P4 promoter activity (Klein *et al*., 2016). Such altered activity of the *rpoE*P4 promoter is in accordance with its positive regulation by RpoS. Thus, these results allow us to conclude that *in vivo* the P4 promoter behaves in a typical RpoS-dependent manner. Hence, direct recognition of the *rpoE*P4 promoter by RpoS was examined in the *in vitro* run-off assay (Fig. 23).

For run off assays, purifed RpoS (Fig. 22) RNA polymerase core and DNA template of 105 bp carrying -218 TSS were used. The results showed that only RNA polymerase core supplemented with RpoS was able to initiate transcription from the *rpoE*P4 promoter. The addition of housekeeping sigma factor to the core RNAP with or without phosphorylated RcsB did not resulted in the synthesis of expected 45-nt product. These results show that the *rpoE*P4 promoter is recognized and transcribed by the RpoS sigma factor acting as a positive regulator and this promoter responds to the modulators that regulate RpoS activity/amounts.

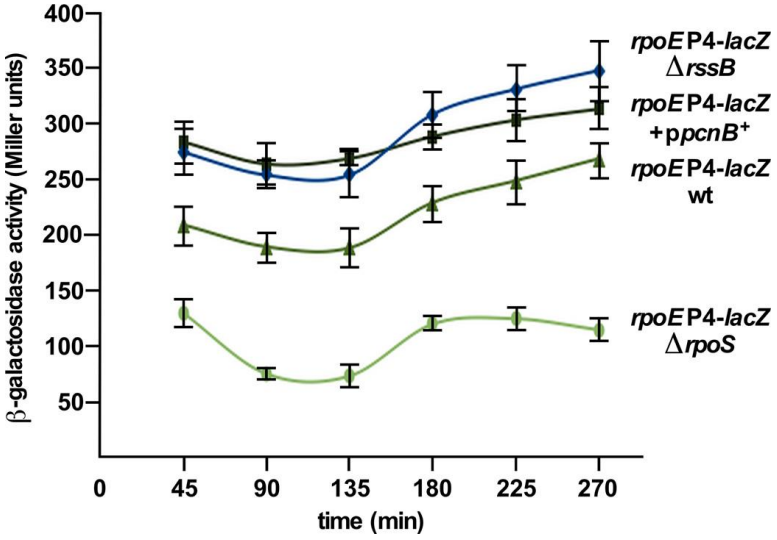

Fig. 21. *β*-Galactosidase assay showing the reduction of the *rpoE*P4-*lacZ* promoter fusion activity upon the *rpoS* gene deletion. The induction of the *rpoE*P4-*lacZ* promoter fusion occurs upon deletion of the *rssB* gene and when the *pcnB* gene is overexpressed from a plasmid in the presence of 50 μM IPTG. These results are consistent with a positive regulation of the *rpoE*P4-*lacZ* promoter by RpoS. Cultures were grown in LB medium at 30°C. Samples from four independent cultures were analyzed for the *β*-galactosidase activity at different growth intervals.

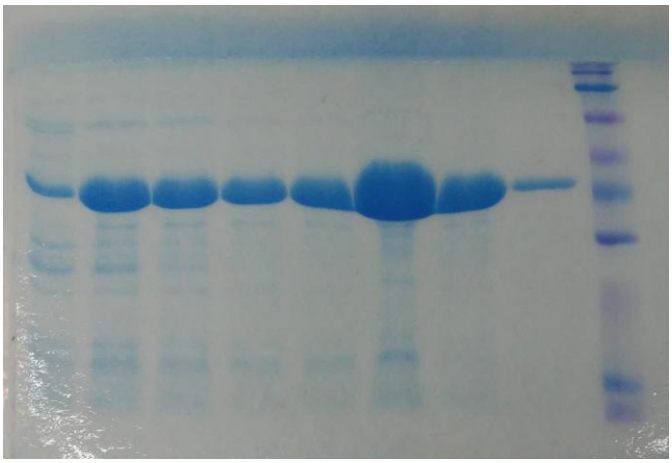

Fig. 22. RpoS purification. Strain carrying pET24b plasmid with cloned *rpoS* gene was grown in LB medium at 30°C and induced by addition of 0.5 mM IPTG. Purification was performed as described in paragraph 4.2.16. Samples were resolved on 12.5% SDS-PAGE. Line 1- fraction eluted with 100 mM imidazole; lines 2-4 - elution with 250 mM imidazole; lines 5-8 - fractions eluted with 500 mM imidazole; line 9 - Prestained Protein Ladder from Bioline (migration pattern with apparent molecular weights 10, 15, 20, 25, 40, 50, 80, 125, 190 kDa).

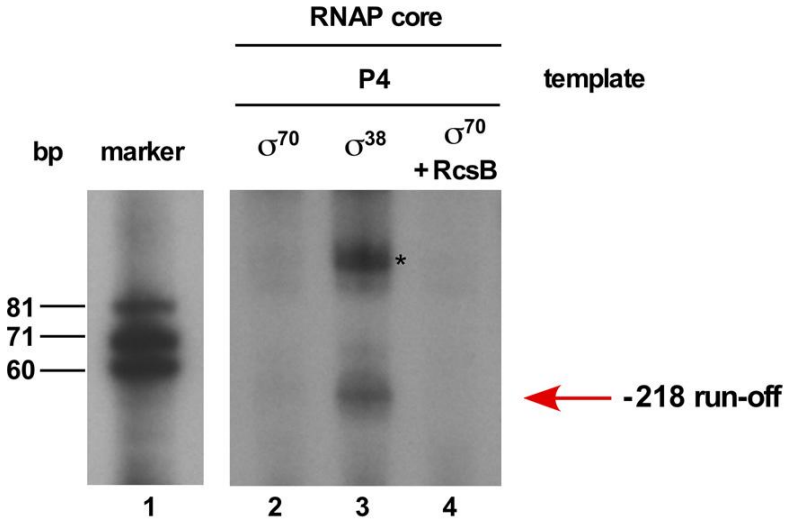

Fig. 23. *In vitro* transcription run-off assay showing recruitment of RNA polymerase complexed with RpoS (σ^{38}) for the P4 promoter of the *rpoE* gene. DNA template of 105 bp was used. Lane 1 corresponds to the size standard. Lane 2 shows the incubation reaction with RpoD (σ^{70}), lane 3 the incubation with RpoS (σ^{38}) resulting in the synthesis of expected 45-nt product marked with the arrow. Lane 4 was incubated with RpoD (σ^{70}) in the presence of phosphorylated RcsB. Band marked with an asterisk (*) symbol indicates nonspecific end-to-end transcription reaction products.

3.7. Sensing of LPS defects

Initial mutational hunt with Tn*10* transposon mutagenesis, performed with strains carrying a single-copy chromosomal fusion with the *lacZ* gene and the 529-bp region upstream of the promoter regulated by RpoE, showed that majority of mutations that conferred Lac activity of mapped to LPS biosynthetic genes. To investigate which of newly discovered promoters responds to LPS defects, deletions of LPS core biosynthetic genes were systematically introduced to strains carrying different promoter fusions. Additionally, the impact of the addition of polymyxin B (PMB) on *rpoE*P-*lacZ* activity was investigated. Furthermore, parallel studies revealed that the addition of ammonium metavanadate, chemical compound causing LPS modifications (Zhou *et al*., 1999), induces the *rpoE* transcription, and at the same time activates the BasS/R two-component system. The BasS/R two-component system may also positively regulates the *rpoE* promoter activity. In order to obtain more specific response polymyxin B was used as other chemical compound that may cause LPS changes. It has been shown that sublethal concentrations of polymyxin B do not induce the BasS/R two-component system (Froelich *et al*., 2006).

Polymyxin B is a cationic antimicrobial peptide produced by *Bacillus polymyxa* (Raetz *et al*., 2007). Polymyxin B resistant mutants have LPS substituted with 4-amino-4-deoxy-L-arabinose (L-Ara4N) and phosphoethanolamine (P-EtN) (Trent *et al*., 2001). L-Ara4N substitutions cause partial neutralization of a negative charge of lipid A, which decreases electrostatic interaction with polymyxin B and provide resistance (Raetz *et al*., 2007).

Lipid A modification by L-Ara4N addition to 4′-phosphate of Kdo_2-lipid IV_A is catalyzed by ArnT, product of the *arnT* gene, which is a part of normally latent *arnB-arnC-arnA-arnD-arnT-arnE-arnF* operon. Transcription of this operon is under a positive control of the BasS/R two-component system. It is also known that mutants lacking the *basR* gene are hypersensitive to PMB (Froelich *et al*., 2006), showing significance of BasS/R in lipid A modification by L-Ara4N. Previous studies carried out in our laboratory revealed overlapping control of LPS modifications by σ^E and BasS/R two-component system (Klein *et al*., 2009; Klein *et al*., 2011). Thus, various experiments were carried out in order to verify whether σ^E response is activated upon exposure to PMB.

β-galactosidase assay with wild-type strains carrying various single-copy chromosomal promoter fusions of the *rpoE* gene to the *lacZ* gene was performed. Polymyxin B (Sigma) was added to LB medium at concentrations ranging from 0.2 to 0.3 μg/ml. The effect of the polymyxin B addition on the *rpoE* transcription induction was observed when bacteria cultures were treated at A_{600} of 0.05, but not at/or above of 0.4 (Klein *et al*., 2016). These experiments showed that the PMB addition increases the *rpoE*P3 promoter activity. Those results indicate that binding of polymyxin B to lipid A (Morrison & Jacobs, 1976) activates σ^E response by increasing of transcription by the recognition of its *rpoE*P3 promoter. Taken together, these results suggest that alterations in the structure of LPS in *E. coli* are sensed by the *rpoE*P3 promoter.

Next, the *waaC* deletion was introduced into strains SR18987 (to measure *rpoE*P3 promoter activity, described in paragraph 3.3), SR19089 (to measure P2 promoter activity, described in paragraph 3.3) and SR18874 carrying the *rpoE*P4-*lacZ* fusion. The *waaC* gene encodes heptosyltransferase I, which mediates the addition of the first Hep residue in the LPS core. The highest response was observed upon deletion of the *waaC* gene in the strain carrying the *rpoE*P3-*lacZ* fusion. Approximately a 7-fold induction of the *rpoE*P3 activity was observed as compared to the wild-type strain carrying the same promoter fusion (Fig. 24). The activity of the *rpoE*P2-*lacZ* fusion was also effected by deletion of this LPS core biosynthetic gene. However, in case of the *rpoE*P2 promoter the induction of the activity in Δ*waaC* mutant was only from 70% to a 2-fold upon entry into the stationary phase (Fig. 25). No differences in the activity of the RpoS-regulated P4 promoter were observed when the *waaC* gene was deleted in the strain carrying the *rpoE*P4-*lacZ* fusion.

Further, the impact of deletion of genes whose product mediate further steps in the LPS core biosynthesis were investigated in the strain carrying the *rpoE*P3 promoter fusion (SR18987). During experiment, cultures of strain carrying the single copy chromosomal *rpoE*P3-*lacZ* promoter fusion and its derivatives with non-polar deletion of various LPS core biosynthetic genes, were grown in LB medium at 30°C and analyzed for the *β*-galactosidase activity after different time intervals. Four independent derivatives in each case were analyzed and averaged data after a 250-min incubation are presented in Fig. 24. As mentioned above, the highest induction of the *rpoE*P3 occurred upon deletion of the *waaC* gene. Mutants with deletion of the *waaF* gene, encoding product that mediate addition of Hep I in LPS biosynthesis exhibited about 2-3 fold higher

activity of the *rpoE*P3 than the wild-type strain. Also strains with deletion of either *waaG*, or *waaP*, or *waaO* genes showed more than 2-fold increase in the P3 promoter activity (Fig. 24). Δ*waaQ*, Δ*waaS*, Δ*waaY*, and Δ*waaZ* mutants did not exhibit any major changes in the *rpoE*P3-*lacZ* promoter fusion activity as compared to the wild-type strain.

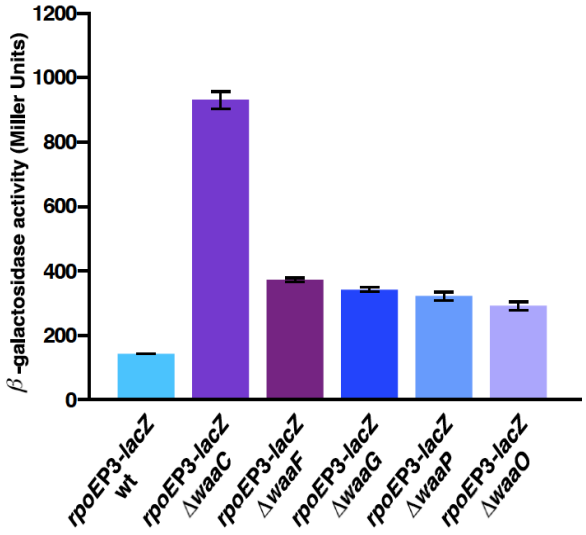

Fig. 24. Deletion of LPS core biosynthetic genes caused increased transcription of the *rpoE*P3-*lacZ* fusion. The highest induction of this promoter occurred upon deletion of the *waaC* gene which encodes heptosyltransferase that mediates the addition of the first Hep residue in the LPS core. Mutants with deletion of genes encoding products that mediate further steps of LPS biosynthesis have activity of the *rpoE*P3 promoter about 2-fold higher than the wild-type strain.

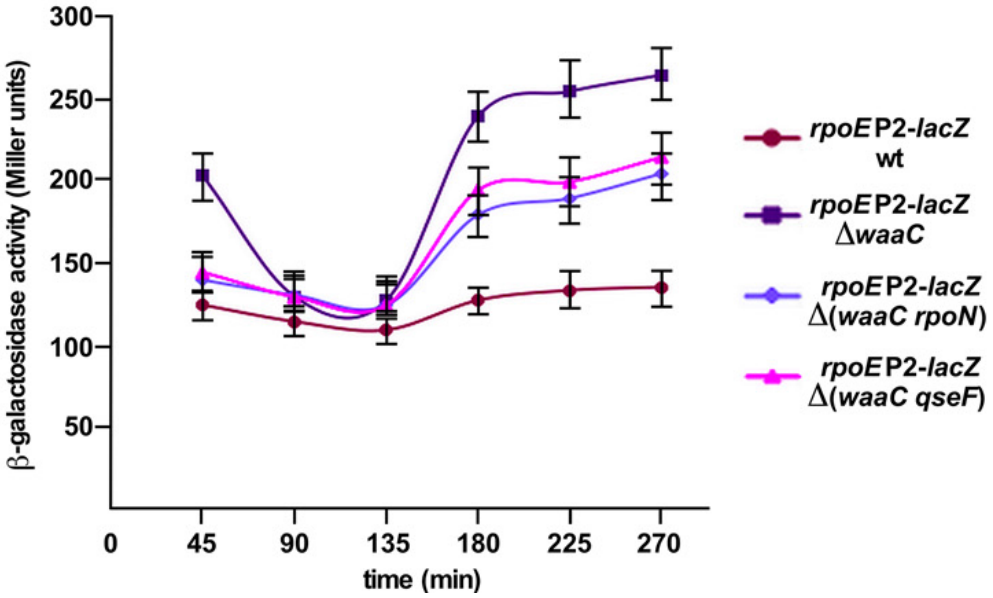

Fig. 25. *β*-Galactosidase assay showing the induction of the *rpoE*P2-*lacZ* promoter fusion activity upon the *waaC* gene deletion. The *rpoE*P2 promoter induction upon the *waaC* gene deletion was decreased by the *rpoN* or *qseF* deletion, showing a partial role of RpoN in sensing such LPS defect. The *β*-galactosidase activity was analyzed after different growth intervals from four independent cultures of each derivative.

In order to check the authenticity of examined *waa* mutants, the mass spectrometric analysis was performed with the help of Dr. Klein. For these experiments, LPS was obtained from isogenic strains grown under conditions that induce lipid A and inner core modifications (Klein *et al*., 2009; Klein *et al*., 2011; Klein *et al*., 2013). As it was described in paragraph 2.1.4, upon induction of RpoE, constitutive transcriptional induction of the *eptB* gene, encoding phosphoethanolamine transferase occurs, which adds P-EtN to the second Kdo can be observed (Klein *el al*., 2011). Therefore, in mutants such as Δ*waaC*, Δ*waaF*, Δ*waaG*, Δ*waaP*, which exhibit elevated transcription of the *rpoE* gene we should be able to observe P-EtN residue addition to LPS.

Mass spectrometric analysis of LPS (Fig. 26) revealed that LPS of the Δ*waaC* mutant mostly consists of lipid A with two Kdo residues (the mass peak at 2,237.3 Da). Such structure of LPS is the minimal LPS required for the *E. coli* viability under optimal growth conditions. Kdo_2-lipid A can be substituted by P-EtN (the mass peak at 2,360.3 Da) and further by L-Ara4N (the mass peak at 2,491.4 Da).

LPS upon deletion of the *waaF* gene, encoding heptosyltransferase mediating addition of the HepII of the inner core, contained mostly Kdo_2-lipid A+HepI (the mass peak at 2,429.4 Da). Similarly to LPS modifications observed in Δ*waaC* mutant, the incorporation of L-Ara4N (the mass peak at 2,560.5 Da) and additional P-EtN residue (the mass peak at 2,683.5 Da) can be observed.

During LPS biosynthesis, the step following WaaF incorporation of HepII is the phosphorylation of the first heptose by WaaP that is needed for the incorporation of the third heptose.

The mass spectra of LPS of Δ*waaP* mutant revealed the presence of mass peak at 2,783.5 Da corresponding to LPS consist of Kdo_2-lipid A with two heptoses and one hexose residue, which can be further substituted with P-EtN residue (the mass peak at 2,905.5 Da) or L-Ara4N (the mass peak at 2,914.6 Da) and additional P-EtN residue (the mass peak at 3,037.6 Da) and even with additional hexose residues (the mass peaks at 3,199.6 and 3,361.7 Da). No mass peaks corresponding to phosphorylated Hep residues were observed, which is consistent with the lack of WaaP kinase. This mass spectrometric analysis revealed also the presence of pentaacylated form of LPS corresponding to mass peak at 2,573.3 Da and 2,693.3 Da with further incorporation of P-EtN residue.

In the mass spectra of LPS obtained from Δ*waaG* mutant, mass peak corresponding to Kdo_2-lipid A with two heptose residues can be observed, which is consisted with the role of WaaG glycosyltransferase, which mediates incorporation of the first sugar of the LPS outer core of *E. coli* K-12, glucose (GlcI), that is α(1→3) added to HepII (the mass peak at 2,621.5 Da). Further addition of P-EtN residue, followed by phosphorylation can be observed as the mass peaks at 2,744.5 Da and 2,824.4 Da, respectively. However, it seems that LPS of such mutants mostly contained LPS structures with incorporated L-Ara4N (the mass peak at 2,955.5 Da) and additional P-EtN residue (the mass peak at 3,078.5 Da).

The above results revealed that severe LPS defects in the inner core and the lack of GlcI cause induction of the RpoD-dependent *rpoE*P3 promoter activity. Moreover, the induction of this promoter in Δ*waaG* and Δ*waaP* mutants shows the importance of phosphorylation of LPS for OM integrity.

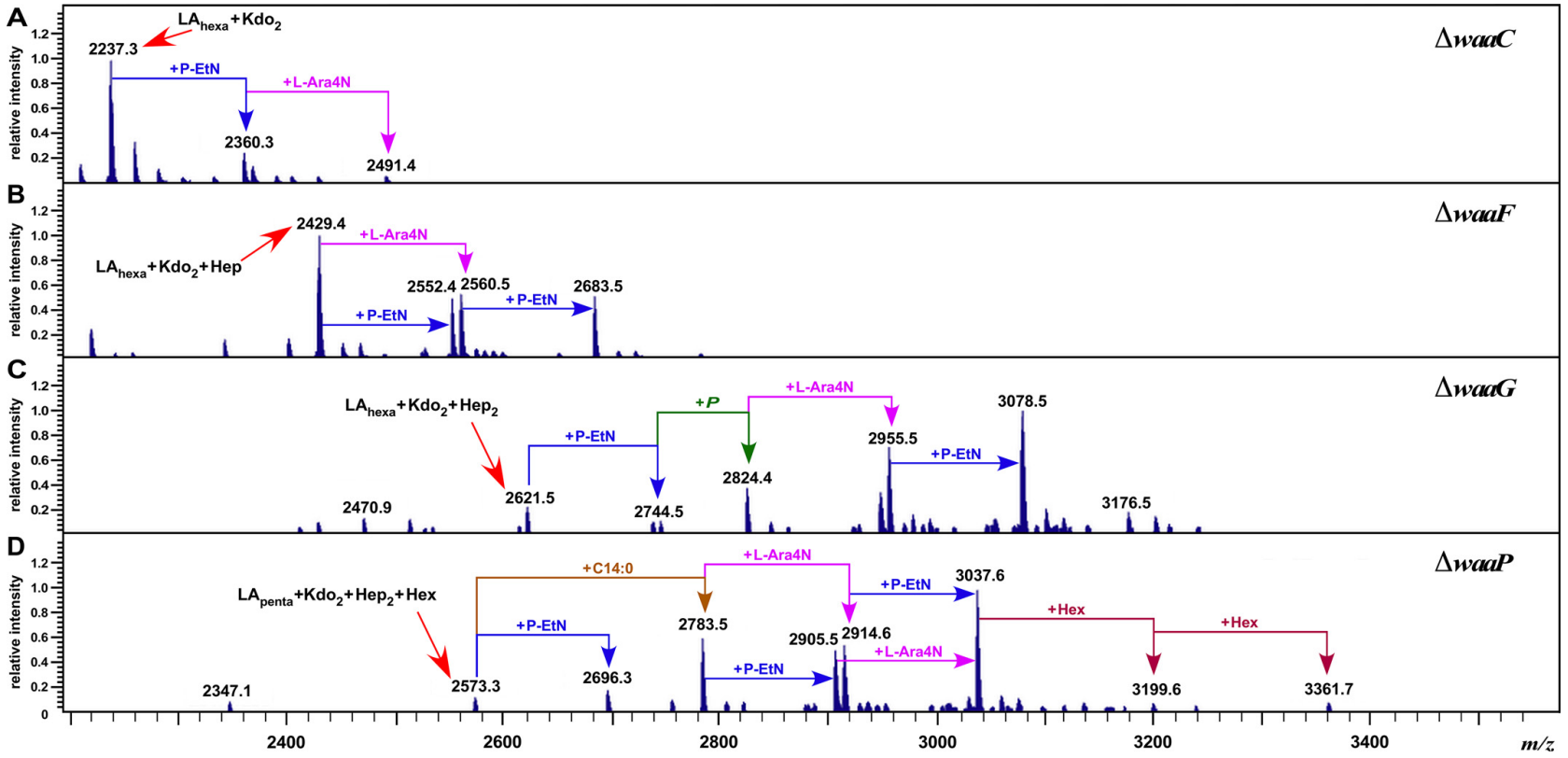

Fig. 26. Charge deconvoluted ES FT-ICR mass spectra in the negative ion mode of native LPS obtained from Δ*waaC*, Δ*waaF*, Δ*waaG* and Δ*waaP* mutants grown in phosphate-limiting medium at 30°C. Mass numbers refer to monoisotopic peaks. The mass peaks corresponding to additional substitutions with P-EtN and/or L-Ara4N due to phosphate-limiting conditions are indicated.

3.8. How response to LPS defects is mediated?

The next step of the research was to find out how LPS defects are sensed that induce *rpoE* transcription? It is likely the signal caused by LPS defects in OM is transmitted via some two-component system. Thus, deletion derivatives of various response regulators were introduced in Δ*waaC* and Δ*waaF* strains carrying a *rpoE*P-*lacZ* fusion. Among these mainly deletion of the *rcsB* was found to abrogate induction of *rpoE*P-*lacZ* activity in either Δ*waaC* or Δ*waaF* mutants. It is known that certain mutations in LPS biosynthetic genes that cause severe defects in LPS composition, lead to synthesis of colanic acid. Colanic acid synthesis requires the induction of the Rcs[2] complex signal transduction system. The spectrum of LPS defective mutants that cause an induction of the *rpoE*P transcriptional activity was confined to only those *waa* genes, whose mutational inactivation exhibit a concomitant induction of synthesis of colanic acid (mucoid colonies) as it was not observd in either *waaQ* or *waaR* mutants that do not form mucoid colonies. Thus, it provides the rationale to suggest Rcs system could transmit signal of LPS defect. Thus, a null mutation in the *rcsB* gene was introduced in *waaC* mutants and analyzed for the *rpoE*P3 activity.

Cultures of SR18987 strain carrying the *rpoE*P3-*lacZ* fusion, its Δ*waaC* and Δ(*waaC rcsB*) derivatives were grown in LB medium at 30°C and analyzed for *β*-galactosidase activity after different intervals of time. Averages from four cultures in each case are plotted and shown in the Fig. 27 below. As is evident mutation of the *rcsB* gene encoding principal response regulator of Rcs system, abolished the *rpoE*P3 induction in a Δ*waaC* mutant.

[2] Rcs signal transduction pathway consists of a histidine kinase sensor RcsC, a principal response regulator RcsB, an auxiliary activator RcsA, a phospho-transfer protein RcsD and a signal transductor RcsF (Majdalani & Gottesman, 2005; Clarke, 2010). As name indicates, Rcs (regulator of capsule synthesis) controls the expression of the genes encoding products responsible for the biosynthesis of the capsular polysaccharide colanic acid. Rcs response to a variety of signals, including changes of temperature, osmolarity and membrane proteins (Sledjeski & Gottesman, 1996; Mouslim *et al.*, 2003). RcsB can act as homodimers or heterodimers with RcsA, what results in enhanced transcription of regulated genes. However, under normal conditions, RcsA synthesis is low and it is rapidly degraded by a Lon protease, but yet, at low temperatures synthesis of RcsA increases, leading to colanic acid production and a mucoid phenotype (Majdalani & Gottesman, 2005).

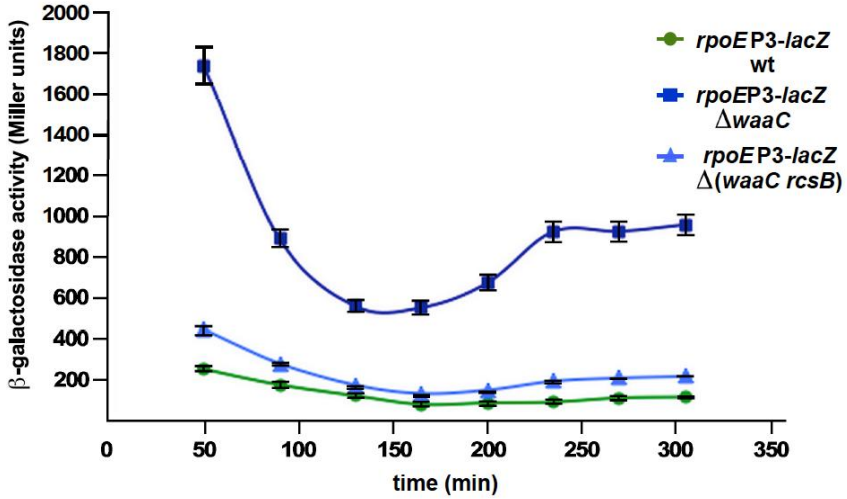

Fig. 27. *β*-Galactosidase assay showing that the deletion of the *rcsB* gene encoding principal response regulator of Rcs system, abolished the *rpoE*P3 induction in the *waaC* mutant. *β*-galactosidase activity was analyzed after different growth intervals from four independent cultures of each derivative.

In order to show the direct regulation of the *rpoE*P3 promoter activity by the Rcs system, a principal response regulator RcsB was purified (Fig. 28) and used in the electrophoretic mobility shift assay after phosphorylation by acetylphosphate.

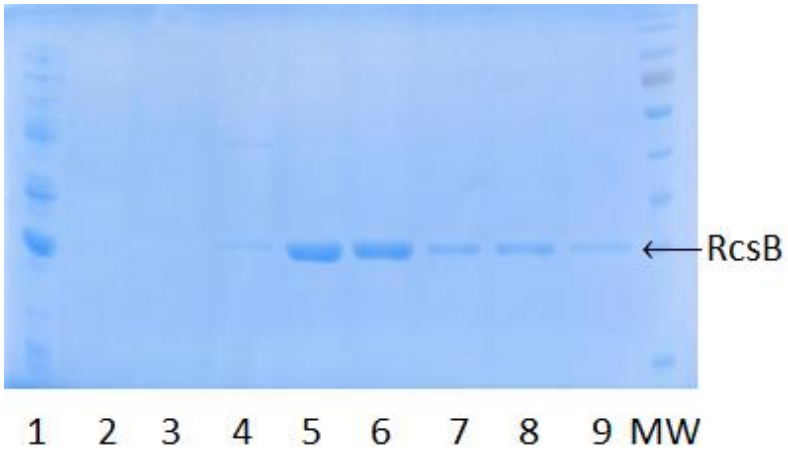

Fig. 28. RcsB purification. Purification was performed as described in paragraph 4.2.16. Samples after the induction of RcsB and after elution with indicated concentrations of imidazole, were resolved on 12.5% SDS-PAGE. Line 1 - RcsB induction; line 2 - fraction eluted with 50 mM imidazole; line 3 - elution with 100 mM imidazole; lines 4-7 elution with 250 mM imidazole; lines 8-9 elution with 500 mM imidazole; MW - Prestained Protein Ladder (migration pattern with apparent molecular weights 15, 25, 35, 40, 55, 70, 100, 130, 170 kDa).

The gel shift analysis using phosphorylated RcsB showed that this known transcription activator indeed binds DNA fragment that covers DNA sequence upstream of the *rpoE*P3 TSS as shown by an efficient shift in the mobility of such DNA (Fig. 29 below). The DNA shift did not occur when RcsB consensus box was deleted. Above results established that Rcs system directly positively regulates the P3 promoter in response to LPS defects.

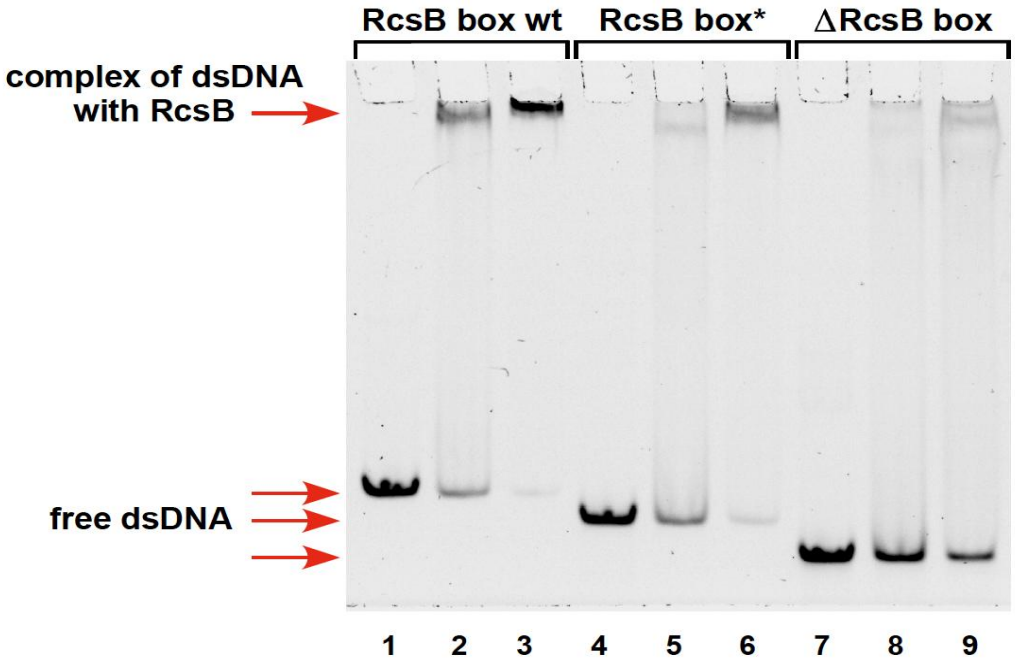

Fig. 29. RcsB binding DNA fragment of the P3 promoter. Three different DNA fragments were used. Lines 1-3: 81 bp DNA fragment of the P3 promoter with the wild-type RcsB sequence; lines 4-6: DNA fragment lacking conserved CAT trinucleotide residues of RcsB consensus; lines 7-9: DNA fragment lacking nucleotides CATGGTTTGG of RcsB consensus. Lanes 1, 4 and 7 represent DNA fragment alone. Lines 2, 5, 8 and 3, 6, 9 represent samples of DNA incubated with 120 and 180 ng of RcsB, respectively. After incubation reaction mixes were analyzed on a 6% native gel.

3.9. The overexpression of genes encoding lipoproteins induces the *rpoE*P3 promoter.

During the multicopy approach, the major class of genes identified, which when overexpressed in strains carrying the *rpoE*P1-P5-*lacZ* fusion encode lipoproteins (*csgG, pgaB, spr*, *yhdV*, *yceB, yddW* and *yghB*) suggesting that such proteins are part of signal transduction controlling *rpoE* promoters activity. From these, the *yhdV* gene was repeatedly isolated. Thus, the impact of its overexpression was analyzed. Measurement of *β*-galactosidase activity revealed that the *rpoE*P3-*lacZ* activity was nearly 3.4-fold increased when the *yhdV* gene was overexpressed as compared to the vector alone (Fig. 30).

The YhdV lipoprotein, similarly to YddW, YghB, and Spr, also identified in these experiments, is in the bottom 25% range abundance in the cell (based on pax-db.org). The increased synthesis of such lipoproteins could cause limitation of the Lol system responsible for lipoproteins sorting and moreover defective lipoprotein sorting is known to induce the Rcs pathway (Tao *et al*., 2012). Thus, the *rcsB* deletion was introduced in the strain carrying a single copy chromosomal *rpoE*P3-*lacZ* fusion and the *yhdV* gene cloned on the plasmid, in order to verify if mechanism of activation of the *rpoE*P3 promoter by lipoproteins was also Rcs-dependent. The results shown that the activation of the *rpoE*P3 promoter upon overexpression of the *yhdV* gene was significantly reduced when the *rcsB* gene was deleted (Fig. 30). Hence, the Rcs system senses imbalances in the OM due to either LPS defects or overexpression of lipoproteins causing activation of the *rpoE*P3 transcription. However, these results do not show if the overexpression of every lipoprotein will induce the *rpoE*P3 transcription.

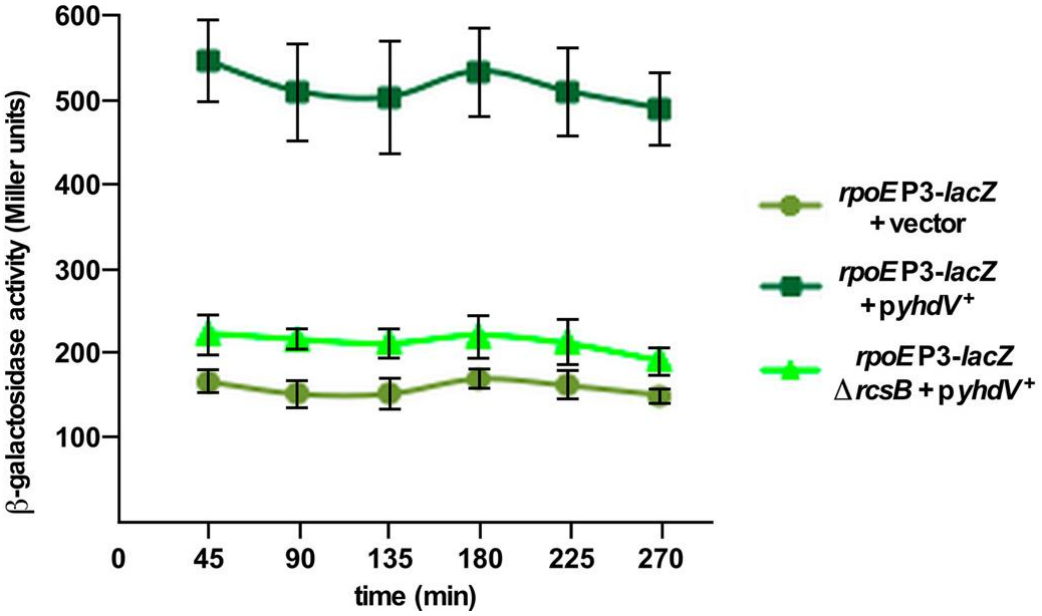

Fig. 30. *β*-Galactosidase assay showing increased *rpoE*P3-*lacZ* activity upon the overexpression of the *yhdV* gene. This induction is significantly reduced by the simultaneous deletion of the *rcsB* gene. Four independent cultures of SR18987 (carrying a single copy chromosomal *rpoE*P3-*lacZ* fusion) with empty vector, or carrying plasmid with cloned *yhdV* gene and its Δ*rcsB* derivative were analyzed for *β*-galactosidase activity in the presence of 50 μM IPTG after different growth intervals.

3.10. Identification of a novel sRNA RirA that regulates LPS biosynthesis by controlling RfaH activity and also regulates the *rpoE*P3 promoter activity.

During the search for potential regulators of *rpoE*P transcription using multicopy approach, several plasmids were isolated that contained partial coding sequence of the *waaQ* gene and intergenic region between *waaA* and *waaQ* genes. Such plasmids were found to specifically induce the activity of LPS responsive *rpoE*P3 promoter. However, since no complete ORF is present in such plasmids, a potential existence of a non-coding sRNA was examined. The first DNA sub-cloning to identify the minimal DNA region that in multicopy induces the *rpoE*P3 promoter was undertaken. Total RNA was extracted from strains carrying such a minimal DNA insert in the plasmid was used in the RACE analysis to identify 5′ and 3′ ends of RNA, using various oligonucleotides specific to *waaQ* 5′UTR. Cloning of cDNA products revealed the presence of a 73 nt DNA sequence that could encode a small non-coding RNA. All such clones revealed that this 73 nt sRNA shares its 5′ end with the *waaQ* mRNA (Fig. 31). Thus, this novel sRNA uses the same promoter as used by the *waaQ* gene. Analysis of the nucleotide sequence revealed the presence of conserved 2 JUMPstart sites and 8 nt *ops* (operon polarity site) (Fig. 32). The *ops* and JUMPstart sites are recognized by RfaH transcriptional factor that acts by suppressing pausing, increasing transcriptional elongation rates and couple transcription with translation. RfaH recognizes only those promoters that contain *ops* site in the 5′UTR and hence it is needed for transcription of *waaQ* operon, *rfb* operon and genes whose products are needed for conjugation (*tra* genes) and hemolysin biosynthetic genes. The 73 nt sRNA identified in this work was named RirA (RfaH interacting RNA), based on confirmed experimentally proved interaction with RfaH.

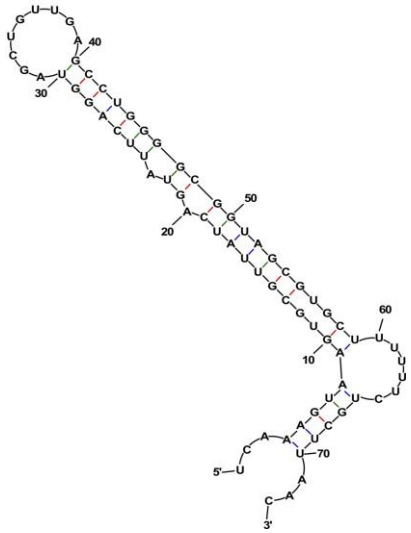

Fig. 31. Model of RirA generated using M-fold

Fig. 32. The nucleotide sequence of the gene encoding the RirA with its promoter region. The arrow indicates the TSS of the *rirA* sRNA. The -10 and extended -10 promoter elements, the JUMPstart, and *ops* sites are marked.

Next, quantitative effect on transcriptional activity of the *rpoE*P3 promoter was examined (Fig. 33). These experiments revealed approximately 2-fold induction of the *rpoE*P3 promoter activity when the *rirA* sRNA was expressed in a medium-copy plasmid from its own promoter. Consistent with these results and in line with model of RirA interaction with RfaH, Δ*rfaH* mutants also exhibited a constitutive induction of the *rpoE*P3 promoter.

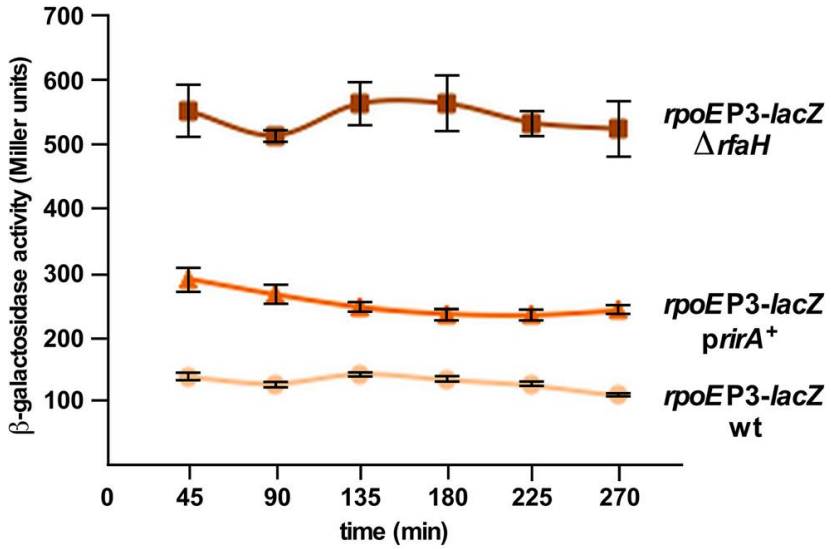

Fig. 33. *β*-Galactosidase assay showing the induction of the *rpoE*P3-*lacZ* promoter fusion activity (SR18987 with the pRS551 vector) upon overexpression of the *rirA* gene from its own promoter in pRS551 or upon *rfaH* gene deletion. The *β*-galactosidase activity was analyzed after different growth intervals from four independent cultures of each derivative in LB medium at 30 °C.

To understand molecular basis of overexpression of RirA that leads to the induction of the *rpoE*P3 promoter, LPS properties were examined with the help of Dr. G. Klein. These experiments showed that *rirA* overexpression causes truncation of LPS, reduction in LPS amount and abrogation of incorporation of *O*-antigen, thus mimicking phenotype of Δ*rfaH* mutants. Further experiments indeed showed that RirA binds RfaH in the presence of RNA polymerase and this interaction requires the presence of *ops* site in RirA (Klein *et al*., 2016).

Described results revealed a complex network in the regulation of the *rpoE* transcription by multiple sigma factors. Despite the well-known regulation of the downstream promoter by RpoE, it was shown how the *rpoE* transcription is regulated in response to different stresses like nitrogen-limitation, entry into stationary phase, challenges with osmolarity and defects in the LPS composition, which constitutes the essential major component of outer membrane.

Isolation of *trans* acting Tn*10* insertion mutations that either increase or decrease the *rpoE*P activity complemented by screen of complete library of knockout non-polar deletions identified several candidate genes, whose products could directly or indirectly

regulate transcription from the distal promoter region. This was complemented by usage of multicopy genomic libraries to identify genes which when overexpressed alter the *rpoE*P transcriptional activity. Additionally, different growth conditions and challenges with various membrane stress causing factors were used to monitor the *rpoE*P transcriptional activity. Combination of these three parallel approaches revealed participation of different regulators, interconnected network of transcriptional factors and composition of LPS that either control the *rpoE*P activity or induce signal of activation of transcription.

Mapping of 5′ ends of *rpoE* mRNA identified five new transcriptional initiation sites (P1 to P5) located distal to promoter regulated by RpoE (now named as the *rpoE*P6 promoter). Experiments showed that three major promoters, P2, P3 and P4, are activated in response to unique signals. The *rpoE*P4 promoter exhibits the highest basal activity, which is regulated by modulators of RpoS or exposure to increased osmolarity in accordance to its positive regulation by RpoS. The RpoN-recognized P2 promoter was identified and shown to be positively regulated by the QseE/F two-component system and the NtrC activator.

In this work it was also for the first time shown that transcription from the RpoD-regulated P3 promoter is induced upon severe defects in the LPS composition and the overproduction of certain lipoproteins. Experiments carried out established that the P3 promoter is positively regulated by Rcs system and by the global regulator CRP. Hence, a mechanism of sensing of LPS alterations at the transcription level in *E. coli* was established. The *rpoE*P3 promoter was also shown to respond to imbalance in the amounts of lipoproteins and this signal transduction was also found to require Rcs two-component system.

Finally, a novel sRNA RirA was identified, since its overexpression causes defects in LPS core and *O*-antigen biosynthesis and hence the activation of expression of the *rpoE*P3 promoter. This sRNA, was shown to be a 73 nt non-coding RNA located in the 5'UTR of *waaQ* mRNA and to contain *ops* site that is recognized by RfaH. This sRNA was shown to bind RfaH transcriptional factor in the presence of RNA polymerase and this binding limits the ability of RfaH to act as transcriptional elongation/antiterminator for its target operons.

The results presented in this chapter are the part of the publication in *Journal of Biological Chemistry* titled "Multiple transcriptional factors regulate transcription of the *rpoE* gene in *Escherichia coli* under different growth conditions and when the lipopolysaccharide biosynthesis is defective" included with this thesis.

Following section described results published in *Journal of Biological Chemistry* titled "Assembly of lipopolysaccharide in *Escherichia coli* requires the essential LapB heat shock protein" also included with this thesis.

3.11. Regulation of lipopolysaccharide synthesis and transport

Due to the fact that LPS is essential for the *E. coli* viability, during growth after each cell division, LPS molecules have to be properly assembled at the cell surface at a speed estimated as 70 000 molecules per minute (Whitfield & Trent, 2014). It means that LPS synthesis and its translocation to the cell surface should be efficient and tightly regulated. Proper execution of LPS synthesis requires the presence at the inner membrane of fully active stoichiometric amounts of all LPS biosynthetic enzymes. Moreover, only completely synthesized LPS molecules should be subsequently translocated. This coupling of LPS synthesis and its translocation requires newly discovered heat shock proteins named LapA and LapB (lipopolysaccharide assembly proteins) (Klein *et al*., 2014). LapB was shown to be essential for viability of *E. coli* under standard laboratory growth conditions. LapB contains six tetratricopeptide repeats (TPR) and a C-terminal rubredoxin-like domain that was found to be essential for its activity. LapB ensures that LPS assembly is completed before its translocation by acting as a scaffold-like protein for LPS biosynthetic enzymes. LapB may control that only the mature LPS is delivered to MsbA and because LapA and LapB proteins were found to co-purify with Lpt complex they may function together in subsequent steps of LPS translocation. The pull down experiments also revealed that LapA and LapB co-purified with LPS, heptosyltransferase WaaC, chaperones (DnaK and DnaJ) and protease FtsH. FtsH is known to control the levels of two LPS biosynthetic enzymes, LpxC and WaaA (Katz & Ron, 2008). Co-purification of LapB with LPS and Lpt components suggests that LapB acts as a scaffold for LPS assembly/translocation and might signal LPS accumulation at the cytoplasmic side of the IM and/or activate the Lpt system. Importantly, Δ*lapB* or Δ(*lapA lapB*) mutants synthesize excess of LPS due to defects in turnover of unstable LpxC enzyme. Characterization of suppressors that allow deletion of the *lapB* gene, identified factors that either reduce LPS amounts, or increase synthesis of phospholipids or repress activation of *rpoE* induction, thereby allowing us to address the essential function of LapB.

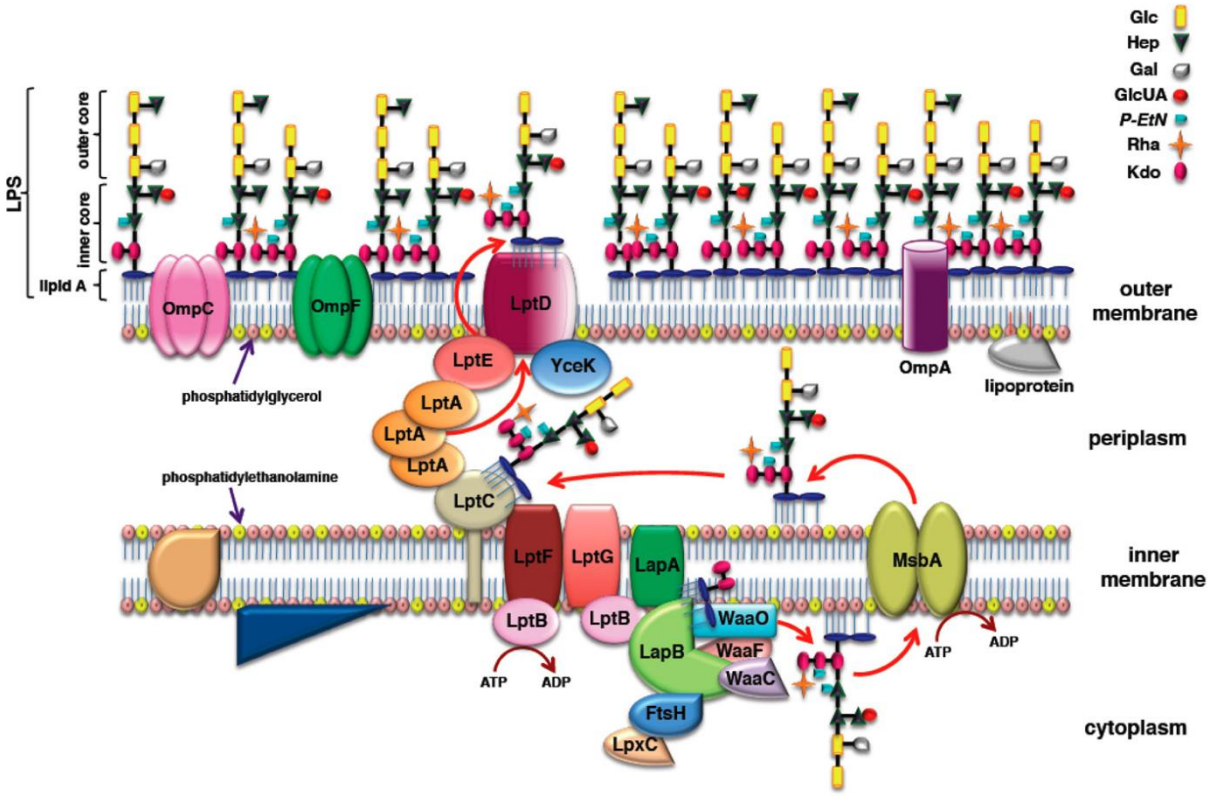

Fig. 34. Transport of LPS from the place of its synthesis to the outer leaflet of outer membrane.

The *lapB* gene was identified during a screen for mutants that exhibit increased activity of *rpoE* sigma factor with a concomitant increase in permeability defects but not mapping to genes whose products are involved in LPS biosynthesis. The aim was to target regulators of LPS assembly. The *lapB* gene encodes an essential heat shock protein, which can be only deleted under slow growth conditions (minimal medium at 30°C) in some, but not in all genetic backgrounds. *lapA* and *lapB* mutants have an increased envelope stress response. Such stress responsive pathways respond to defects in the LPS assembly. Hence, the LPS content of Δ*lapB* or Δ(*lapA lapB*) mutants was elevated, as compared to the wild type. LPS from such mutants is enriched with truncated as well as pentaacylated and LPS derivatives with carbon chain polymorphism. Such accumulation of LPS precursors is a consequence of increased stability of LpxC (hence more LPS) and aggregation of several LPS biosynthetic enzymes in Δ(*lapA lapB*) mutants (Klein *et al*., 2014).

The Δ*lapB* mutant accumulated extragenic suppressors that could be mapped either to genes whose products either are involved in LPS biosynthesis *gmhA*, *lpxC*, *waaC*, the *waaQ* operon or factors that dampen the envelope stress response response, or cause

abrogation of synthesis of most abundant lipoprotein, Lpp (Braun's lipoprotein, which contains three fatty acid chains). The reduction in Lpp amounts could restore a balance between phospholipid and LPS amount by increasing the availability of free fatty acids (Hantke & Braun, 1973).

Essentiality of the *lapB* gene allowed us to perform extensive multicopy suppressor analysis. Such analysis revealed that some of the Δ(*lapA lapB*) defects can be suppressed by the overexpression of genes encoding products predicted to be involved in LPS assembly (*yceK*) or LPS synthesis (*yeaD*), peptidoglycan biogenesis (*murA*, *ydhA*, *yffH*), chaperones (*dnaK* and *dnaJ*), toxin HicA, stress response regulator *rcsF*, as well as phospholipid biosynthesis (*fabB*, *fabZ*).

FabZ is the primary dehydratase involved in the phospholipids synthesis pathway. A balance between phospholipids and LPS content is required to maintain the proper execution of OM biosynthesis and thereby ensure the bacterial viability. Both LPS and phospholipids biosynthetic pathways share the same precursor molecule, *R*-3-hydroxymyristoyl-ACP (Fig. 35). It is used as a substrate for LpxA and FabZ enzymes known to be key factors in the balanced synthesis of LPS and phospholipids (Ogura *et al*., 1999; Führer *et al*., 2006). Induced activity of one of these enzymes causes the depletion of the pool of *R*-3-hydroxymyristoyl-ACP, and favors production of one of the OM components, causing toxic imbalance between LPS and phospholipids. In the absence of LapB, the LPS levels were elevated. Lethality of such mutants was shown to be caused by reduced synthesis of phospholipids as a consequence of the depletion of the common precursor of both LPS and phospholipids (Klein *et al*., 2014). Consequently, the overexpression of the *fabZ* gene product can restore this essential balance and prevent lethality of Δ(*lapA lapB*) mutants (Klein *et al*., 2014).

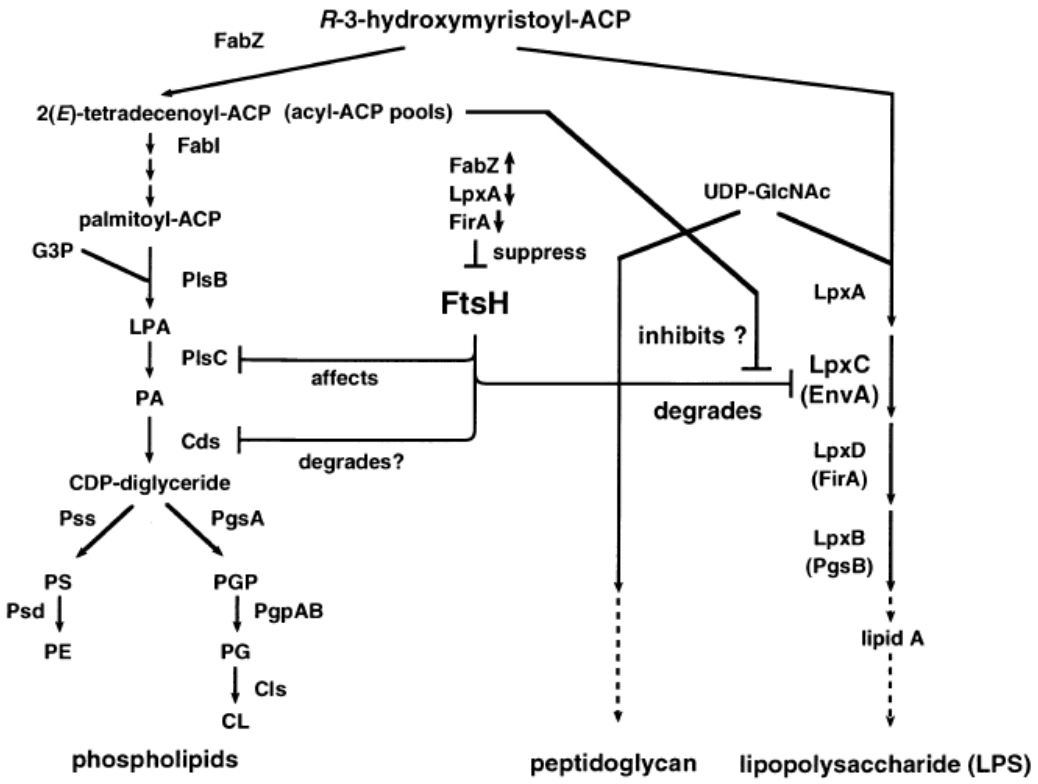

Fig. 35. LPS and phospholipid biosynthetic pathways share the same precursor molecule, *R*-3-hydroxymyristoyl ACP (Ogura *et al*., 1999).

Moreover, LpxC levels are regulated by ATP-dependent metalloprotease, FtsH (Ogura *et al*., 1999), which as it was mentioned before co-purified with LapA and LapB proteins (Klein *et al*., 2014). The FtsH protease is involved in the degradation of some membrane and cytoplasmic proteins such as the heat shock sigma factor RpoH (Tomoyasu *et al*., 1993; Herman *et al*., 1995), the membrane proteins YccA and SecY (Kihara *et al*., 1995; Akiyama *et al*., 1996), proteins tagged with the SsrA degradation peptide (Herman *et al*., 1998). FtsH also controls the abundance of phage proteins, such as lambda cIII, cII and Xis, required for excision of lambda phage from the chromosome (Herman *et al*., 1997, Shotland *et al*., 2000, Leffers & Gottesman, 1998).

Above described results suggest that LapB contributes to ensure balanced biosynthesis of LPS and phospholipids, by participating in the tight control of the LpxC and FabZ levels and activity by controlling of the LpxC turnover in concert with FtsH (Klein *et al*., 2014).

During the suppressor analysis of deletion derivatives of a key LPS assembly factor (LapB), a novel RpoE-regulated non-coding sRNA was identified. This multicopy suppressor was shown to be located within the coding sequence of the *cutC* gene and

the downstream 3' untranslated region (3'UTR). The *cutC* gene belongs to the RpoE regulon and is required for copper homeostasis (Dartigalongue *et al*., 2001). Subcloning of minimal DNA fragments that can cause suppression revealed that the promoter region of the *cutC* gene is not required, which suggested a potential non-coding region within the *cutC* gene and its terminator region (Fig. 36). Further analysis by mapping of 5' and 3' ends by RACE technique, revealed the expression of 307 nucleotides primary RNA transcript that is processed to a 80 nt mature non-coding RNA (Fig. 38). This led to the discovery of a new *trans*-acting small non-coding RNA, which was designated as *slrA* (suppressing *lap* deficiency RNA) and was characterized further as a part of my PhD thesis.

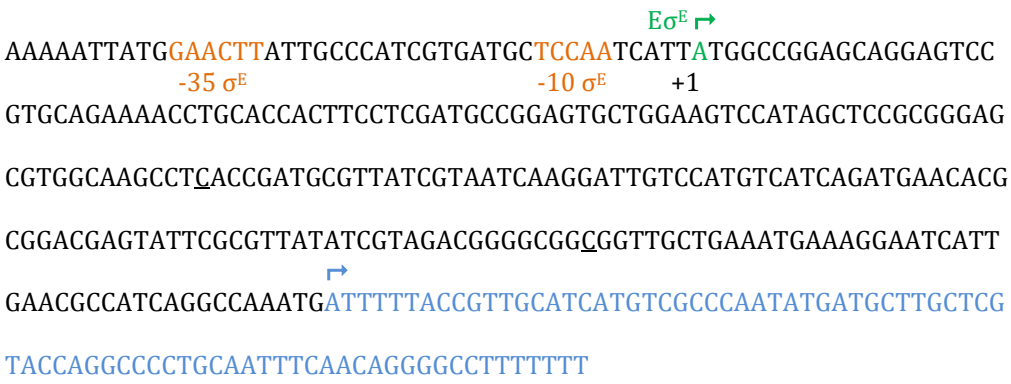

Fig. 36. Nucleotide sequence of the gene encoding *slrA* RNA obtained by mapping of 5' and 3' ends by RACE technique. The beginning of the primary transcript and mature transcript are indicated by green and blue arrows, respectively. Nucleotides in blue show the region encoding the SlrA mature form. The -35 and -10 promoter elements are marked in orange (adapted from Klein *et al*., 2014).

slrA	AAAAATTATGGAACTTATTGCCCATCGTGATGCTCCAATCATTA
*rpoE*P2	ATGCGTTACGGAACTTTACAAAAACGAGACACTCTAACCCTTTG**C**
*rpoH*P3	AGCTTGCATTGAACTTGTGGATAAAATCACGGTCTGATAAAAC**A**
*clpX*P	GCGCATGAATGAACTTATGGCGCTTCATACGGGTCAATCATTA**G**
*htrA*P	CTTTAGTTCGGAACTTCAGGCTATAAAACGAATCTGAAGAACA**C**
*lpxP*P	CTCTTTACAGGAACCATTGTCGTACATGATGGCCCAACCA**A**

Fig. 37. The alignment of -35 and -10 promoter elements of the gene encoding *slrA* RNA with conserved RpoE-regulated promoters (adapted from Klein *et al*., 2014).

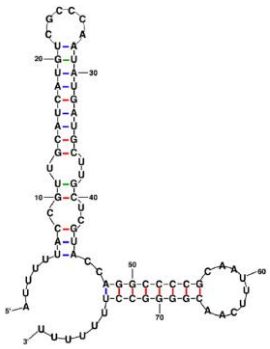

Fig. 38. Mature form of *slrA* RNA (Klein *et al*., 2014)

The sequence shown above revealed that the -35 and -10 regions and their spacing of the *slrA* transcription initiation site resembled the consensus sequence of RpoE-regulated promoters. The -35 region perfectly matches the -35 consensus of promoters recognized by RpoE (Fig. 37). These observations suggested that transcription of *slrA* RNA encoding gene is most likely regulated by the recognition of *slrA* promoter by RNA polymerase with an associated RpoE sigma factor.

This regulation was experimentally verified by the examination of the activity of a generated single-copy promoter fusion of *slrA* and *lacZ* gene (encoding *β*-galactosidase) in the wild type strain and in Δ*rseA* mutant. The Δ*rseA* mutant exhibits a constitutive induction of RpoE caused by the deletion of an anti-sigma factor for RpoE, which negatively regulates RpoE activity. As shown in Fig. 39, *β*-galactosidase activity is highly increased in Δ*rseA* mutant as predicted for RpoE-regulated promoters.

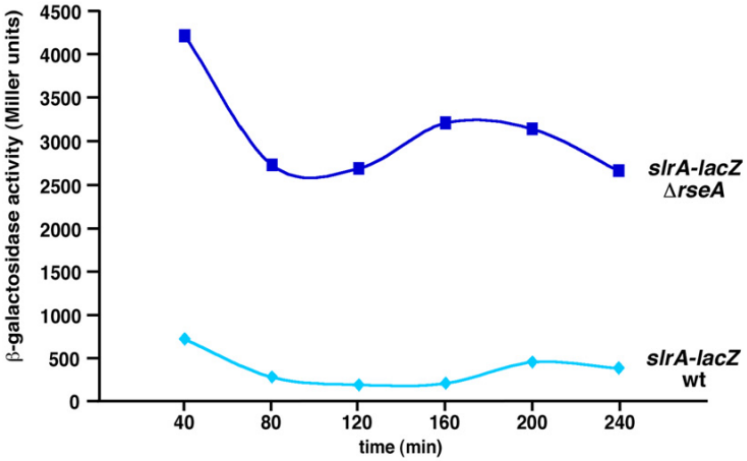

Fig. 39. *β*-Galactosidase assay showing the activity of *slrA* promoter measured using strains carrying single-copy chromosomal promoter fusions with *lacZ* gene in Δ*rseA* mutant as compared with wild type (wt) strain (Klein *et al*., 2014).

The aim of my research was to examine the molecular basis of the suppression of Δ(*lapA lapB*) mutants by the overexpression of this novel *slrA* non-coding RNA. In order to do that, specific defects of Δ(*lapA lapB*) mutants were analyzed and verified if the overexpression of RpoE-regulated *slrA* sRNA could suppress some of these defects. Initially, the inability of growth in the presence of detergents as well as the accumulation of LpxC was examined.

Experiments revealed that the minimal plasmid clone in a medium copy plasmid, carrying the gene encoding the *slrA* RNA restored growth of a Δ(*lapA lapB*) mutant on MacConkey agar and rich medium (LA) even up to 42°C (Fig. 40).

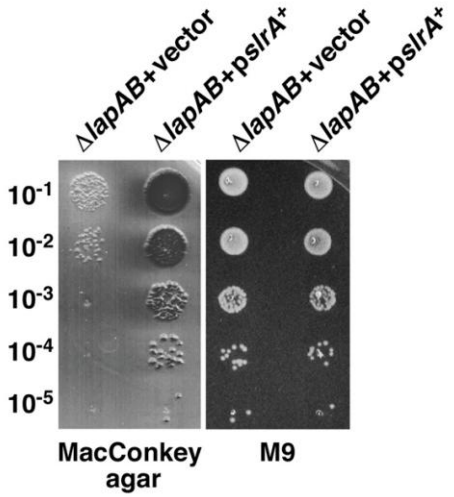

Fig. 40. Serial dilution spot analysis of Δ(*lapA lapB*) mutant carrying plasmid with *slrA* RNA or empty vector. The cultures were grown in M9 liquid broth at 30°C up to A_{600} of 0.2 and then spotted on MacConkey agar and M9 agar as control.

LapB essentiality could be explained by the accumulation of toxic high amounts of LpxC in mutants lacking the *lapB* gene. Levels of LpxC were analyzed in Δ(*lapA lapB*) mutant by the immunoblotting of whole cell extracts with LpxC specific antibody. Cultures were grown at different temperatures with and without overexpression of the *slrA* RNA. This experiment showed that the overproduction of *slrA* non-coding RNA caused a modest decrease in the accumulation of LpxC at 30°C and 37°C (Fig. 41).

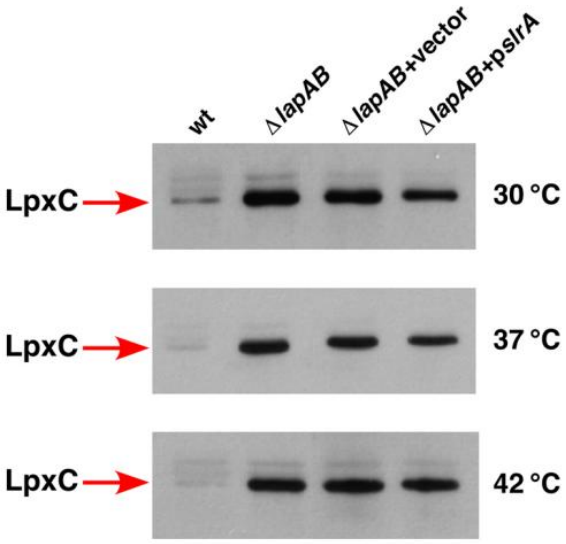

Fig. 41. The immunoblotting analysis of LpxC level in Δ(*lapA lapB*) mutant and its derivatives carrying plasmid with *slrA* RNA or empty vector. The cultures were grown in M9 liquid broth at 30°C up to A_{600} of 0.2 and portions were shifted to LB medium at 37°C and 42°C for 30 minutes. An equivalent amounts of total proteins were examined on 12% SDS-PAGE and then immunoblotted with LpxC antibodies. A modest decrease in the accumulation of LpxC can be seen when *slrA* RNA expression is elevated.

Further, to fully understand the mechanism of the suppression of Δ(*lapA lapB*) mutants defect by *slrA* sRNA, proteomic changes were analyzed. The whole cell extract from Δ(*lapA lapB*) mutant was compared to that obtained from wild type, both after overexpression of the *slrA* RNA. Proteins whose amounts differed significantly in analyzed strains were identified by MALDI-TOF. This experiment revealed reduced amounts of Lpp lipoprotein after overexpression of the *slrA* sRNA. It is consistent with the isolation of a loss of function mutation in the *lpp* gene in the previous experiments as an extragenic suppressor of growth defect of Δ(*lapA lapB*) mutants. Thus, the main substrate for SlrA was identified as the most abundant protein Lpp (Braun's lipoprotein). Predicted base-paring region between SlrA and *lpp* mRNA was identified and hence it was concluded that SlrA functions in *trans* to repress Lpp synthesis.

It is known that mutations in the *lpp* gene also suppress the temperature-sensitive phenotype of *degP* mutants (Strauch *et al.*, 1989; Baird *et al.*, 1991). DegP is a periplasmic serine protease required for survival at high temperatures. In order to examine if overexpression of *slrA* RNA has similar effect, a strain with a chromosomal constitutive elevated expression of *slrA* was constructed by the replacement of the

processed RNA region upstream of mature *slrA* with usage of appropriate oligonucleotides. Next, Δ*degP* mutation was introduced in the wild-type and its isogenic derivative with chromosomal constitutive expression of the *slrA* sRNA (designated $slrA^C$) by bacteriophage-mediated P1 transduction. These isogenic strains along with controls were analyzed for growth on LA medium at 30 °C (permissive for Δ*degP*) and 42 °C (non-permissive for Δ*degP*). Analysis of their growth revealed that elevated expression of *slrA* enables the growth of a Δ*degP* mutant strain at 42°C (Fig. 42).

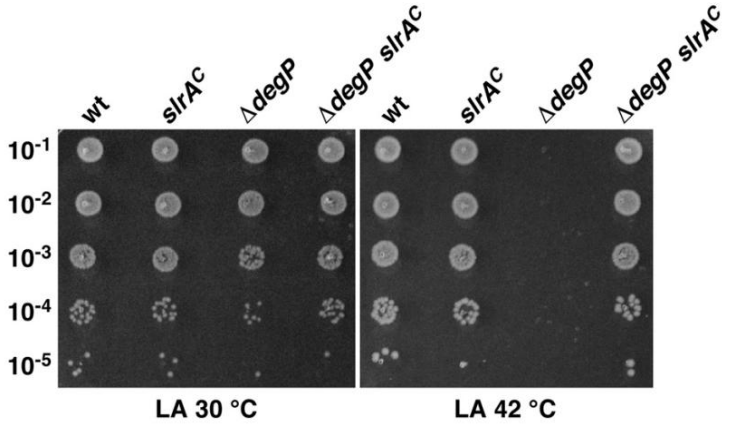

Fig. 42. Serial dilution spot analysis of Δ*degP* mutant with a chromosomal constitutive elevated expression of *slrA* RNA (Δ*degP* $slrA^C$), comparing to Δ*degP* mutant with normal *slrA* RNA expression (Δ*degP*), and strain with a chromosomal constitutive elevated expression and wild type strain. The cultures were grown in LB medium at 30 °C, adjusted to A_{600} of 0.1 and then serial dilution were spotted on LA plates at 42 °C and 30 °C as control. The inability to grow of Δ*degP* mutants at 42 °C is suppressed by elevated expression of *slrA* RNA.

Mutations in the *lapB* gene were first isolated in the search for induction of the envelope stress response of *E. coli*. Hence, experiments were carried out to address if overexpression of the *slrA* sRNA dampens this RpoE-dependent stress response. These studies revealed a significant reduction in the activated pathway of RpoE-dependent stress response. In these experiments the amount DegP were measured by immunoblotting of samples from whole cell lysates of obtained from isogenic strains of Δ(*lapA lapB*) with or without overexpression of *slrA* using anti-DegP antibodies. Transcription of the gene encoding DegP is regulated via its RpoE-dependent promoter. Consequently, down-regulation of the RpoE-dependent stress response of Δ(*lapA lapB*) mutant by *slrA* sRNA could be examined by comparison of levels of DegP in Δ(*lapA*

lapB) derivative with *slrA* sRNA overexpressed from a plasmid and carrying the vector alone. Results from such experiments show that elevated levels of DegP in Δ(*lapA lapB*) mutant are reduced upon overexpression of *slrA* sRNA (Fig. 43).

Thus, the suppression upon by the *slrA* RNA could also be explained by the *slrA*-mediated negative feedback mechanism ascribed to the down-regulation of elevated levels of the RpoE-dependent stress response in Δ(*lapA lapB*) mutants. It is worth mentioning elevated induction of RpoE is toxic accompanied by cell lysis.

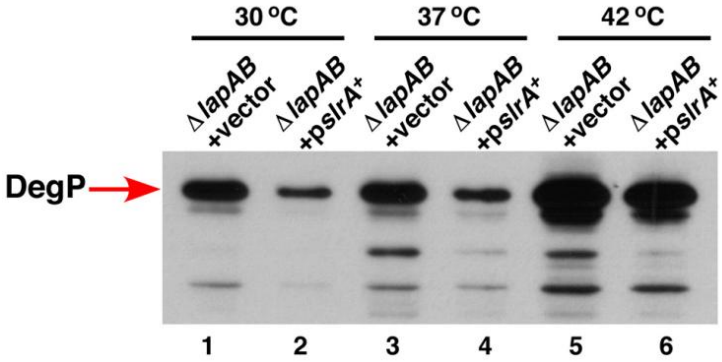

Fig. 43. The immunoblotting analysis of DegP level in Δ(*lapA lapB*) mutant and its derivatives carrying plasmid with *slrA* RNA or empty vector at different temperatures. The cultures were grown in M9 liquid broth at 30 °C up to A_{600} of 0.2 and aliquots were shifted to LB medium at 37 °C and 42 °C for 30 minutes. An equivalent amounts of proteins were examined on 12% SDS-PAGE and then immunoblotted with DegP antibodies. Elevated expression of *slrA* RNA decreased DegP content at all of examined temperatures.

The reduction of Lpp amounts, upon mild overexpression of the *slrA* sRNA, may restore a balance between phospholipids and LPS in Δ(*lapA lapB*) mutants, due to an increased availability of fatty acids when not incorporated in Lpp. The restoration of balanced cell envelope biogenesis was also investigated by comparing the aggregation of WaaC glycosyltransferase in Δ(*lapA lapB*) carrying the plasmid overexpressing *slrA* sRNA or the vector alone and the wild type strain at 30 °C and 42 °C. A chromosomal single-copy C-terminal 3xFLAG epitope fused to WaaC was constructed in the wild type and its Δ(*lapA lapB*) derivative. Cultures were grown at 30 °C in M9 medium up to an A_{600} of 0.2, then shifted to LB medium at 42 °C for 90 minutes. Proteins were fractionated to obtain aggregates. Samples of aggregates obtained after fractionation were analyzed on 12% SDS-PAGE and subjected to immunoblotting with FLAG antibody. Western blots of samples from WaaC-FLAG derivatives revealed a reduction

in the WaaC aggregation at 42 °C upon overexpression of the *slrA* sRNA (showed below on Fig. 44).

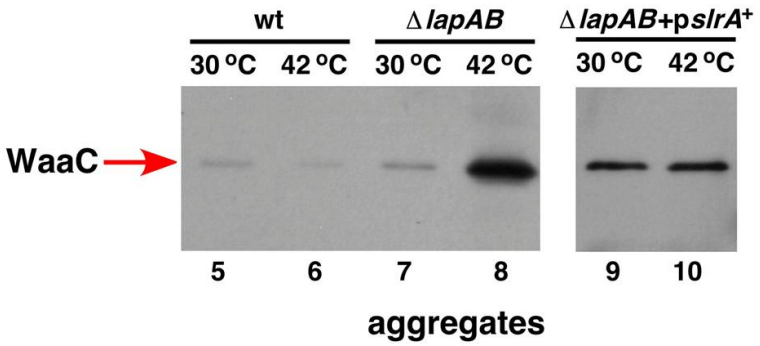

Fig. 44. The immunoblotting analysis of WaaC level in Δ(*lapA lapB*) mutant and its derivative carrying plasmid with *slrA* RNA at different temperatures. The aggregation of WaaC in Δ(*lapA lapB*) mutant at 42 °C is reduced by a mild overexpression of *slrA* RNA.

Summing up, during this work a new small non coding sRNA, *slrA* was identified (Klein *et al*., 2014). The *slrA* gene was shown to have promoter elements that are recognized by RpoE and its activity was found to be increased when RpoE activity is maximal for example in Δ*rseA* mutants. Overexpression of the *slrA* sRNA gene was shown to suppress specific defects of Δ(*lapA lapB*) mutant via three mechanisms. Firstly, the overexpression of this novel sRNA reduces toxic amounts of LpxC. Secondly, the suppression by the *slrA* RNA could also be explained by the *slrA*-mediated negative feedback mechanism ascribed to the down-regulation of elevated levels of the RpoE-dependent stress response. Finally, upon overexpression of the *slrA* sRNA amounts of Lpp were reduced. This protein carries three fatty acyl chains and is the most abundant protein in *E. coli* (Hantke & Braun, 1973; Nikaido, 1996). The reduction of Lpp amounts increased availability of fatty acids not incorporated in Lpp, and by that can restore a balance between phospholipids and LPS. Taken together, decrease in LpxC and Lpp amounts and a reduced levels of stress response can explain the mechanism of action of the *slrA* sRNA as a multicopy suppressor of a Δ(*lapA lapB*) mutant.

3.11.1. Structure function analysis of LapA and LapB proteins

In order to gain insights in the structure and function of LapB, the Phyre 2 server was used to perform the modeling of LapB protein (Fig. 45). The LapB was predicted to share structural similarity to the TPR-containing N-terminal domain of eukaryotic *O*-linked GlcNAc transferases, eukaryotic APC/C subunit Cdc/Cut9, MamA protein required for magnetosome assembly, and some other TPR-containing proteins (Klein *et al*., 2014). The presence of TPR repeats suggested that LapB could be involved in mediating protein-protein interactions and could serve as a docking site in the IM for LPS assembly.

The C-terminal domain of LapB contains a rubredoxin-like domain (RYCQKCGXXWHCPSCXP), highly conserved among LapB homologs. LapB is the first reported protein to contain both TPR motifs and a rubredoxin domain. Indeed, the crystal structure of LapB (without the N-terminal transmembrane helix) showed the presence of nine TPR motifs, including two TPR folds that were not predicted from sequence, and a rubredoxin-type metal binding domain bound intimately to the TPR motifs (Prince & Jia, 2015).

The LapA protein was predicted to have within its C-terminal domain (amino acids 61–89), a structural fold resembling connector region LβH in the C-terminal domain of LpxD. In the crystal structure of LpxD, this connector loop is located in proximity to the active site residues (Bartling & Raetz, 2009).

To address critical amino acids residues that are required for the LapB function the error-prone mutagenesis was carried out using low-copy plasmid carrying the cloned *lapB* gene. Plasmid were transformed back into Δ(*lapA lapB*) strain and non-complementing clones identified. DNA sequence analysis of such mutated *lapB* gene identified several amino acid residues that are required for LapB function. Some of non-functional mutations identified in this approach were located within the rubredoxin-like domain (Cys-371→Arg, Ser-378→Pro, and Pro-372→Leu), indicating essentiality of this domain for the LapB function.

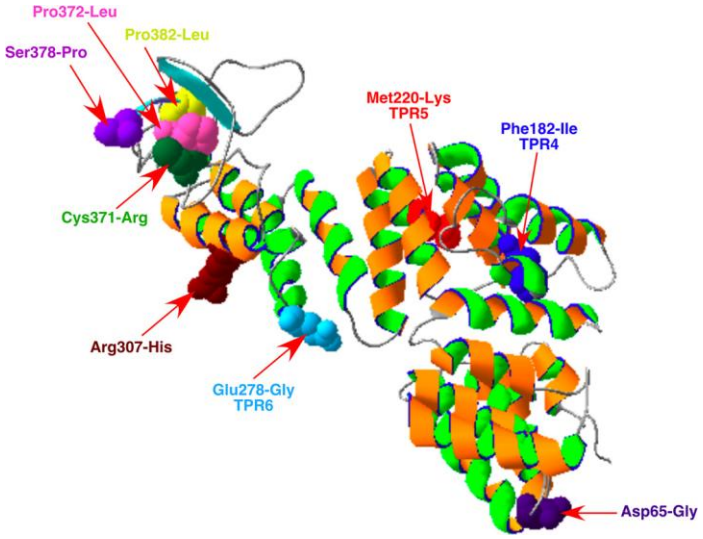

Fig. 45. The model of LapB from *E. coli* performed with the best fitting model on the Phyre 2 server. Mutations critical for the LapB function are indicated.

3.11.2. Defects in LPS assembly in Δ(*lapA lapB*) mutants are also sensed by RpoE via the activation of Rcs pathway that induce the activity of the *rpoE*P3 promoter.

In the work described in Klein *et al*., 2014, I and co-investigators, identified two new heat shock regulated genes *lapA* and *lapB*. Among these genes the *lapB* was shown to be essential and Δ*lapB* mutants were shown to synthesize excess of LPS and accumulate precursor forms of LPS. In this study it was also shown that in the absence of LapA and LapB proteins, transcription of the *rpoE* gene is significantly induced (Klein *et al*., 2014). Thus, in this study, I transduced Δ*lapB* derivatives under permissive growth conditions in strains carrying *rpoE*P1-P5-*lacZ* and *rpoE*P3-*lacZ* (with mutated RpoN recognition site) fusions in the wild-type background and in Δ*rcsB*. It is worth mentioning that a deletion of the *lapB* gene can be tolerated only at 30°C on minimal medium and hence strain construction was performed under such conditions. Quantification of *β*-galactosidase activity of strains with Δ(*lapA lapB*) mutation, revealed a more than a 5-fold increase in the *rpoE*P1-P5-*lacZ* activity and a 3-fold induction of the *rpoE*P3-*lacZ* activity, when the Rcs system was intact (Fig. 46 and Fig. 47). However, this activation of the *rpoE* transcriptional activity in Δ(*lapA lapB*) mutants was significantly reduced in strains lacking RcsB. Thus, these results demonstrated that the transcriptional activation of the *rpoE* gene caused by severe defects in the LPS synthesis and assembly is driven from the *rpoE*P3 promoter, which specifically responds to such defects. Furthermore, this signal activation due to defects in LPS amounts and biogenesis requires the RcsB response regulator.

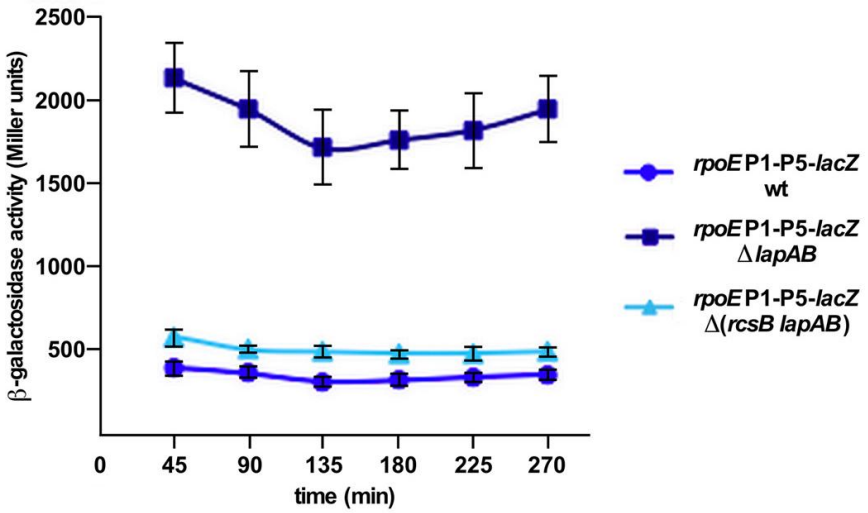

Fig. 46. *β*-Galactosidase assay showing increased *rpoE*P1-P5-*lacZ* activity upon deletion of the *lapA* and *lapB* genes. This induction is significantly reduced by the additional deletion of the *rcsB* gene. The overnight cultures of SR7917 (carrying a single copy chromosomal *rpoE*P1-P5-*lacZ* fusion), its Δ(*lapA lapB*) and Δ(*rcsB lapA lapB*) derivatives were grown in M9 medium at 30 °C. *β*-galactosidase activity was analyzed after different growth intervals from four independent cultures of each derivative.

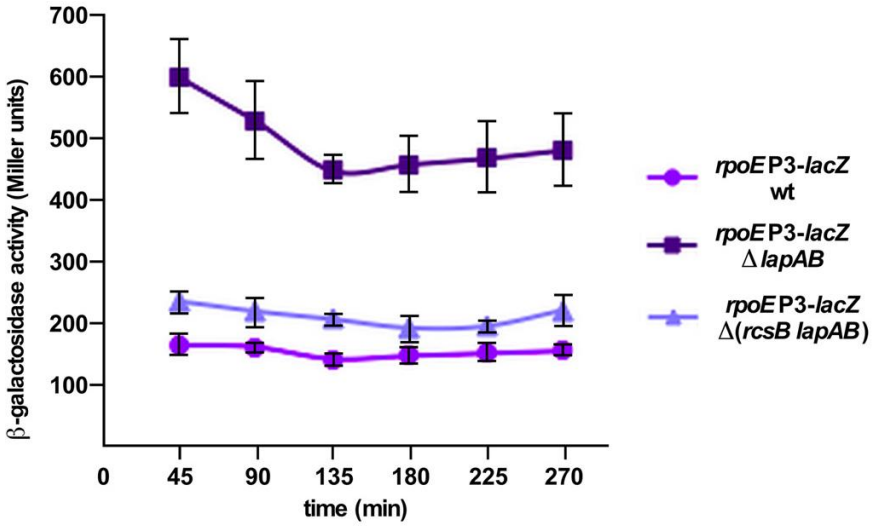

Fig. 47. *β*-Galactosidase assay showing increased *rpoE*P3-*lacZ* activity upon deletion of the *lapA* and *lapB* genes. This induction is significantly reduced by the additional deletion of the *rcsB* gene. The overnight cultures of SR18987 (carrying a single copy chromosomal *rpoE*P3-*lacZ* fusion), its isogenic Δ(*lapA lapB*) and Δ(*rcsB lapA lapB*) derivatives were grown in M9 medium at 30 °C. The *β*-galactosidase activity was analyzed after different growth intervals from four independent cultures of each derivative.

CONCLUSIONS

The *rpoE* gene in *E. coli* was identified more than two decades ago and shown to be essential for bacterial viability. RpoE function has been very well addressed. Several studies revealed that its regulon members include genes encoding protein folding factors, whose function is to ensure correct assembly of OMPs and also genes, whose products are required for assembly and transport of LPS. RpoE also transcribes some small non-coding sRNAs that act as negative feed-back arm as such sRNAs repress the expression of abundant porins. Previous analysis of regulation of the *rpoE* gene revealed a transcriptional and post-transcriptional control. At the post-transcriptional level, mechanism of regulation of RpoE activity has been addressed at the level of sequestration of RpoE by its anti-sigma factor RseA under non-stress conditions. However, when OMP assembly is impaired, RseA is subjected to degradation mediated by a cascade of proteases, liberating RpoE from RseA and allowing the transcription of genes, whose promoters are recognized by $E\sigma^E$ polymerase. Concerning the transcriptional regulation, it was shown transcription of the *rpoE* gene is regulated from a proximally located autoregulated promoter (designated then "P2" promoter) recognized by $E\sigma^E$ polymerase itself and by an unknown mechanism from a distal promoter designated "P1" promoter (Raina *et al*., 1995; Roverie *et al*., 1995). However, the regulation of distal promoter could not be addressed since, neither RNA polymerase containing house-keeping sigma factor RpoD nor when RNA polymerase was supplemented with RpoE could initiate transcription and recognize the distal promoter (Fig. 48).

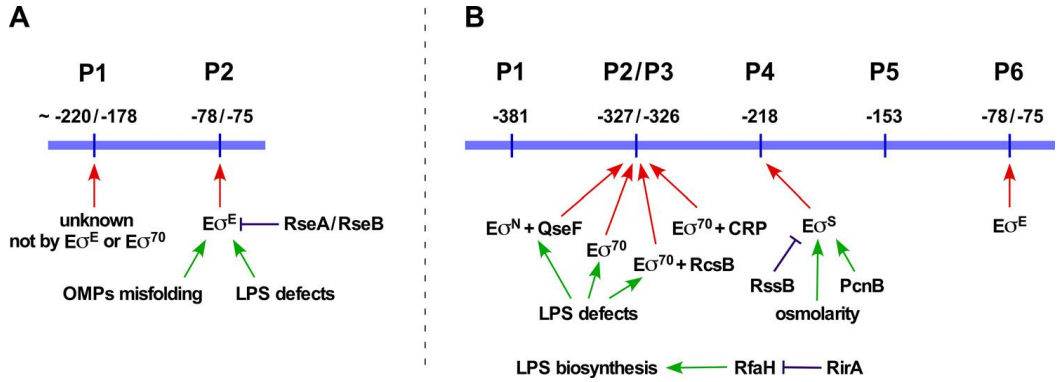

Fig. 48. Transcriptional regulation of the *rpoE* gene. Transcriptional start sites of the *rpoE* gene, as well as major regulators and factors controlling the *rpoE* transcription identified previously (A) and those identified in this work (B) (Klein *et al*., 2016).

In these studies the DNA covering the potential P1 promoter region contained only a limited length of DNA. Thus, the transcriptional regulation of distal promoter region remained a mystery. Several studies in our laboratory have repeatedly shown that severe defects in LPS or defects in its assembly induce *rpoE* transcriptional activity (Missiakas *et al*., 1996; Dartigalongue *et al*., 2001, Klein *et al*., 2009; Klein *et al*., 2011; Klein *et al*., 2014). However, how transcription of the *rpoE* gene is induced, when LPS is defective also remained elusive. Furthermore, transcription of the *rpoE* gene was also known to be induced by several factors that do not change to any major extent OMP composition, like treatment with cold shock or osmolarity changes or upon entry into stationary phase and yet again mechanism of transcriptional activation remained unknown. Thus, the main aim of my PhD thesis was to address: (i) regulation of transcription of the distal promoter region, (ii) identification of mechanism of sensing LPS alterations that induce *rpoE* transcription, (iii) regulatory mechanisms that cause transcription induction of the *rpoE* gene upon entry into stationary phase, changes in carbon sources and other stresses.

Thus, first a new single-copy chromosomal promoter fusion was constructed covering more than 500 bp region located upstream of the autoregulated proximal promoter and for simplicity called *rpoE*P-*lacZ* fusion. Such single-copy promoter region was analyzed for: (a) which growth or stress factors induce or repress its activity and (b) isolate *trans* acting factors (genes), which when either absent or when present in multicopy alter the activity of *rpoE*P-*lacZ* fusion. Thus, it was aimed to identify either sigma factors or activators or two-component systems that regulate activity of *rpoE*P-*lacZ* fusion. Thus, libraries of random transposon insertions and a complete set of knock-out non-polar deletion derivatives were screened for alteration in the activity of *rpoE*P-*lacZ* fusion. In parallel, two multicopy libraries covering all essential and non-essential genes were introduced into *rpoE*P-*lacZ* fusion to identify genes, whose overexpression changes its activity. Combination of all these approaches revealed a complex network in the regulation of the *rpoE*P transcription by multiple sigma factors and involvement of different two-component systems and transcriptional factors.

Analysis of different growth conditions or challenge with factors that impact LPS, revealed induction upon entry into stationary growth phase, nitrogen starvation and upon addition of ammonium metavanadate or treatment with polymyxin B to growth medium. The latter two stresses are associated with LPS alterations. Mutational screen

revealed that defects in LPS core biogenesis due to, either the absence of either conserved early heptosyltransferases or in the absence of major LPS regulatory transcriptional factor RfaH, cause a significant increase in the *rpoE*P-*lacZ* fusion activity. Multicopy approach identified a positive role for Rcs and QseE/F two component systems. Further, factors that modulate the activity of RpoS were identified in both multicopy and mutational approaches. However, *rpoE*P-*lacZ* fusion activity was reduced when growth medium was supplemented by glucose suggesting an additional regulation by catabolite repression mediated by global regulator CRP. Thus, these results suggested that upstream regulatory region of transcription must contain multiple promoters that recruit different sigma factors and transcriptional regulators. Thus, a new strategy was to identify 5′ ends of *rpoE* mRNA in mutants with LPS defects or when two-component system like QseE/F is induced.

Mapping of 5′ ends of *rpoE* mRNA identified five new transcriptional initiation sites (P1 to P5) located distal to promoter regulated by RpoE (now named as the *rpoE*P6 promoter) (Fig. 48). Experiments showed that three major promoters, P2, P3 and P4, are activated in response to unique signals and recognized by RpoN, RpoD and RpoS sigma factors, respectively. The *rpoE*P4 promoter exhibits the highest basal activity, which is regulated by modulators of RpoS or exposure to increased osmolarity in accordance to its positive regulation by RpoS sigma factor. The RpoN-recognized *rpoE*P2 promoter was shown to be positively regulated by QseE/F two-component system and NtrC activator. Most importantly, transcription from the RpoD-regulated *rpoE*P3 promoter was shown to be induced upon severe LPS defects in the inner core and the lack of GlcI (the Δ*waaQ*, Δ*waaS*, Δ*waaY*, and Δ*waaZ* mutants did not exhibit any major changes in the *rpoE*P3-*lacZ* promoter fusion activity as compared to the wild type strain), or the imbalance in the amounts of lipoproteins, and these signal transductions were found to require Rcs two-component system. Hence, a mechanism of sensing of LPS alterations in *E. coli* was established. The RpoD-regulated *rpoE*P3 promoter was also shown to be positively regulated by the global regulator CRP. These aspects of transcriptional regulation were established using *in vitro* run-off assays in the presence and absence of activators and by EMSA using specific DNA fragments.

In depth analysis of the activation of expression of the *rpoE*P3 promoter lead to identification of a novel sRNA, RirA, since its overexpression causes defects in LPS core and *O*-antigen biosynthesis. This sRNA, was shown to be a 73 nt non-coding RNA

located in the 5′UTR of *waaQ* mRNA and to contain *ops* site that is recognized by RfaH. This sRNA was shown to bind RfaH transcriptional factor in the presence of RNA polymerase and this binding limits the ability of RfaH to act as transcriptional elongation/antiterminator for its target operons.

The induced activity of *rpoE*P3 promoter was also found in Δ(*lapA lapB*) mutants, where defects in LPS assembly are also sensed by RpoE via the activation of Rcs pathway.

LapB controls LPS synthesis by controlling the first committed step in LPS biosynthesis by regulating turnover of unstable LpxC enzyme in concert with FtsH. LapB-mediated regulation ensures balanced biosynthesis of LPS and phospholipids, consequently guarantying outer membrane integrity and hence bacteria viability.

The extensive multicopy suppressor analysis of *lapB* mutants led to the discovery of a new *trans*-acting small non-coding RNA *slrA* (suppressing *lap* deficiency RNA). The mechanism of action of the *slrA* sRNA as a multicopy suppressor of a Δ(*lapA lapB*) mutants can be explained by the decrease in LpxC and Lpp amounts and by acting in negative feed back manner on RpoE that reduces elevated levels of stress response in Δ(*lapA lapB*) mutants.

4. MATERIALS AND METHODS

4.1. Materials

LA, LB Agar – per liter

Agar-Agar	15 g
Sodium chloride	10 g
Tryptone	10 g
Yeast extract	5 g

pH adjusted to pH 7.0 with addition of 300 µl of 10 N NaOH

LB Medium – per liter

Sodium chloride	10 g
Tryptone	10 g
Yeast extract	5 g

pH adjusted to pH 7.0 with addition of 300 µl of 10 N NaOH

M9 Minimal Media with 0.3% glucose – per liter

2x Agar-Agar	500 ml
10 mg/ml B1	1 ml
1M $CaCl_2$	0.1 ml
10% Casamino acids	5 ml
0.01 M $FeCl_3$	1 ml
20% Glucose	15 ml
5x M9 salts	200 ml
1 M $MgSO_4$	1 ml

MacConkey Agar – per liter

Agar-Agar	13.5 g
Bile salts	1.5 g
Crystal Violet	0.001 g
Gelatin peptone	17.0 g
Lactose monohydrate	10.0 g
Meat and casein peptone	3.0 g
Neutral red	0.03 g
Sodium chloride	5.0 g

SOC - per liter

Bacto tryptone	20 g
Glucose	18 g
KCl	1.86 g
$MgCl_2$	19 g
$MgSO_4$	1.2 g
NaCl	0.5 g
Yeast extract	5 g

Nitrogen-limiting salts

KH_2PO_4	33.8 mM
K_2HPO_4	77.5 mM
K_2SO_4	5.74 mM
$MgSO_4$	0.41 mM
NH_4Cl	3 or 10 mM

Whenever necessary media were supplemented with ampicillin (100 μg/ml), carbenicillin (100 μg/ml), kanamycin (50 μg/ml) or chloramphenicol (20 μg/ml).

2x binding buffer - per 10 ml

2 M imidazol	0.1 ml
8x phosphate buffer, pH 7.4	2.5 ml

6x sample buffer (lysis buffer)

Bromophenol blue	0.001%
Glycerol	10%
SDS	2%
Tris-HCl, pH 6.8	0.0625 M
β-mercaptoethanol	5%

10x SDS-PAGE running buffer - per liter

glycine	144.0 g
Tris MW 121.1	30.3 g
SDS	10.0 g

Buffer A

Imidazole	100 mM
NaCl	300 mM
NaH_2PO_4	50 mM

Coomassie Brilliant Blue staining solution

Acetic acid	10%
Coomassie Brilliant Blue	0.25%
Methanol	50%

Fixing solution

Acetic acid	10%
Methanol	50%

Z buffer - per liter

$Na_2HPO_4x7H_2O$	16.1 g
$NaH_2PO_4xH_2O$	5.5 g
KCl	0.75 g
$MgSO_4x7H_2O$	0.246 g
β-mercaptoethanol	2.7 ml

The pH was adjusted to 7.0

4.2. Methods

4.2.1. RNA purification

Isogenic cultures of wild-type, its Δ*waaC* derivative and the wild-type strain carrying the *qseG* on plasmid were grown in LB medium at 30°C until an absorbance of 0.2 at 600 nm. When required the expression of the *qseG* gene was induced by the addition of 75 μM IPTG. Cultures were centrifuged (10000 rpm, 10 min). Typically, the pellet from a 5-ml culture was resuspended in 1 ml cold TRIsure solution (Bioline UK). RNA was purified according to the manufacturer's protocol and digested with RQ1 DNase (Promega) to remove any chromosomal DNA, followed by phenol extraction and RNA precipitation.

4.2.2. RACE

For full-length, RNA ligase-mediated rapid amplification of 5' and 3' cDNA ends GeneRacer Kit from Invitrogen was used. RNA with and without treatment with CIP (calf intestinal phosphatase) was used to identify primary and processed products according to manufacturer's instructions.

Two and a half μg of total RNA after DNase I treatment, obtained from cultures was treated with tobacco acid pyrophosphatase at 37°C for 1 hr to remove the 5′ cap structure from mRNA. After the incubation, RNA was treated with phenol:chloroform and RNA ethanol precipitated. Then, dephosphorylated RNA was ligated at 37°C for 1 hr to the GeneRacer™ RNA oligonucleotide using T4 RNA ligase. This ligation reaction was carried out at 37°C for 1 hr, followed by treatment with phenol:chloroform and RNA ethanol precipitated. Then, after RNA precipitation, half of the product was reverse transcribed with SuperScript III RT with use of Race I Primer (gene specific) and the second half was reverse transcribed with SuperScript III RT with use of Race 2 Primer (gene specific). Reverse transcription reaction was carried out for 20 min at 50°C and then shifted for another 40 min to 55°C. After the completion of RT reactions, the enzyme was inactivated by incubating reactions at 70°C for 15 min followed by treatment with RNase H for 20 min at 37°C. The cDNA obtained from both reactions was separately amplified by PCR using GeneRacer 5′ primer and gene-specific primers. An additional round of PCR was employed, using GeneRacer Nested primer and gene-specific oligonucleotides. PCR products from the first amplification and the second

round nested PCR were purified (with use of Thermo Scientific kit) and cloned into the pCR-4-TOPO vector. Plasmids were isolated (with use of Promega kit) and sequenced using M13 forward primer supplied by Invitrogen.

4.2.3. Single-copy chromosomal fusions with the *lacZ* gene

DNA fragment of a proper length was PCR amplified, purified and digested with appropriate restriction enzymes and ligated with digested vector (pRS415, pRS550, pRS551). Fast ligation kit (Thermo Scientific) was used for cloning purposes. Ligations were performed at 23°C and the ligation mixture was transformed into competent cells of the wild type Lac^- strain and plated on medium supplemented with either ampicillin (100 μg/ml) alone or ampicillin+kanamycin antibiotics. Lac^+ transformants were selected or when required selection was made only for kanamycin resistance. Cultures from selected candiadtes were used to extract plasmid DNA. Such DNA was digested with restriction enzymes to verify the presence of insert. Cultures of verified Lac+ or kanamycin resistant were grown to early exponential phase. Subsequently, maltose was added up to concentration 0.2%, shaken for another 20 min at 37°C and warm soft agar was added and mixture was poured on LA plates. Such plates were then used to spot bacteriophage λRS45 (Simons *et al.*, 1987). After overnight incubation at 37°C phage spots were collected in 10mM $MgCl_2$ solution and incubated at RT then chloroform was added to kill phage-resistant bacteria and after incubation at RT and centrifugation at 7000 rpm for 10 minutes supernatant containing phage was collected and titrated. Such phage lysates were used to infect wild type strain in order to obtain single chromosomal fusions. In order to do that excess of phage (multiplicity of infection around 5) was mixed with wild type culture grown with 0.2% maltose and incubated for 30 minutes without shaking. Phage adsorption was stopped by addition of sodium citrate (up to concentration 10 mM). After cultures were centrifuged at 7000 rpm for 5 min, pellet was resuspended in LB medium supplemented with 10mM sodium citrate and plated on medium supplemented with 10 mM sodium citrate and X-Gal or when required directly selected for kanamycin resistance (when pRS550 or pRS551 were used as promoter probe vectors).

4.2.4. Chromosomal DNA isolation

Overnight culture was centrifuged (10000 rpm, 3 min). Pellet was resuspended in lysis buffer RNase A was added and incubated for 5 min. Afterwards Proteinase K was added and incubated at 56°C till complete lysis occurred. After the lysis, binding buffer was added and mixture was transferred on the column and centrifuged (10000 rpm, 2 min, RT). Column was washed twice by adding 500 µl of washing solution, incubating at room temperature for 3 minutes and centrifuging (10000 rpm, 1 min, RT). Then, additional 3 minutes centrifugation at 10000 rpm was performed. Column was dried at 69 °C for 3 minutes, then elution buffer (10 mM Tris-HCl, 0.5 mM EDTA, pH 9.0) was added and incubated for another 3 minutes at 69 °C, and centrifuged (8000 rpm, 4 min, RT). Electrophoresis in 0.7% agarose gel with ethidium bromide was performed and the integrity of isolated DNA was observed under the UV light.

4.2.5. PCR

PCR was performed in 25 µl volume. Each mixture contain 12.5 µl of Master Mix, 5.0 µl of Q-buffer, 1 µl of each primer and 1.5 µl of chromosomal DNA. 25 cycles of denaturation at 94 °C for 45 seconds, annealing at 50 °C for 1 minute and elongation at 72 °C for 1.5 minute were performed.

4.2.6. PCR product purification

After PCR amplification, three volumes of QG buffer (5.5 M guanidine thiocyanate, 20 mM Tris HCl pH 6.6) and one volume of isopropanol were added to PCR product. The liquid was transferred on the Qiagen spin column and incubated for 5 minutes at room temperature (RT) and centrifuged at 13000 rpm for 1 min at RT. 750 µl of washing solution (80% ethanol, 10 mM Tris-HCl pH 7.5) was added on the column and after 5 minutes incubation at RT column was centrifuged at 13000 rpm for 1 min at RT, followed by the second centrifugation at 13000 rpm for 3 min at RT. Column was dried at 69 °C for 3 minutes, then elution buffer was added and incubated for another 3 minutes at 69 °C, and centrifuged (13000 rpm, 4 min, RT). Electrophoresis in 1.5% agarose gel with ethidium bromide was performed and PCR product was observed under the UV light.

4.2.7. Competent cells

Overnight culture was diluted 1:100 in LB and if necessary with the addition of appropriate antibiotic and incubated at 30°C or 37°C with shaking. When culture was properly grown (OD 600 nm ≈ 0.4), it was centrifuged (7000 rpm, 10 min, 4 °C). The pellet was washed three times with ice-cold 10 mM $MgCl_2$. Then, pellet was resuspended in of ice-cold 100 mM $CaCl_2$ (half a volume of centrifuged bacterial culture) and incubated on ice for 20 min. After incubation cells were centrifuged (7000 rpm, 10 min, 4 °C) and after removing of supernatant completely, pellet was resuspended in ice-cold 100 mM $CaCl_2$ and portioned by 100 µl and frozen at -80 °C with the addition of 15 µl of 80% glycerol.

4.2.8. Transformation

Two to six µl of plasmid DNA was added to one portion of competent cells. After 45 minutes incubation on ice heat shock was performed at 43 °C for 45 seconds. Then, after 15 seconds on ice 700 µl of LB (if necessary supplemented with 0.2% glucose) was added and expression was done at 30 °C or 37 °C for 1 hour. After this time mixture was centrifuged (7000 rpm, 3 min, RT) and after supernatant removal, pellet was resuspended in 100 µl of LB and plated on LA medium plates supplemented by appropriate antibiotic and incubated at appropriate temperature.

4.2.9. Plasmid DNA isolation

Five ml of overnight culture was centrifuged (7000 rpm, 10 min) and after removal of supernatant, pellet was resuspended in 250 µl of resuspention solution (containing 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 µg/ml RNaseA). Then the lysis buffer (200 mM NaOH and 1% SDS) was added and mixture was inverted gently till lysis. 350 µl of neutralization solution (0.9 M potassium acetate, pH 4.8, 4.2 M guanidine hydrochloride) was added and after 3 minutes incubation on ice. Centrifugation was performed (13000 rpm, 10 minutes) and liquid was transferred on the silica DNA-binding column and centrifuged (13000 rpm, 1 min). Next, washing of the column was performed by adding of the first washing solution (5 M guanidine hydrochloride, 30% isopropanol) and 3 minutes incubation at room temperature and centrifugation (13000 rpm, 1 min, RT). The second wash was performed by the addition of the second

washing solution (80% ethanol, 10 mM Tris-HCl pH 7.5), 3 minutes incubation at room temperature and centrifugation (13000 rpm, 1 min, RT). Then, after liquid removal, additional 3 minutes centrifugation at 13000 rpm was performed. Column was dried at 69 °C for 3 minutes, then elution buffer (TE buffer) was added and incubated for another 3 minutes at 69 °C, and centrifuged (8000 rpm, 4 min, RT). Electrophoresis in 0.8% agarose gel with ethidium bromide was performed and isolated DNA was observed under the UV light.

4.2.10. Digestion of plasmid DNA

Digestion with restriction enzymes was performed in 20 µl volume (1 µl of each enzyme, 2 µl of 10x digestion buffer, 6-12 µl of plasmid DNA, depending of its concentration). Digestions were performed at 37 °C. For routine purpose Fast digestion restriction enzymes were obtained from Thermo Scientific. Reaction products were analyzed on 0.8 to 1.0% agarose gels.

4.2.11. Electrocompetent cells and electroporation

Culture grown to 0.6 at OD_{600} (in medium supplemented with arabinose) was centrifuged (5000 rpm, 15 min, 4 °C). After supernatant was removed, pellet resuspended in the same volume of ice-cold water and centrifuged (5000 rpm, 15 min, 4 °C). After supernatant was removed, pellet resuspended in the half of the original volume of ice cold water and centrifuged (5000 rpm, 15 min, 4 °C). The supernatant was removed completely and pellet was resuspended in 0.05 of the original volume of 10% ice-cold glycerol and centrifuged (5000 rpm, 15 min, 4 °C). The supernatant was removed completely and pellet was resuspended in proper volume of ice-cold 10% glycerol and divided by 100 µl, to which 1-3 µl of PCR product was added and transferred into cold Gene Pulser Cuvette. Immediately after cells were electroporated using BioRad MicroPulser, 1 ml of ice cold SOC media was added and incubated for 1 hour at 30 °C with shaking and plated on LA plates supplemented with proper antibiotic.

4.2.12. P1 bacteriophage lysates

To 7 ml of bacterial culture (OD 600 nm ≈ 0,2) in of M9 medium or LB medium supplemented with 5 mM $CaCl_2$, 250 µl of bacteriophage P1 stock phage was added.

Phage adsorption was performed during 20 minutes incubation at 30 or 37 °C without shaking. Then mixture was incubated with shaking at 30 °C or 37°C till the lysis occurred (approximately 3 hours). After this time in order to kill phage resistant bacteria 250 µl of chloroform was added. After 20 minutes shaking lysate was centrifuged (3500 rpm, 10 min, 4 °C) and transferred to a sterile tube and stored at 4°C.

4.2.13. Transduction

To 1.2 ml of that properly grown culture (OD 600 nm ≈ 0.8 to 1) 100 µl of P1 phage lysates was added incubated at 37 °C for 20 minutes without shaking. Phage adsorption was stopped by the addition of 110 µl of 1 M sodium citrate. Mixture was centrifuged (7000 rpm, 3 min, RT) and after complete removal of supernatant, pellet was resuspended in 100 µl of LB medium or M9 minimal media supplemented with 10 mM sodium citrate and plated on LA or M9 media plates supplemented with appropriate antibiotic and 10 mM sodium citrate and incubated at appropriate temperature. Transductants were streak purified on LA or M9 media plates supplemented with appropriate antibiotic(s) and 10 mM sodium citrate.

4.2.14. λRed-mediated recombination

In order to delete a gene, PCR was performed with usage of oligonucleotides containing upstream and downstream flanking regions of homology to the target gene with the initiation codon and regions of homology to λRed recombinase expression plasmid. Purified PCR product was electroporated into strain carring λRed recombinase expression plasmid (pKD46) (Datsenko & Wanner, 2000). Transformants were plated on LA plates supplemented with kanamycin, and incubated at 30 °C and after few hours at 37 °C for 30 minutes (in order to lose pKD46 plasmid). Plasmid loss was verified by ampicillin sensitive phenotype. Kanamycin resistance gene insertion in target gene was verified by PCR. Bacteriophage P1 lysate was prepared and transduced into appropriate strain to obtain individual gene deletion.

Then, kanamycin cassette was removed. Firstly, competent cells were transformed with pCP20 plasmid and plated on LA medium supplemented with ampicillin at 30 °C. Then, colonies sensitive to both ampicillin and kanamycin were obtained by multiple streaking.

pCP20 plasmid encodes a yeast FLP recombinase, ampicillin and chloramphenicol resistance and carries temperature sensitive replicon, which enables temporal production of the FLP. It also contains short direct repeats (FRT sites) required for site the specific recombination by FLP, after which antibiotic resistance cassette is removed from the chromosome with no polar effect on downstream genes (Cherepanov & Wackernagel, 1995).

4.2.15. *β*-Galactosidase assay

To measure the activity of various promoters of the *rpoE* gene, single-copy chromosomal promoter fusions to the *lacZ* gene were constructed and *β*-galactosidase assays were performed.

To measure *β*-galactosidase activity, culture carrying promoter fusion was grown in appropriate medium to OD_{600} = 0.02. Cultures were grown at 30 °C and samples were taken at different growth intervals. Sample for measuring the bacterial growth was measured at 600 nm and sample for measuring β-galactosidase activity was added to Z buffer (to obtain volume equal 1 ml). Next, 20 µl of 10% SDS, 20 µl of chloroform was added, and vortexed. 200 µl of 4 mg/ml *ortho*-nitrophenyl-*β*-galactoside (ONPG) was added and vortexed and incubated at 30 °C for 10 minutes. After this time reaction was stopped by adding 500 µl of 1 M Na_2CO_3. Next, mixture was vortexing and kept for 3 minutes in dark. Then, water phase of the liquid was poured to cuvette and absorbance was measured at 420 nm and 550 nm and *β*-galactosidase recorded in Miller units using equation below.

$$\frac{OD_{420} - 1.75 \cdot OD_{550}}{OD_{600} \cdot V \cdot T} \cdot 1000$$

Where,

T – time of the reaction (min),

V – volume of the sample (ml),

OD_{420}, OD_{550}, OD_{600} – absorbance measured at 420 nm, 550 nm and 600 nm, respectively.

4.2.16. Protein purification

Proteins were purified as described in Klein *et al.*, 2014. Firstly, plasmid carried gene encoding protein of interest with hexa-His-tag was transformed into wild type strain. Cultures were grown to an optical density of 0.1 at 600nm at 28 °C and expression was induced by the addition of 0.15-0.7 mM IPTG. After about 4 hours long incubation, cells were harvested by centrifugation at 7000 rpm for 20 minutes at 4 °C. The pellet was gently resuspended in cold in B-PER reagent (Bacterial Protein Extraction Reagent from Pierce Company), supplemented with benzonase, binding buffer, lysozyme, protease inhibitor mixture (Sigma). The lysates were centrifuged at 14000 rpm for 90 minutes at 4 °C. In case of soluble protein, supernatant was directly applied over nickeL-nitrilotriacetic acid (Ni-NTA) column pre-equilibrated and washed with buffer A. In case of IM or OM protein, the pellet was resuspended in B-PER reagent mixed with 2 volumes of buffer A supplemented with 1% octyL-*β*-D-glucoside and mixture of protease inhibitors. Pellet was stirred at 4 °C and centrifuged at 45000x g for 30 min at 4 °C. Supernatant was applied over nickel-nitrilotriacetic acid beads pre-equilibrated and washed with buffer A. Proteins were eluted with buffer A, using a step gradient ranging from 50, 100, 250, and 500 mM imidazole and analyzed by SDS-PAGE.

4.2.17. SDS-PAGE (polyacrylamide gel electrophoresis)

A resolving gel (12.5%) was prepared by mixing 1.83 ml of water, 0.75 ml of 1.5 M Tris-HCl (pH 8.8), 1.875 ml of 30% Acrylamide with 0.5% Bis-acrylamide solution, 45 µl of 10% SDS, 22.5 µl of 10% APS and 2.25 µl of TEMED. The mixture was poured between two glass plates in a gel caster, cover with water and polymerization initiated by a source of free radicals and a stabilizer (APS and TEMED) was carried out for 60 minutes. The stacking gel solution (4%) was prepared by mixing 1.47 ml of water, 0.25 ml of 1 M Tris-HCl (pH 6.8), 0.26 ml of 30% Acrylamide with 0.5% Bis-acrylamide solution, 20 µl of 10% SDS, 15 µl of 10% APS and 2 µl of TEMED. Gel polymerization was performed for 45 minutes.

Samples were preparated by resuspention or mixing with 6x sample buffer (lysis buffer) and heating for 10 minutes at 95 °C. Prepared samples and protein molecular weight marker (PageRuler Presteined Protein Ladder from Thermo Scientific Company) were loaded into wells. Electrophoresis was run for about 40-45 minutes at 200 V and gel was washed, fixed with fixing solution and stained with Coomassie Brillant Blue.

4.2.18. Western Blot Analysis

Depending on the experiment either whole cell lysates were used or proteins were precipitated by the addition of 10% TCA. Proteins were applied to a by 12% SDS-PAGE. Then, proteins were blotted to a PVDF membrane and detected with specific antibodies as described in Raina *et al*., 1995 and Dartigalongue & Raina, 1998.

4.2.19. λTn*10*::kan transposon mutagenesis

To 1 ml of bacterial culture grown at 30°C, maltose was added to obtain 0.2 % concentration. After 25 minutes incubation with shaking at 30°C, 15 µl of λTn*10* and 1 µl of 1 M IPTG (isopropyl *β*-D-1-thiogalactopyranoside) was added. After 25 minutes incubation at 37°C without shaking mixture was centrifuged (7000 rpm, 5 min, RT), resuspended in 400 µl LB with 10 mM sodium citrate and incubated for 25 minutes at 37°C. After centrifugation (7000 rpm, 5 min, RT) pellet was resuspended in proper volume of LB and plated on plates with appropriate antibiotic and 5 mM sodium citrate and incubated at 37°C.

4.2.20. Inverse PCR

PCR was performed in 25 µl volume in three rounds. Each mixture contain 12.5 µl of Master Mix, 5.0 µl of Q-buffer, 1 µl of each primer and for first round 1.5 µl of chromosomal DNA was used and for second round 2.0 µl od first round product was used and for third round 2.0 µl of second round product was used. Primers complementary to Tn*10*::kan transposon was used along with random primers.

Twenty five cycles of denaturation at 94°C for 45 seconds, annealing at 50°C for 1 minute and elongation at 72°C for 1.5 minute were performed.

4.2.21. *In vitro* run-off assay

The reaction was carried in volume of 20µl in a buffer containing 40 mM Tris-HCl, pH 7.5, 150 mM KCl, 10 mM $MgCl_2$, 1 mM DTT. Fifty ng of DNA template was incubated with the core RNAP and the specific purified wild-type σ factor at a molar ratio of 4:1. To allow the formation of the open complex, DNA templates were first incubated with RNAP. The nucleotides were added to final concentrations: 0.5 mM each of ATP, GTP,

CTP, and 0.1 mM UTP plus 3.7 kBq of [α-^{32}P]UTP. After 20 min incubation 37°C, reaction was ended by formamide stop solution.

During *in vitro* run-off assays with RpoN two different DNA templates were used (the wild-type and with mutations at the -12 and -24 elements). Since RpoN requires ATP-dependent activator, NtrC or QseF was added and after the open complex formation, the reaction mixture was incubated at 37 °C for 5 min with 5 mM ATP before the addition of nucleotides.

Four µl of each RNA sample labeled with [α-^{32}P]UTP were electrophoresed on a 5% acrylamide gel containing 7 M urea as described previously (Raina *et al*., 1995).

4.2.22. Electrophoretic mobility shift assay (EMSA)

DNA fragments were synthesized by PCR with use of specific oligonucleotides. Reactions were performed in binding buffer from Invitrogen. 35 ng of DNA probe was incubated with an increasing amount of purified protein. Whenever required, RcsB and QseF were phosphorylated with acetyl-phosphate at 30 °C for 30 min and purified CRP was incubated with 1 mM cAMP in buffer containing 10 mM Tris-HCl (pH 7.8 at 4°C), 150 mM NaCl, and 3mM $Mg(OAc)_2$ to generate the CRP-cAMP complex. Products of reactions were analyzed on native acrylamide gels (4 or 6%) and stained with SYBR Green (Invitrogen).

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