

MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

„Novel roles of the RNase E adaptor protein RapZ in
E. coli-K12“

verfasst von / submitted by

Lara Veronika Perko Budja, dipl.biol. (UN)

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of
Master of Science (MSc)

Wien, 2023 / Vienna, 2023

Studienkennzahl lt. Studienblatt /
degree programme code as it appears on
the student record sheet:

UA 066 830

Studienrichtung lt. Studienblatt /
degree programme as it appears on
the student record sheet:

Molekulare Mikrobiologie, Mikrobielle Ökologie und
Immunbiologie

Betreut von / Supervisor:

Assoz. Prof. Dipl.-Biol. Dr. Boris Görke

TABLE OF CONTENTS

1. Acknowledgments	7
2. List of abbreviations	8
3. Abstract.....	11
4. Introduction	12
4.1. The cell envelope of Gram-negative bacteria	12
4.1.1. Role of the Hexosamine Biosynthesis Pathway for synthesis of UDP-GlcNAc – an essential precursor for envelope synthesis	13
4.1.2. Cell envelope biogenesis.....	15
4.1.2.1. Transport of OM proteins	15
4.1.2.2. Biosynthesis and transport of LPS	15
4.1.2.2.1. Lpt machinery	18
4.1.3. Cell envelope stress response systems in <i>E. coli</i>	20
4.1.3.1. Causes of cell envelope stress.....	21
4.1.3.2. The σ^E -mediated envelope stress response	21
4.1.3.2.1. σ^E responds to LPS damage.....	23
4.1.3.3. Additional specialized ESR systems	23
4.2. The <i>E. coli</i> <i>rpoN</i> operon	26
4.2.1. Connections between the <i>rpoN</i> operon and cell envelope stress	27
4.2.2. Phosphotransferase systems (PTSs).....	28
4.2.2.1. The nitrogen-related Phosphotransferase system (PTS ^{Ntr})	30
4.2.2.2. Regulatory targets of PTS ^{Ntr}	32
4.2.2.3. The role of PTS ^{Ntr} on GlmS enzymatic activity.....	34
4.2.3. RNase adaptor protein RapZ and regulation of GlmS	35
4.2.3.1. Two small RNAs GlmY and GlmZ regulate <i>glmS</i> expression	36
4.2.3.2. RapZ senses glucosamine-6-phosphate levels	38
4.2.3.3. Nva-FMDP targets enzymatic activity of GlmS	39
4.3. Aims of this study	41
4.3.1. Revealing the consequences of σ^E -driven upregulation for downstream processes regulated by RapZ.....	41
4.3.2. Identification of mutations and compounds that activate the <i>lptAp1</i> promoter	42
4.3.3. Identification of mutations that lead to the σ^E -dependent upregulation of <i>sixA</i> expression.....	42
4.3.4. Elucidation of the role of the RapZ protein for expression of cold-shock related transcripts.....	43
5. Materials and Methods	44
5.1. Materials	44
5.1.1. Media and plates	44

5.1.2. <i>Escherichia coli</i> -K12 strains.....	45
5.1.3. Plasmids.....	48
5.1.4. Oligonucleotides	49
5.2. Methods.....	53
5.2.1. Cultivation of <i>E. coli</i>	53
5.2.1.1. General growth conditions of <i>E. coli</i>	53
5.2.1.2. Determination of optical density	53
5.2.1.3. Strain storage / Preparation of cryo cultures.....	53
5.2.1.4. Plate spotting assay	54
5.2.2. Construction of <i>E. coli</i> -K12 strains.....	54
5.2.2.1. General transduction of genetic markers using phage <i>T4-GT7</i>	54
5.2.2.1.1. Preparation of <i>T4-GT7</i> phage lysates	54
5.2.2.1.2. Transduction of <i>E. coli</i> using phage <i>T4-GT7</i>	54
5.2.2.2. Removal of antibiotic resistance markers from <i>E. coli</i> -K12 strains (Datsenko and Wanner, 2000)	55
5.2.2.3. Integration of DNA fragments into the chromosomal λ -attachment site <i>attB</i> of <i>E. coli</i>	56
5.2.2.3.1. The ectopic integration of the <i>lptA'</i> - <i>lacZ</i> reporter construct into the chromosomal λ - <i>attB</i> -site.....	57
5.2.3. Transposon mutagenesis screen using a miniTn10 delivery system.....	57
5.2.4. Methods for working with recombinant DNA	59
5.2.4.1. Preparation of competent <i>E. coli</i> cells	59
5.2.4.2. Transformation of competent cells with plasmid DNA.....	59
5.2.4.3. Polymerase chain reaction (PCR)	60
5.2.4.3.1. DNA amplification with <i>PfuS</i> (for cloning).....	60
5.2.4.3.2. DNA amplification with <i>Taq</i> (colony PCR).....	61
5.2.4.3.3. Semi-random two-step PCR	61
5.2.4.4. Agarose gel electrophoresis.....	63
5.2.4.5. Extraction of DNA from agarose gels	64
5.2.4.6. DNA clean-up following PCR or restriction digests	64
5.2.4.7. Molecular cloning	64
5.2.4.7.1. Restriction of DNA	64
5.2.4.7.2. Dephosphorylation of 5'-ends of DNA vectors	65
5.2.4.7.3. Ligation of DNA fragments.....	65
5.2.4.8. Isolation of plasmid DNA.....	65
5.2.4.9. DNA sequencing (according to Sanger)	66
5.2.4.10. Construction of plasmid pFS35	66
5.2.5. RNA methods	67

5.2.5.1. RNA extraction from bacterial cells for Northern Blot.....	67
5.2.5.2. <i>In vitro</i> transcription to generate DIG-labeled probes for Northern Blot.....	68
5.2.5.3. Denaturing urea-PAA gel electrophoresis	69
5.2.5.4. Analysis of specific RNA species by Northern blotting.....	69
5.2.6. Protein methods	71
5.2.6.1. Preparation of total cell extracts for SDS-PAGE	71
5.2.6.2. Denaturing polyacrylamide gel electrophoresis (Cleveland <i>et al.</i> , 1977).....	71
5.2.6.3. Coomassie staining of SDS-PAA gels	72
5.2.6.4. Analysis of proteins by Western blotting	72
5.2.7. Reporter gene assays	74
5.2.7.1. Quantitative β -galactosidase activity assay (Miller, 1972).....	74
5.2.8. Kits	75
5.2.9. Used softwares and websites	75
5.2.10. Size markers	76
6. Results	77
6.1. Role of GlmY/GlmZ and RapZ for the expression of cold-shock related transcripts....	77
6.1.1. A possible role of RapZ for abundance of cold-shock related transcripts <i>cspB</i> and <i>cspH</i>	78
6.2. Impact of σ^E on the adaptor protein RapZ and expression of <i>glmS</i>	82
6.2.1. Influence of the σ^E overexpression on growth of <i>E. coli</i> -K12 under standard laboratory growth conditions.....	83
6.2.2. σ^E overexpression triggers accumulation of protein RapZ.....	85
6.2.2.1. RapZ accumulates upon ectopic <i>rpoE</i> expression.....	86
6.2.2.2. Further dissection of the regulatory elements responding to envelope stress in the <i>lptAB</i> and the <i>rpoN</i> operon using ectopic <i>lacZ</i> fusions	88
6.2.2.3. Pilot experiment for determination of an appropriate inducer concentration for the P_{tet} promoter	91
6.2.2.4. Impact of the σ^E -dependent accumulation of RapZ on the GlmY/GlmZ/ <i>glmS</i> circuit using the arabinose-controlled <i>rpoE</i> expression plasmid	100
6.2.2.5. Impact of the σ^E -dependent accumulation of protein RapZ on the GlmY/GlmZ/ <i>glmS</i> circuit using the anhydrotetracycline-controlled <i>rpoE</i> expression plasmid	106
6.2.2.6. Impact of the σ^E -dependent accumulation of protein RapZ under GlcN6P depletion conditions	109
6.3. Compounds eliciting expression from the <i>lptAp1</i> promoter.....	113
6.3.1. Thanatin activates the <i>lptAp1</i> promoter	113
6.3.2. Impact of thanatin on the RapZ/GlmY/GlmZ/ <i>glmS</i> circuit.....	116
6.3.3. Impact of thanatin on the RapZ/GlmY/GlmZ/ <i>glmS</i> circuit in combination with the GlmS inhibitor Nva-FMDP	122
6.4. Genetic alterations activating the <i>lptAp1</i> promoter	126

6.4.1. Construction of a suitable <i>lptA'</i> - <i>lacZ</i> reporter gene fusion for screening.....	126
6.4.2. Finding signals affecting activity of the <i>lptAp1</i> promoter by transposon mutagenesis screen using a miniTn10 delivery system	128
6.4.3. Confirmation of the transposon screening candidates by measuring <i>lptA'</i> - <i>lacZ</i> in strains with corresponding clean deletions	131
6.4.4. Validation of the effects caused by <i>surA</i> mutations by complementation	136
6.5. Screening for genes that activate the <i>sixA'</i> - <i>lacZ</i> reporter gene fusion	139
6.5.1. Construction of a <i>sixA'</i> - <i>lacZ</i> reporter gene fusion suitable for screening	139
6.5.2. Finding signals affecting activity of the <i>sixA'</i> - <i>lacZ</i> reporter gene fusion by transposon mutagenesis screen using a miniTn10 delivery system.....	142
7. Discussion.....	145
7.1. Investigation of a possible role of GlmY/GlmZ and RapZ for the expression of cold-shock related transcripts	145
7.2. Impact of σ^E on the <i>rpoN</i> operon	147
7.2.1. Dissection of regulatory elements responding to envelope damage in the <i>lptAB</i> and <i>rpoN</i> operon	149
7.2.1.1. Activation of the <i>lptAp1</i> promoter	150
7.2.1.2. Read-through transcription from <i>lptAp1</i> into the <i>rpoN</i> operon.....	157
7.2.2. The consequences of the σ^E -dependent accumulation of protein RapZ for the GlmY/GlmZ/ <i>glmS</i> circuit	159
7.3. Impact of σ^E on the PTS ^{Ntr}	167
7.4. Final conclusions and outlook	168
8. Supplementary data	170
9. Zusammenfassung.....	184
10. References	185

1. Acknowledgments

First of all, I would like to thank my supervisor Assoz. Prof. Dipl.-Biol. Dr. Boris Görke for giving me the opportunity to join his lab and to work on this exciting project. I am extremely grateful to him for his continuous support and guidance throughout this project. I very much appreciate your valuable advice, suggestions and estimable knowledge. Thank you for always finding the time to discuss.

I want to especially thank my mentor Florian Sikora who provided me with encouragement and patience throughout this project and never wavered in his support. I am immensely grateful for all the helpful discussions and that you always took the time to answer my questions. Your guidance and constructive advice shaped this thesis and I could not have asked for a better supervisor.

I am also thankful to my lab colleagues, Alexandra and Laura. I had great pleasure of working with you and I want to thank you for creating a good working atmosphere and always being there with helpful suggestions and advices.

I am thankful to Prof. Dr. Alessandra Polissi from the Department of Pharmacological and Biomolecular Sciences at the University of Milano for providing our laboratory with Thanatin and the conditional *E. coli* FL905 and FL907 strains, and to Prof. Dr. Slawomir Milewski from the Department of Pharmaceutical Technology and Biochemistry at the University of Technology in Gdansk for providing the Nva-FMDP.

I am grateful to my parents, Darja and Simon, who supported me in everything and repeatedly motivated me throughout my studies, and my brothers, Lan and Gal, who never ceased to express their love and support. A very special thank you goes to my boyfriend Stefan and my friends for always being there for me.

2. List of abbreviations

List of abbreviations	
% (v/v)	volume percent
% (w/v)	mass volume percent
α-KG	α -ketoglutarate
A	ampere
AA/Bis	Acrylamide/bisacrylamide
Amp	Ampicillin
AMP	Adenosine monophosphate
AMPs	antimicrobial peptides
AP	Alkaline Phosphatase
appr. / ~	approximately
APS	Ammonium persulfate
ara	arabinose
ATc	anhydrotetracycline
ATP	Adenosine triphosphate
<i>bEBP</i>	bacterial enhancer binding protein
bp	base pair
°C	degrees Celsius
cAMP	cyclic Adenosine monophosphate
CAMP	cationic antimicrobial peptide
CAP	catabolite activator protein
Cm	Chloramphenicol
Csp	cold-shock proteins
ddH₂O	double distilled water
dH₂O	distilled water
DIG	Digoxigenin
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide / Deoxy nucleoside triphosphate
ECA	Enterobacterial common antigen
EDTA	Ethylenediaminetetraacetic acid
ESR	envelope stress response
EtOH	ethanol
FLP	flippase recombination enzyme
FRT	FLP recognition target
Fru6P	Fructose-6-phosphate
g	gram
GlcN	glucosamine

GlcNAc	N-acetyl glucosamine
GlcNAc6P	N-acetyl-D-glucosamine-6-phosphate
GlcN1P	glucosamine-1-phosphate
GlcN6P	glucosamine-6-phosphate
GlmS	glucosamine-6-phosphate synthase
Gln	L-glutamine
Glu	glutamate
h	hour
IM	inner membrane
OM	outer membrane
K⁺	potassium
Kan	Kanamycin
kdo	2-keto-3-deoxy-D-manno-octulosonic acid
kb	kilo base
kDa	kilo Dalton
L	liter
<i>lacZ</i>	gene coding for β -galactosidase
LB	Luria Bertani
LPS	Lipopolysaccharide
M	Molar
mA	milliampere
MetOH	methanol
mg	milligram
MIC	minimal inhibitory concentration
min	minute
mL	milliliter
mM	millimolar
mRNA	messenger RNA
MurNAc6P	N-acetylmuramic acid 6-phosphate
ng	nanogram
nm	nanometer
nt	nucleotide
Nva-FMDP	L-norvalyl-methylester N ³ -(4-methoxyfumaryl)-(S)-2,3-diaminopropanionic acid
OD_x	Optical density at a wavelength x nm
OM	outer membrane
OMP	outer membrane protein
ON	overnight
ONPG	ortho-Nitrophenyl- β -galactoside
ori	origin of replication

P	promoter
PAA	polyacrylamide
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEP	phosphoenolpyruvate
PTS	phosphotransferase system
PTS^{Ntr}	nitrogen-related phosphotransferase system
PG	Peptidoglycan
PMB	Polymyxin B
PMF	proton motive force
pmol	picomole
PVDF	polyvinylidene difluoride
RBS	ribosomal binding site
RNA	Ribonucleic acid
RNAP	RNA polymerase
rpm	rotations per minute
RT	room temperature
SD	Shine-Dalgarno sequence
SDS	sodium dodecyl sulfate
s	second
Spec	Spectinomycin
sRNA	small non-coding RNA
T_A	annealing temperature
TCS	Two-component system
TEMED	Tetramethylethylenediamine
T_m	melting temperature
Tris	Tris-(hydroxymethyl)-aminomethane
TSS	transcriptional start site
U	Unit
UAS	upstream activating sequence
UDP-GlcNac	Uridine diphosphate N-acetyl D-glucosamine
UMP	Uridine monophosphate
UTR	untranslated region
UV	ultraviolet
V	Volt
VC	vector control / empty vector
wt	<i>wild-type</i>
X-Gal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside
µg	microgram
µL	Microliter

3. Abstract

The cell envelope of Gram-negative bacteria is a crucial barrier protecting the cells from changing environmental conditions and detrimental chemicals, such as antibiotics. To overcome damages to the envelope, bacteria have evolved complex signal transduction pathways. In *Escherichia coli*, a key player of the envelope stress response is the σ factor σ^E . However, the full extent of the σ^E response in *E. coli* is not understood.

Recently, it was discovered that induction of the σ^E response leads to a burst of *rpoN* operon expression via activation of the *lptAp1* promoter located upstream. In this thesis, the activation of *lptAp1* was characterized by transposon mutagenesis and addition of the antimicrobial peptide thanatin. Taken together, the data suggests that this promoter specifically responds to lipopolysaccharide defects.

In addition, the results of this thesis indicate transcriptional readthrough from the *lptAp1* promoter into the *rpoN* operon. Consequently, all proteins encoded by this operon, including RapZ, are more abundant upon activation of σ^E . The RNA binding protein RapZ controls biosynthesis of cell envelope precursors by regulating levels of enzyme GlmS in response to the abundance of its product, glucosamine-6-phosphate, via two small RNAs, GlmY and GlmZ. The results show that envelope stress increases RapZ levels and therefore also affects processing of the sRNA GlmZ, which impacts the regulatory GlmY/GlmZ/*glmS* circuit, likely preventing *de novo* biosynthesis of cell envelope components when the envelope is defective. Moreover, a putative role of RapZ in the regulation of cold-shock related transcripts was analyzed, but results from a previous RNA sequencing analysis could not be confirmed.

4. Introduction

4.1. The cell envelope of Gram-negative bacteria

The cell envelope of Gram-negative bacteria is a complex multilayered structure that is composed of the inner membrane (IM), the periplasm containing a thin layer of peptidoglycan (PG) and the outer membrane (OM) (Figure 1) (Silhavy *et al.*, 2010).

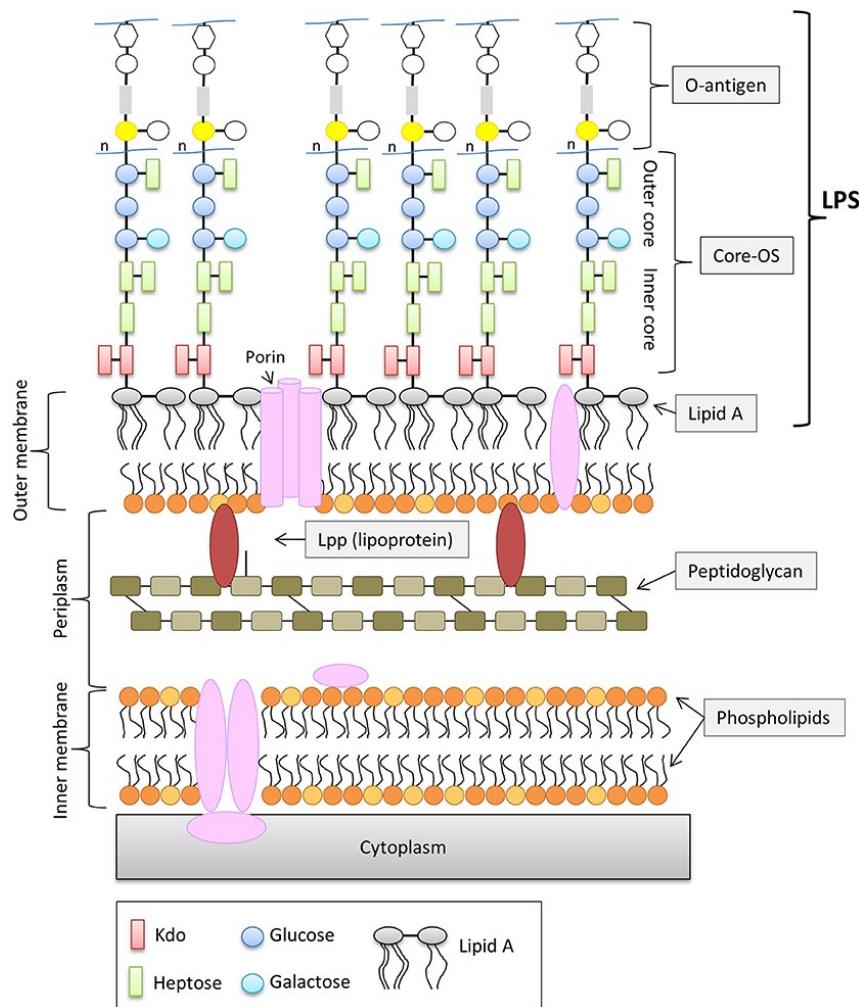


Figure 1. The cell envelope structure of *Escherichia coli* (Ebbensgaard *et al.*, 2018).

The IM of *E. coli* is a symmetric lipid bilayer composed of glycerophospholipids that provides the final barrier between the environment and the cytoplasm. It contains various membrane proteins that are important for cellular functions and play a role in energy production, protein transport and secretion. The periplasm is an aqueous cellular compartment delimited by the IM and the OM that contains different proteins involved in transport and metabolic functions (Mitchell and Silhavy, 2019). It includes a layer of peptidoglycan (PG) which provides mechanical stability and protects the cell from osmotic lysis (Silhavy *et al.*, 2010; Martorana *et al.*, 2021). The PG is connected to the OM by a murein lipoprotein (Lpp), or Braun's

lipoprotein (Braun, 1975) which is the most abundant protein in *E. coli* (Silhavy *et al.*, 2010). The outer membrane is a distinctive feature of Gram-negative bacteria that works as a selective permeability barrier against various toxic compounds (e.g. antibiotics, AMPs or detergents) (Nikaido, 2003) and is essential for maintaining the turgor pressure and resisting different environmental conditions (Klein and Raina, 2019). At the same time, it also represents the bacterium's Achilles heel as it is targeted by a wide range of different antibiotics. The OM of *E. coli* is an asymmetric lipid bilayer that is composed of glycerophospholipids in its inner leaflet and lipopolysaccharides (LPS) in its outer leaflet. LPS of Gram-negative bacteria is a complex glycolipid that consists of three moieties: the conserved lipid A that anchors the molecule in the membrane, the core oligosaccharide that contributes to membrane stability, and the highly variable O-antigen polysaccharide chain (Raetz and Whitfield, 2002). Hydrophilic lateral interactions between LPS molecules provide the permeability barrier function of the OM (Raetz and Whitfield, 2002; Nikaido, 2003). LPS is synthesized in the cytoplasm and further transported from the IM, across the periplasm, to the cell surface where it is inserted into the OM. The proteins associated with the OM are either lipoproteins, embedded in the inner leaflet of the OM by a lipid anchor, or outer membrane β -barrel proteins (OMPs), forming pores in the OM to allow the passive diffusion of small hydrophilic molecules through the OM (Nikaido, 2003; Silhavy *et al.*, 2010). The toxic molecules that are able to penetrate the OM are removed by the efflux pumps that contribute to OM's impermeability and are to some extent responsible for antibiotic resistance in bacteria (Silhavy *et al.*, 2010; Mitchell and Silhavy, 2019). Due to its importance, the biosynthesis and translocation of the cell envelope components is a complex and tightly regulated process.

4.1.1. Role of the Hexosamine Biosynthesis Pathway for synthesis of UDP-GlcNAc – an essential precursor for envelope synthesis

One essential common precursor required for biosynthesis of peptidoglycan and LPS is uridine diphosphate N-acetyl D-glucosamine (UDP-GlcNAc), which is the end product of the hexosamine biosynthesis pathway (Figure 2) (Milewski, 2002).

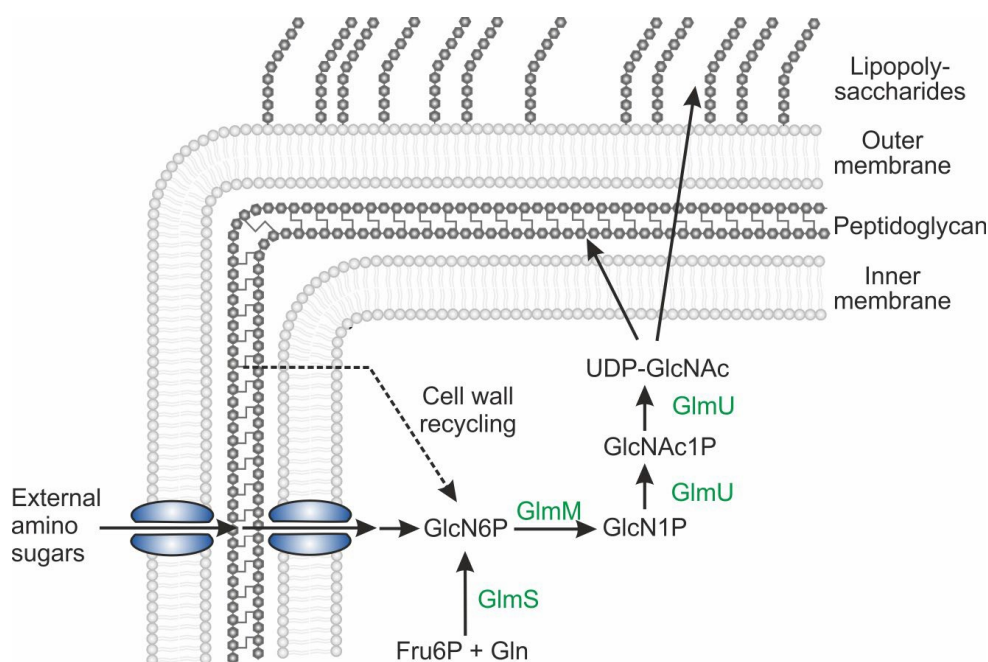


Figure 2. Biosynthetic pathway of UDP-N-acetylglucosamine in *Escherichia coli* (Figure provided by Görke B., unpublished).

The hexosamine biosynthesis pathway is initiated with the synthesis of glucosamine-6-phosphate (GlcN6P) from fructose-6-phosphate (Fru6P) and L-glutamine (Gln) which is the first committed step towards the synthesis of UDP-GlcNAc and cell envelope biogenesis (Teplyakov *et al.*, 2002; Khan *et al.*, 2016). The reaction is catalyzed by the enzyme glucosamine-6-phosphate synthase GlmS (Milewski, 2002; Göpel *et al.*, 2014; Klein and Raina, 2019). GlmS is the key enzyme of the hexosamine biosynthesis pathway and is essential for bacterial viability, unless exogenous amino sugars (e.g. N-acetylglucosamine) are present in the environment (Wu and Wu, 1971; Postma *et al.*, 1993; Khan *et al.*, 2020). In that case, exogenous amino sugars can be internalized and converted to GlcN6P, thereby bypassing the need for the *de-novo* GlcN6P biosynthesis by the enzyme GlmS (Wu and Wu, 1971; Khan *et al.*, 2020). Additionally, GlcN6P can be generated by cell wall recycling (Johnson *et al.*, 2014). Once generated, GlcN6P is either degraded by the enzyme NagB to Fru6P and ammonium, or it is converted into glucosamine-1-phosphate (GlcN1P) by the enzyme phosphoglucosamine mutase GlmM (White, 1968; Göpel *et al.*, 2014; Johnson *et al.*, 2014). Finally, UDP-GlcNAc is synthesized from GlcN1P in the two final reactions of the hexosamine biosynthesis pathway that are catalyzed by the bifunctional enzyme GlmU, which contains two domains that carry out each reaction independently (Mengin-Lecreux and Van Heijenoort, 1996). UDP-GlcNAc is the last common precursor required for the biosynthesis of cell wall peptidoglycan and LPS in bacteria. As the enzyme GlmS is essential for the cell wall homeostasis and bacterial viability, it represents a good target for antimicrobial compounds (Khan *et al.*, 2016).

4.1.2. Cell envelope biogenesis

Outer membrane biogenesis is a complex, highly regulated process. Synthesis of all the components of the Gram-negative cell envelope begins in the cytoplasm or at the inner surface of the IM (Silhavy *et al.*, 2010) and further transport of those components to their designated place faces several challenges. Initially, all the cell envelope components must be translocated across the IM to the aqueous periplasmic space between the IM and the OM and the hydrophobic components of the OM must be inserted into the OM. While the transport across the IM is energy-dependent and generally driven by ATP hydrolysis, the periplasmic environment is devoid of ATP (Konovalova *et al.*, 2017).

4.1.2.1. Transport of OM proteins

The translocation of OM proteins across the IM is achieved by either the general secretion pathway (Sec-pathway) or by the twin-arginine-translocation pathway (Tat-pathway). The Sec-pathway only transports unfolded proteins in an ATP-dependent manner while the Tat-pathway can transport already folded proteins or proteins bound to a co-factor by utilizing the PMF (Berks, 1996; Berks *et al.*, 2005). All the OM proteins, such as lipoproteins or β -barrel proteins (OMPs), are initially made in precursor form with a signal sequence (signal recognition peptide – SRP) that targets them for translocation from the cytoplasm (Driessen and Nouwen, 2008; Silhavy *et al.*, 2010). The translocation reaction is catalyzed by the IM protein complex SecYEG (Van Den Berg *et al.*, 2004). In the periplasm, OM proteins are bound by the soluble periplasmic chaperones which deliver them to their respective OM acceptor (Silhavy *et al.*, 2010). Lipoproteins are bound by the chaperone LolA that delivers them to the OM acceptor protein LolB and OMPs are bound by a redundant network of chaperons (i.e. SurA, Skp, DegP) that deliver them to the β -barrel assembly machine (Bam complex) in the OM (Konovalova *et al.*, 2017). Subsequently, LolB and Bam complex facilitate the insertion of OM proteins into the OM.

4.1.2.2. Biosynthesis and transport of LPS

Lipopolysaccharides are a major component of the Gram-negative outer membrane that to a large extent contribute to the permeability barrier properties of the OM (Raetz and Whitfield, 2002). In *E. coli*, more than 100 genes are required for the synthesis, assembly and translocation of LPS (Klein and Raina, 2019). LPS of Gram-negative bacteria is a complex glycolipid that consists of three moieties (Figure 1): the conserved hydrophobic lipid A that anchors the molecule in the membrane, the phosphorylated core oligosaccharide that contributes to membrane stability, and the highly variable O-antigen polysaccharide chain that extends away from the cell surface (Raetz and Whitfield, 2002; Klein and Raina, 2019). The lipid A moiety is mostly conserved among the species of Gram-negative bacteria and the

core oligosaccharide moiety is genus specific, while the O-antigen polysaccharide chains significantly vary in their structure (Raetz and Whitfield, 2002). The core oligosaccharide moiety consists of two parts, the inner core oligosaccharide that is critical for permeability barrier function of the OM and the outer core oligosaccharide that provides the attachment site for the O-antigen moiety (Raetz and Whitfield, 2002). Smooth-form LPS is a mature form of LPS that comprises all three moieties, while rough-form LPS consists of the lipid A and the core oligosaccharide but lacks the O-antigen (Kalynychn *et al.*, 2014). *Escherichia coli* K-12 strains generally form only the rough-form LPS due to mutations in the O-antigen operon that developed during cultivation of this strain in the laboratory (Stevenson *et al.*, 1994).

The LPS biosynthesis begins in the cytoplasm with the synthesis of lipid A (Figure 3). In *E. coli*, lipid A is synthesized at the inner (cytoplasmic) leaflet of the IM by a conserved pathway of nine constitutive enzymes, each encoded by a single structural gene (Raetz and Whitfield, 2002). The first step in this pathway is the fatty acylation of the UDP-GlcNAc, the final product of the hexosamine biosynthesis pathway, which is catalyzed by the enzyme LpxA (Anderson *et al.*, 1985). In a series of subsequent reactions which are catalyzed by the enzymes LpxC, LpxD, LpxH, LpxB, respectively, the characteristic β -1'-6 linked lipid A disaccharide is generated (Figure 3). Following its insertion into the inner leaflet of the IM, the enzyme LpxK phosphorylates the disaccharide to form the kinase lipid IV_A (Ray and Raetz, 1987). Next, the bifunctional enzyme KdtA (WaaA) transfers two Kdo (2-keto-3deoxy-D-manno-octulosonic acid) residues to lipid IV_A (Belunis and Raetz, 1992). Finally, in the last steps of lipid A biosynthesis, hexaacylated lipid A is generated by the addition of the secondary lauroyl and myristoyl residues to the distal glucosamine unit of lipid IV_A (Brozek and Raetz, 1990). The two reactions are catalyzed by the acyltransferases LpxL and LpxM consecutively (Brozek and Raetz, 1990) and the completed Kdo₂-lipid A serves as the acceptor for the core oligosaccharide and lipo-oligosaccharide chains (Bertani and Ruiz, 2018). In *E. coli*, the chromosomal *waa* region contains the major core-oligosaccharide assembly operons (Whitfield *et al.*, 2003).

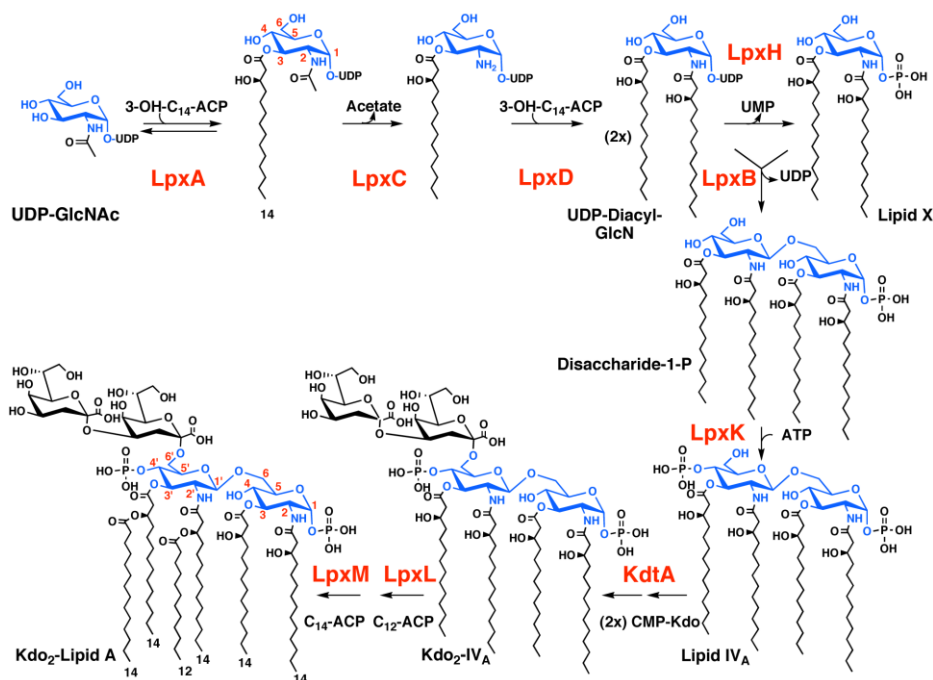


Figure 3. Biosynthetic constitutive pathway of Kdo₂-lipid A synthesis in *Escherichia coli* (Raetz *et al.*, 2007).

Upon the completion of Kdo₂-lipid A synthesis, the rough-form LPS (lipid A-core oligosaccharide) is synthesized by a conserved pathway involving sequential glycosyl transfer from nucleotide sugar precursors. At the inner (cytoplasmic) leaflet of the IM, the coordinated complex of membrane-associated glycosyltransferases (Waa proteins) assembles the core oligosaccharide chains to the Kdo₂-lipid A (Figure 4) (Whitfield *et al.*, 2003). First, the heptosyltransferases WaaC and WaaF sequentially catalyze the addition of two heptose residues to the Kdo₂-lipid A (Gronow *et al.*, 2000). The first heptose moiety that was initially added by WaaC is then phosphorylated by the LPS kinase WaaP. Subsequently, another heptose moiety is attached to the second heptose moiety that was initially added by WaaF in a reaction that is catalyzed by the LPS heptosyltransferase WaaQ (Yethon *et al.*, 1998). Next, the first step in the synthesis of the outer core is carried out by the LPS glucosyltransferase WaaG that adds a glucose residue to the second heptose moiety that was initially added by WaaF (Whitfield *et al.*, 2003). The second heptose moiety that was initially added by WaaF is then phosphorylated by the LPS kinase WaaY. The LPS glycosyltransferase WaaB then adds a galactose residue to the glucose residue that was previously added by WaaG and the glycosyltransferase WaaO subsequently adds one more glucose residue to the same glucose residue (Whitfield *et al.*, 2003). Furthermore, another glucose residue is added by the LPS glycosyltransferase WaaJ to the second glucose residue that was previously added by WaaO (Whitfield *et al.*, 2003). Finally, the LPS heptosyltransferase WaaU adds a heptose group the last glucose moiety that was previously added by WaaJ and the synthesis of the rough-form LPS is complete (Whitfield *et al.*, 2003).

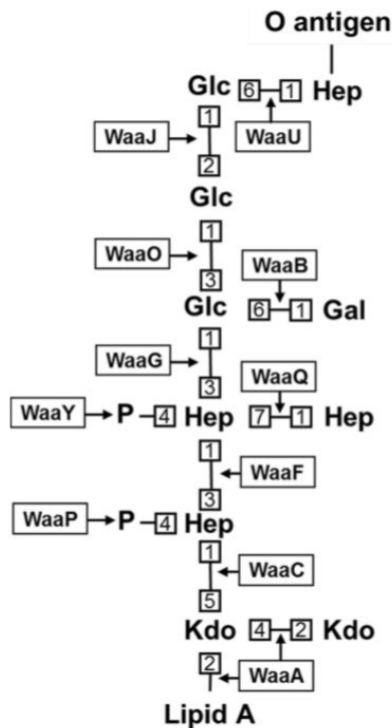


Figure 4. The pathway of core-oligosaccharide synthesis in *Escherichia coli* (Bertani and Ruiz, 2018).

LPS is synthesized in the cytoplasm and further transported from the IM, across the periplasm, to the cell surface where it is inserted into the OM. Rough-form LPS (lipid A-core oligosaccharide) is synthesized at the inner leaflet of the IM from where it is translocated to the outer leaflet of the IM by the ATP-binding cassette (ABC) transporter MsbA (Mi *et al.*, 2017). MsbA-driven flipping of rough LPS to the periplasmic leaflet of the IM is driven by ATP hydrolysis (Mi *et al.*, 2017). As MsbA exhibits a preference for hexa-acylated LPS transport, it provides the first checkpoint for LPS selectivity, preventing the transport of premature LPS intermediates (Ho *et al.*, 2018). At the periplasmic leaflet of the IM, the O-antigen (undecaprenyl-diphosphate-linked intermediate) is ligated to a terminal sugar of the rough-form LPS (lipid A-core oligosaccharide) (Ruan *et al.*, 2012). The reaction is catalyzed by the enzyme WaaL which plays a key role in the formation of a mature, smooth-form LPS (Valvano, 2011).

4.1.2.2.1. Lpt machinery

Subsequently, the translocation of mature LPS to the outer leaflet of the OM is achieved by a dedicated LPS transport (Lpt) machinery that spans from the IM to the OM as a so-called Lpt bridge (Wu *et al.*, 2006). The LPS machinery in *E. coli* is comprised of seven essential proteins that are organized in three sub-complexes, each located in one cell envelope compartment (Figure 5A) (Sperandeo *et al.*, 2008). The ATP-binding cassette (ABC) transporter LptB₂CFG extracts LPS from the IM, pushing it into the Lpt bridge (Okuda *et al.*,

2012) that spans the periplasm and protects the hydrophobic LPS acyl chains from the hydrophilic environment during the transport (Sperandeo *et al.*, 2008). Subunit LptC of the LptB₂CFG transporter drives the ATP hydrolysis and provides the docking site for the periplasmic protein LptA (Sperandeo *et al.*, 2011). LptA monomers connect the LptB₂CFG subcomplex at the IM to the LptDE subcomplex at the OM (Sperandeo *et al.*, 2007) but the exact number of LptA monomers comprising the periplasmic bridge is still unknown. LPS is transported through the Lpt bridge to the OM where the LptDE translocon inserts it into the outer leaflet at the cell surface (Tran *et al.*, 2010). The LptDE translocon is comprised of the β -barrel protein LptD and the small lipoprotein LptE that is buried inside the barrel (Freinkman *et al.*, 2011). To avoid LPS mistargeting, the assembly of the Lpt machinery is carefully monitored at key regulatory checkpoints: unless the LptB₂CFG complex is correctly formed at the IM, the recruitment of LptA by the LptC protein does not occur (Villa *et al.*, 2013) and furthermore, unless the LptDE translocon is correctly assembled at the OM, LptA fails to interact and the Lpt bridge does not form (Martorana *et al.*, 2021). The periplasmic domains of LptF, LptG, LptC, LptA and LptD proteins all share a conserved β -jellyroll fold domain (Suits *et al.*, 2008; Tran *et al.*, 2010; Qiao *et al.*, 2014) that mediates the interactions between them and enables the formation of a hydrophobic Lpt bridge that spans the periplasm and protects the LPS acyl chains during transport (Villa *et al.*, 2013; Okuda *et al.*, 2017; Sperandeo *et al.*, 2019). For a functional Lpt machinery, the interaction between the Lpt proteins is of critical importance and the depletion of any component results in the accumulation of a colanic-acid modified LPS at the outer leaflet of the IM (Sperandeo *et al.*, 2011). LptA undergoes degradation upon incorrect assembly of the Lpt bridge, suggesting that LptA stability and appearance of modified forms of LPS are reflecting Lpt defects (Sperandeo *et al.*, 2011). Due to its importance in Gram-negative bacteria, LPS and proteins involved in its transport and biosynthesis represent crucial antibiotic targets.

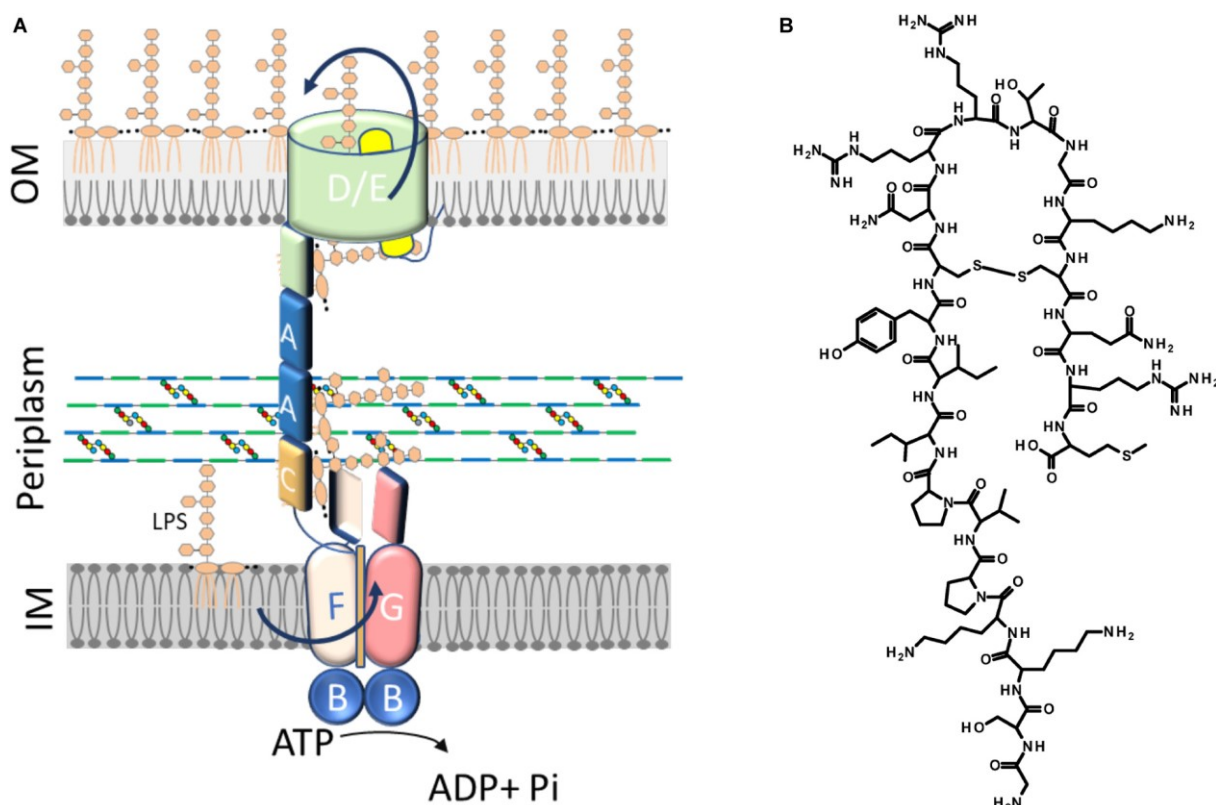


Figure 5. The Lpt machinery in *Escherichia coli* (Moura *et al.*, 2020). A. The lipopolysaccharide transport system in *Escherichia coli*. **B.** The structure of thanatin.

Thanatin is a naturally occurring 21-residue long cationic antimicrobial peptide (CAMP), isolated from the insect *Podisus maculiventris* (spined soldier bug), that contains one disulfide bond and exhibits antimicrobial activity against a broad spectrum of organisms, including *E. coli* (Figure 5B) (Fehlbaum *et al.*, 1996). The minimal inhibitory concentration (MIC) of thanatin against *E. coli* was reported to be below 1,5 μM (Vetterli *et al.*, 2018). Thanatin is one of the few known inhibitors of the LPS transport machinery. It binds LptA and LptD, disrupting the interactions required for the correct Lpt complex assembly (Vetterli *et al.*, 2018), resulting in LptA degradation as well as the accumulation of colanic-acid modified LPS (Moura *et al.*, 2020). The disassembly of the Lpt machinery leads to interruption of LPS transport to the OM and defects in membrane assembly (Moura *et al.*, 2020).

4.1.3. Cell envelope stress response systems in *E. coli*

The cell envelope of Gram-negative bacteria is a complex permeability barrier which is constantly exposed to harmful molecules that can cause damage within the cell, but also to the cell envelope itself. To adapt and survive under these deleterious conditions, bacteria possess envelope stress response (ESR) systems that respond to different types of stressors, helping to limit and repair the damages in order to maintain the cellular homeostasis. Each ESR is triggered by the activation of sensor proteins which in turn causes alterations of gene expression, achieved through the corresponding signaling cascades

(Mitchell and Silhavy, 2019). These alterations of gene expression generally include quality control factors or lead to up- or downregulation of biosynthesis of the respective envelope components (Grabowicz and Silhavy, 2017). While the ESR systems are often tightly intertwined with each other, it is believed that each system responds to a certain type of envelope stress (Laloux and Collet, 2017).

4.1.3.1. Causes of cell envelope stress

Different changes in the cell's environment, toxic molecules and antibiotics can cause stress to the cell envelope. Temperature changes influence membrane fluidity and biosynthesis of the cell envelope components (Pogliano and Beckwith, 1993). Changes in pH induce misfolding of proteins and lead to disruption of enzymatic reactions (Mitchell and Silhavy, 2019). Furthermore, changes in osmolarity influence the permeability barrier function of the cell envelope and unfavorable redox conditions cause oxidative damage to proteins and membranes (Arts *et al.*, 2015; Wood, 2015). Nutrient limitations as well impact the folding of proteins and synthesis of the cell envelope components, leading to a loss of OM integrity (Mitchell and Silhavy, 2019). In addition, the components of the cell envelope can be damaged by toxic molecules (e.g. ethanol, detergents like bile salts, SDS or EDTA) and antibiotics (e.g. β -lactams targeting PG, polymyxins targeting LPS) that are present in the environment (Merritt and Donaldson, 2009; Farris *et al.*, 2010; Mitchell and Silhavy, 2019). There are also intrinsic sources of stress that lead to accumulation of unfolded protein in the envelope and the cytoplasm. This translational stress is caused either by the lack of specific amino acids or by mutations in the protein translation machinery (Mohler and Ibba, 2017).

4.1.3.2. The σ^E -mediated envelope stress response

The sigma factor RpoE (σ^E) represents the major cell envelope stress response system of Gram-negative bacteria, including *Escherichia coli* (Mitchell and Silhavy, 2019). Its regulon comprises more than 100 genes that are mostly linked to functions of the envelope (Dartigalongue *et al.*, 2001). The σ^E responds to the buildup of misfolded OMPs in the periplasm and to perturbations in LPS biosynthesis and trafficking problems (Walsh *et al.*, 2003; Lima *et al.*, 2013). The production of altered forms of LPS (Mitchell and Silhavy, 2019) or depletion of the LPS transport machinery (Lpt) components cause activation of the σ^E ESR (Klein and Raina, 2019) which regulates the extracytoplasmic functions of LPS translocation and some of its modifications (Klein *et al.*, 2016). Possibly, the activating signals responding to various stresses, all causing the σ^E activation, are sensed differently. There are also other ESR systems that are often tightly intertwined with the σ^E ESR.

The σ^E response is achieved through a proteolytic cascade that leads to cleavage of the anti-sigma factor RseA and the release of σ^E from the inner leaflet of the cytoplasmic membrane

(Walsh *et al.*, 2003), allowing it to associate with RNAP and activate its regulon (Figure 6). The σ^E regulon comprises genes encoding periplasmic chaperones and proteases as well as small RNAs, stopping the ongoing synthesis of abundant OMPs and lipoproteins (Klein and Raina, 2017). In the absence of stress, σ^E is associated with an IM protein anti-sigma factor RseA and sequestered at the inner (cytoplasmic) leaflet of the IM to prevent binding to the core polymerase and, thus, transcription of its target genes (Ades *et al.*, 1999). Accumulating unfolded OMPs in the periplasm activate the IM protease DegS through direct interaction which causes a conformational change in DegS, initiating a proteolytic cascade that leads to cleavage of RseA and removal of its periplasmic domain (Walsh *et al.*, 2003). The anti-sigma factor stabilizing protein RseB is a negative regulator of σ^E activity (Missiakas *et al.*, 1997) that binds to the periplasmic region of RseA and inhibits the cleavage of RseA by DegS (Cezairliyan and Sauer, 2007). Possibly, RseB is displaced either through the interaction with unfolded OMPs (Chaba *et al.*, 2011) or with the acyl chains of LPS (Lima *et al.*, 2013), but the exact mechanism is still unknown. Additionally, in the absence of stress, the periplasmic domain of RseA causes steric hindrance that prevents another IM protease, RseP, from cleaving RseA within the transmembrane domain (Akiyama *et al.*, 2004; Akiyama *et al.*, 2015). Once the periplasmic domain of RseA is removed by DegS, RseP carries out a second cleavage of RseA that releases σ^E , which is still bound to a small soluble fragment of RseA, into the cytoplasm (Flynn *et al.*, 2004). Afterwards, the cytoplasmic proteases remove the soluble fragment of RseA and free σ^E is able to direct the RNA-polymerase to transcribe genes from σ^E -dependent promoters that generally lead to the upregulation of OMP folding pathways (Rhodius *et al.*, 2006). The σ^E regulon comprises genes encoding periplasmic chaperones that assist in correct OMP folding, periplasmic proteases that degrade misfolded OMPs (Rhodius *et al.*, 2006) and small RNAs MicA, RybB and MicL that control ongoing synthesis of abundant OMPs and lipoproteins (Gogol *et al.*, 2011; Klein and Raina, 2017). Additionally, σ^E also regulates genes involved in LPS biosynthesis and its translocation (Rhodius *et al.*, 2006). The OMP homeostasis is consequently restored by decreased production of OMPs and increased quality control factors that prevent mislocated or misfolded OMPs and insert them into the OM (Mitchell and Silhavy, 2019).

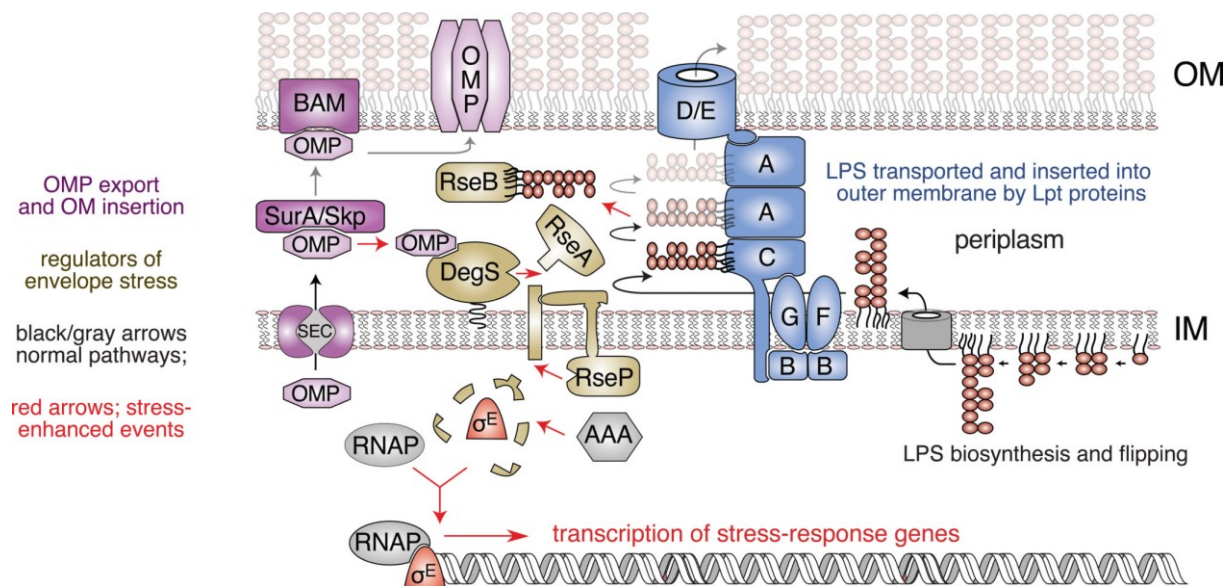


Figure 6. The σ^E ESR in *Escherichia coli* (Lima *et al.*, 2013).

4.1.3.2.1. σ^E responds to LPS damage

The sigma factor σ^E also responds to LPS damage as the production of altered forms of LPS (Mitchell and Silhavy, 2019) or depletion of the LPS transport machinery (Lpt) components (Klein and Raina, 2019). The induction of σ^E likely occurs by displacement of RseB through the interaction with the LPS acyl side chains that accumulate in the periplasm (Lima *et al.*, 2013). Six promoters direct the transcription of *rpoE* and as two of them respond to LPS defects (Klein *et al.*, 2016), transcriptional regulation may additionally contribute to the activation of σ^E by LPS stress. The *rpoE-P3* promoter is σ^{70} (RpoD)-dependent and directly controlled by the Rcs system in response to LPS core biosynthesis defects and overproduction of certain lipoproteins (Klein and Raina, 2019). This could explain why certain LPS defects that induce the Rcs system, also induce the σ^E ESR (Klein and Raina, 2019). Furthermore, the *rpoE-P2* promoter is σ^{54} (RpoN)-dependent and positively controlled by the TCS QseE/QseF in response to defects in the core oligosaccharide of LPS (Klein *et al.*, 2016). The TCS QseE/QseF employs the OM-anchored lipoprotein QseG that is essential for signal transduction (Göpel and Görke, 2018; Khan *et al.*, 2020), reminiscent of the Rcs system that employs the OM-lipoprotein sensor RcsF (Laloux and Collet, 2017). Consequently, induction of σ^E may involve different signaling pathways depending on the nature of LPS defect. LPS stress activates multiple ESR systems in parallel, resulting in global changes primarily but not exclusively in the envelope (Martorana *et al.*, 2014).

4.1.3.3. Additional specialized ESR systems

Additional ESR systems exist that are often tightly intertwined with the σ^E response. The Rcs (regulator of capsule synthesis) is a two-component system that responds to multiple envelope stresses including LPS alterations, changes in peptidoglycan synthesis and defects

in lipoprotein trafficking (Laloux and Collet, 2017). It is composed of the OM-lipoprotein sensor RcsF that responds to changes in the OM integrity, the histidine kinase RcsC, the phosphotransferase RcsD and the response regulator RcsB. Under non-inducing conditions, Rcs system activation is repressed by the IM protein IgaA that interacts with RcsC or RcsD to prevent RcsB from being phosphorylated (Wall *et al.*, 2018). Upon stress sensing, RcsF interacts directly with the IgaA repressor to remove Rcs signaling inhibition (Cho *et al.*, 2014). Consequently, the histidine kinase RcsC autophosphorylates and then phosphorylates the phosphotransferase RcsD which in turn phosphorylates the response regulator RcsB, activating it for transcriptional regulation (Takeda *et al.*, 2001). RcsB can either act alone as a homodimer or with RscA as a heterodimer to regulate transcription of the Rcs regulon (Wall *et al.*, 2018).

The Psp (phage shock protein) system responds to severe disruptions of the IM that are impairing the proton motive force (Flores-Kim and Darwin, 2016). It is composed of the bacterial enhancer binding protein (bEBP) PspF and the IM proteins PspB, PspC and PspA. Under non-inducing conditions, PspF is repressed by physical interaction with PspA (Dworkin *et al.*, 2000). Upon stress sensing, PspB and PspC bind to PspA to release PspF which is then able to activate the *psp* genes from σ^{54} (RpoN)-dependent promoters (Jovanovic *et al.*, 1996). Consequently, proton leakage is prevented by formation of an oligomeric PspA complex and aerobic respiration is down-regulated (Kobayashi *et al.*, 2007).

The Cpx system responds to IM protein folding stress signals and NlpE-dependent signals (Mitchell and Silhavy, 2019) like increased pH, changes in osmolarity or changes in phospholipid composition (May *et al.*, 2019). The signals are sensed by the canonical Cpx TCS that is composed of the sensor histidine kinase CpxA which autophosphorylates upon stress sensing (Raivio and Silhavy, 1997). Afterwards, it phosphorylates the response regulator CpxR, activating it for transcriptional regulation. Furthermore, the phosphorylated CpxR activates the expression of the periplasmic accessory protein CpxP which in turn inhibits CpxA activation, providing an internal negative feedback mechanism on the system (Raivio *et al.*, 1999). The Cpx regulon comprises a large number of genes that are linked to IM-associated proteins and functions of the envelope (Dorel *et al.*, 2006; Raivio *et al.*, 2013). Once the CpxA/CpxR TCS is activated, it induces the synthesis of periplasmic chaperones (DegP, Skp, DsbA) that are involved in folding and degradation of proteins (Dorel *et al.*, 2006). The expression of periplasmic proteases DegP and Skp is regulated by σ^E and by the CpxA/CpxR TCS (Dartigalongue *et al.*, 2001; Yamamoto and Ishihama, 2005; Rhodius *et al.*, 2006).

The EnvZ/OmpR system responds to changes in environmental osmolality and acid stress (Gerken *et al.*, 2009). The signals are sensed by the canonical EnvZ/OmpR TCS that is composed of the sensor kinase EnvZ which autophosphorylates upon increasing medium osmolality (Forst *et al.*, 1989). Subsequently, it phosphorylates the response regulator OmpR, which acts as the cytoplasmic transcription factor, regulating the expression of the two major OMP porins OmpF and OmpC and the expression of the sRNAs OmrA and OmrB, which in turn regulate the OM protein composition (Mizuno and Mizushima, 1990; Aiba and Mizuno, 1990; Guillier and Gottesman, 2006). While the expression of the OMP porin OmpC is favoured in high osmolality, the expression of OmpF is favoured in low osmolality (Alphen and Lugtenberg, 1977). When osmolality increases, phosphorylated OmpR changes the ratio of two major OMP porins, repressing the expression of *ompF* and activating the expression of *ompC* (Pratt *et al.*, 1996). In addition, the IM protein MzrA regulates the EnvZ/OmpR TCS by interacting with EnvZ and modulating the activity of the system (Gerken *et al.*, 2009; Gerken and Misra, 2010). Furthermore, MzrA connects the EnvZ/OmpR TCS to the CpxA/CpxR TCS in order to enable the cells to appropriately adjust the composition of the cell envelope in response to envelope stress (Gerken *et al.*, 2009).

The Bae (bacterial adaptive response) system responds to exposure to toxic molecules like ethanol, indole and zinc (Raffa and Raivio, 2002; Bury-Moné *et al.*, 2009; Leblanc *et al.*, 2011) from which the cell can be protected by efflux. The signals are sensed by the canonical Bae TCS that is composed of the sensor histidine kinase BaeS which phosphorylates the response regulator BaeR (Nagasawa *et al.*, 1993). BaeR has a small regulon that results in increased levels of the periplasmic chaperone Spy, several efflux pumps and BaeS/R (Nishino *et al.*, 2005).

The PhoPQ system responds to Mg^{2+} limitation (Kato *et al.*, 1999). The environmental Mg^{2+} concentrations are sensed by the sensor histidine kinase PhoQ which autophosphorylates upon Mg^{2+} decreases and in turn phosphorylates the response regulator PhoP, activating it for transcriptional regulation (Kato *et al.*, 1999; Minagawa *et al.*, 2003; Zwir *et al.*, 2005). The PhoP regulon comprises a large number of genes that are involved in magnesium transport, acid resistance and LPS modifications (Kato *et al.*, 1999; Minagawa *et al.*, 2003; Zwir *et al.*, 2005). Furthermore, the phosphorylated PhoP activates the expression of the IM protein MgrB which in turn inhibits PhoQ activation, providing a negative feedback mechanism in the PhoPQ TCS (Lippa and Goulian, 2009; Salazar *et al.*, 2016).

The BasSR system responds to high concentrations of external Fe^{3+} or mild acidity (Hagiwara *et al.*, 2004). The signals are sensed by the sensor histidine kinase BasS which autophosphorylates in the presence of excess Fe^{3+} and subsequently phosphorylates the

response regulator BasR, which in turn induces transcription of the *arnBCADTEF* operon (Nagasawa *et al.*, 1993; Hagiwara *et al.*, 2004). The products of the *arn* operon modify the lipid A of LPS (Hagiwara *et al.*, 2004). The BasS/BasR TCS of *E. coli* is homologous to the PmrA/PmrB TCS of *Salmonella enterica*, which is a major regulator of LPS modification in response to the extracellular iron concentrations (Chen and Groisman, 2013). Furthermore, in *E. coli*, the BasS/BasR TCS regulates susceptibility to the Polymyxin B, a cationic antimicrobial peptide, by activating the transcription of the multidrug efflux pump EmrD (Froelich *et al.*, 2006; Yu *et al.*, 2020). These observations are consistent with the PmrB/PmrA TCS in *Salmonella enterica* (Wösten *et al.*, 2000; Yu *et al.*, 2020).

4.2. The *E. coli* *rpoN* operon

The *E. coli* *rpoN* operon consists of *rpoN*, *hpf*, *ptsN*, *rapZ* and *npr* (Figure 7). It is preceded by *lptA* and *lptB* that are part of the *yrbG-lptB* locus, which encodes genes for OM biogenesis. The gene products of *lptA* and *lptB* are components of the Lpt (LPS) machinery that is dedicated to LPS transport from the IM to the OM in *E. coli* (Sperandeo *et al.*, 2011). According to current knowledge, the *rpoN* operon is transcribed from various promoters: the constitutively active σ^{70} -dependent promoter *rpoNp* (Jones *et al.*, 1994; Studholme and Buck, 2000) which is located between *lptB* and *rpoN* genes, the σ^E -dependent promoter *lptBp* (Rhodius *et al.*, 2006) which is located within the *lptA* gene, and the two closely spaced promoters, σ^E -dependent promoter *lptAp1* and the σ^{70} -dependent promoter *lptAp2* (Martorana *et al.*, 2011), which are located upstream of *lptA* gene (Figure 7). The σ^E -dependent *lptAp1* promoter is activated by specific stress conditions which induce modifications in LPS composition and biogenesis (Martorana *et al.*, 2011) and the second σ^E -dependent promoter, *lptBp*, is more or less inactive *in vitro* and *in vivo* (Mutalik *et al.*, 2009; Rhodius and Mutalik, 2010).

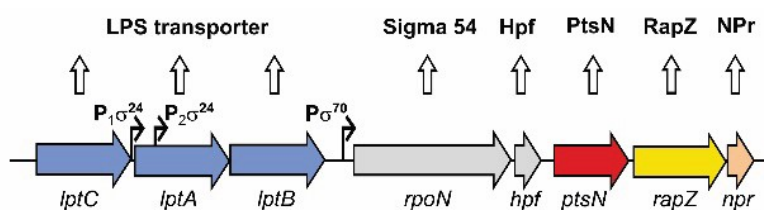


Figure 7. The *Escherichia coli* *rpoN* operon (Figure provided by Görke B., unpublished). The *rpoN* operon consists of *rpoN*, *hpf*, *ptsN*, *rapZ* and *npr*. It is preceded by *lptA* and *lptB* that are part of the *yrbG-lptB* locus.

The gene *rpoN* encodes the alternative RNAP sigma factor RpoN (σ^{54} or σ^N) which controls ~130 genes of seemingly unrelated functions such as nitrogen assimilation during starvation, amino sugar metabolism, flagella synthesis, cell envelope homeostasis and also genes involved in the Psp ESR system (Studholme and Buck, 2000; Bonocora *et al.*, 2015; Riordan

and Mitra, 2017). Contrary to all other σ -factors, σ^{54} has no sequence homology to σ^{70} , but it strictly depends on activator proteins for functionality (Studholme and Buck, 2000). Bacterial enhancer binding proteins (bEBPs) are transcriptional activators that bind far upstream of cognate promoters to upstream activating sequences (UASs) and utilize ATPase activity to form an open promoter complex and trigger σ^{54} -dependent transcription initiation (Reitzer and Schneider, 2001).

The gene *hpf* encodes the ribosome hibernation promoting factor (HPF) which protects ribosomes under stress conditions, likely by blocking ribonuclease from accessing its target sites within the ribosome (Prossliner *et al.*, 2021). HPF together with the ribosome modulation factor (RMF) jointly converts active 70S ribosomes to inactive 100S dimers in a process called ribosome hibernation (Maki *et al.*, 2000; Prossliner *et al.*, 2018). The translationally inactive 100S ribosome dimers are required for survival in the stationary growth phase (Ueta *et al.*, 2008). Generally, ribosome hibernation provides protection by preventing the loss of ribosomal proteins at the dimer interface and by restricting ribonuclease access to rRNA (Feaga *et al.*, 2020). A quick conversion to active 70S ribosomes takes place once the cells are transferred to fresh growth medium and have better growth conditions again (Maki *et al.*, 2000; Ueta *et al.*, 2008).

The genes *ptsN* and *npr* encode proteins PtsN and NPr which are components of the nitrogen-related phosphotransferase system (PTS^{Ntr}) (Powell *et al.*, 1995; Pflüger-Grau and Görke, 2010; Bahr *et al.*, 2011; Lüttmann *et al.*, 2015). The gene *rapZ* encodes the RNase E adaptor protein RapZ which contributes to the cell envelope precursor homeostasis by regulating *glmS* expression in response to the level of glucosamine-6-phosphate (GlcN6P) via sRNAs GlmY and GlmZ (Khan and Görke, 2020). The functions of those genes will be explained in details in the following chapters.

4.2.1. Connections between the *rpoN* operon and cell envelope stress

The σ^E represents the major cell envelope stress response system of Gram-negative bacteria (Lima *et al.*, 2013; Klein and Raina, 2019; Mitchell and Silhavy, 2019) and also responds to LPS damages including defects in LPS synthesis, biogenesis and assembly as well as depletion of the Lpt machinery (Tam and Missiakas, 2005; Klein and Raina, 2019). Recently, it was discovered that activation of the σ^E ESR, achieved by ectopic expression of *rpoE* from a plasmid, leads to burst of *rpoN* operon at the transcriptional level (Sikora and Görke, unpublished). This likely results from the read-through transcription of the σ^E -dependent *lptAp1* promoter upstream of the *rpoN* operon (Martorana *et al.*, 2011) or by another σ^E -responsive element within the *rpoN* operon, or a combination of these regulatory elements, indicating that the *rpoN* operon likely is a member of the σ^E regulon. All gene products of the

rpoN operon (except for *rpoN* itself) exert regulatory functions at the post-transcriptional (RapZ) and post-translational level (Hpf, PTS^{Ntr}). Thus, they might help bacteria to counteract envelope stress through interaction with target proteins or sRNAs.

4.2.2. Phosphotransferase systems (PTSs)

To efficiently take up carbohydrates from the environment, Gram-negative and Gram-positive bacteria possess phosphotransferase systems (PTSs) that use phosphoenolpyruvate (PEP) as an energy source for the sugar transport. The PTS is a phospho-relay system referring to a group of enzymes that transfer phosphoryl-moieties derived from PEP amongst the members of the system in a given order (Pflüger-Grau and Görke, 2010). Two general types of PTSs exist in many proteobacteria: the canonical (sugar/transport) PTS which catalyzes the uptake and phosphorylation of carbohydrates into the cell and exerts regulatory functions by coordinating various proteins and genes in response to carbon source availability (Postma *et al.*, 1993; Deutscher *et al.*, 2014), and the nitrogen-related PTS (PTS^{Ntr}) which exerts diverse regulatory functions such as potassium homeostasis, carbon utilization and secondary metabolite production in *E. coli* (Pflüger-Grau and Görke, 2010; Pflüger-Grau and de Lorenzo, 2014). The PTS^{Ntr} is present only in Gram-negative bacteria and is highly conserved in all proteobacteria, with the exception of the ϵ -proteobacteria (Barabote and Saier, 2005; Pflüger-Grau and Görke, 2010). Both PTS systems are phosphorylated on the expense of PEP and in both systems, the PEP-derived active phosphoryl group is transferred in a cascade from the enzyme I (EI or EI^{Ntr} encoded by *ptsI* and *ptsP*, respectively) to the histidine protein (HPr or NPr encoded by *ptsH* and *ptsO*, respectively) and then further to the enzyme II (EIIA^{Glc} or EIIA^{Ntr} encoded by *crr* and *ptsN*, respectively) (Figure 8). The phosphotransferase enzymes EI and HPr are cytoplasmic proteins in both types of systems, while the phosphotransferase enzymes EII are different. The EII of the canonical PTS is membrane-bound transport protein complex composed of at least three domains (EIIA, EIIB, EIIC) which can be either fused in a single polypeptide or exist as separated interactive chains (Postma *et al.*, 1993; Deutscher *et al.*, 2014). The EIIA^{Glc} subunit of the glucose transporter is a soluble cytoplasmic protein that exerts regulatory functions in addition to the glucose uptake (Görke and Stülke, 2008). By transferring the phosphoryl group from the EIIA domain to the EIIB domain and finally to the sugar that is transported by the EIIC domain, the various EII transporters enable the uptake of specific PTS-sugars (Postma *et al.*, 1993; Deutscher *et al.*, 2006; Deutscher *et al.*, 2014). Numerous bacteria possess various EII transporters, each specific for a certain carbohydrate substrate (Postma *et al.*, 1993). Once the transported sugar gets phosphorylated by the EIIB domain, it cannot leak out of the cell anymore, thus resulting in a one-way concentration gradient of the substrate. With the exception of phosphorylation of the transported sugar, all the other phosphorylation steps in

the PTS phospho-relay system are reversible (Cozzone, 1998). The $EIIA^{Ntr}$ of the nitrogen-related PTS is homologous to the EIIA domain of the canonical EII (Powell *et al.*, 1995). Depending on its phosphorylation state, the $EIIA^{Ntr}$ exerts regulatory functions of the PTS^{Ntr} (Pflüger-Grau and Görke, 2010) that will be explained further in the following chapter.

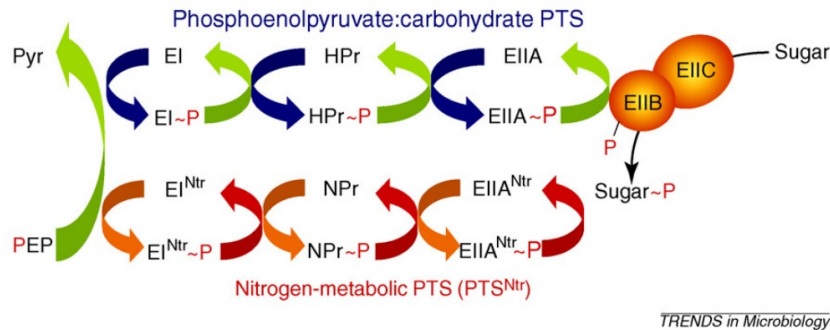


Figure 8. The two parallel phosphotransferase system in bacteria (Pflüger-Grau and Görke, 2010).

As already mentioned, the canonical PTS also exerts regulatory tasks in response to carbon source availability through the phosphorylation state of the $EIIA^{Glc}$ subunit of the glucose transporter (Postma *et al.*, 1993; Deutscher *et al.*, 2014). The phosphorylation state of the latter is determined by the PTS transport activity and the PEP/ pyruvate ratio (Hogema *et al.*, 1998). When enterobacteria utilize a PTS-sugar (e.g. glucose) and/ or when the PEP/ pyruvate ratio is low, the phosphoryl groups are drained from the $EIIA^{Glc}$ transporters during the process of glucose uptake (Hogema *et al.*, 1998; Bettenbrock *et al.*, 2007). This results in a predominantly dephosphorylated form of $EIIA^{Glc}$ which inhibits transporters and enzymes for the metabolism of secondary carbon sources in a process called inducer exclusion (Görke and Stülke, 2008). Furthermore, when enterobacteria utilize less preferred carbon sources, which often are not PTS substrates (e.g. lactose, galactose, glycerol), and/ or when the PEP/ pyruvate ratio is high, the $EIIA^{Glc}$ accumulates in its phosphorylated form which activates the adenylate cyclase (CyaA) (Hogema *et al.*, 1998). CyaA in turn catalyzes the synthesis of cAMP (Harwood *et al.*, 1976; Feucht and Saier, 1980). cAMP is an important signaling molecule that activates the expression of many secondary carbon utilization systems by binding to the CAP protein (Botsford and Harman, 1992).

Both PTSs work in parallel and are independently active, but they can communicate in a process referred to as crosstalk by the exchange of phosphoryl groups amongst the members of both systems (Pflüger and De Lorenzo, 2008; Zimmer *et al.*, 2008). $EIIA^{Ntr}$ (PtsN) can be phosphorylated by either NPr or HPr, but this crosstalk is not possible when the cells are growing on a PTS substrate which is draining the phosphoryl-groups away from HPr (Lüttmann *et al.*, 2015). NPr can as well be phosphorylated by either EI^{Ntr} (PtsP) or EI, but with lower efficiency (Zimmer *et al.*, 2008). Contrarily, EI^{Ntr} (PtsP) is specific for NPr and is

only able to phosphorylate NPr, but not HPr as well (Lüttmann *et al.*, 2015). Taken together, the phosphorylation state of the EIIA^{Ntr} can be additionally modulated by the activity of the canonical PTS via cross-phosphorylation (Rabus *et al.*, 1999; Pflüger-Grau and Görke, 2010).

4.2.2.1. The nitrogen-related Phosphotransferase system (PTS^{Ntr})

Genes *ptsN* and *npr* of the *rpoN* operon encode proteins (EIIA^{Ntr} and NPr, respectively) working in the nitrogen-related phosphotransferase system (PTS^{Ntr}) that regulates various activities through protein-protein interaction which are predominantly exerted by the non-phosphorylated form of PtsN (EIIA^{Ntr}) (Figure 9). The gene *ptsN* encodes the phosphotransferase enzyme EIIA^{Ntr} that is homologous to the EI domain of the canonical phosphotransferase enzyme EII and the gene *npr* encodes the phosphotransfer protein NPr that is homologous to the histidine protein HPr of the canonical PTS. The third member of the PTS^{Ntr}, the phosphotransferase enzyme EI^{Ntr}, is encoded by *ptsP* in a separate operon together with *rppH*. The EI^{Ntr} consists of two domains: a C-terminal domain which is homologous to the canonical phosphotransferase enzyme EI and an N-terminal GAF signaling domain (Reizer *et al.*, 1996). GAF domains bind a variety of diverse small ligands and contribute to signal sensing, thus they are present in many signaling and sensory proteins (Aravind and Ponting, 1997; Pflüger-Grau and Görke, 2010). Several transcriptional regulators of the NifA family contain the GAF domain which is involved in nitrogen sensing (Little and Dixon, 2003; Pflüger-Grau and Görke, 2010). Furthermore, several bacterial activator proteins (bEBPs) that trigger σ^{54} -dependent transcription initiation (e.g. NorR and FhIA) contain a GAF domain (Tucker *et al.*, 2006; Pflüger-Grau and Görke, 2010).

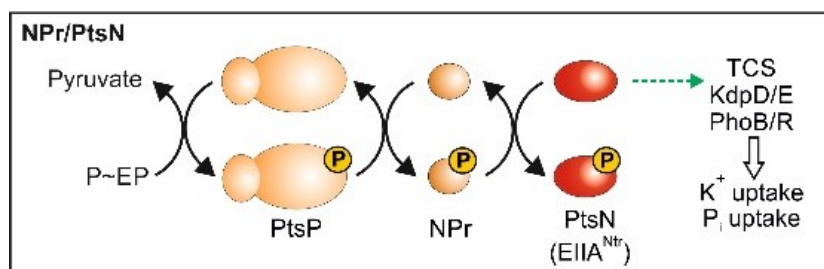


Figure 9. The nitrogen-related phosphotransferase system (Figure provided by Görke B., unpublished). PTS^{Ntr} is phosphorylated on the expense of the PEP from which the phosphoryl group is transferred in a phospho-relay system to the PtsN (EIIA^{Ntr}) which exerts regulatory functions of the PTS^{Ntr}. The non-phosphorylated form of PtsN activates the kinases of the TCSs KdpDE and PhoBR to achieve potassium homeostasis.

Similar to the canonical PTS, PTS^{Ntr} is phosphorylated on the expense of PEP that acts as a phosphoryl group donor. The PEP-derived phosphoryl group is transferred in a cascade from EI^{Ntr} via NPr to histidine residue 73 of the EIIA^{Ntr} (Rabus *et al.*, 1999; Zimmer *et al.*, 2008). The EIIA^{Ntr} is the terminal phosphate acceptor within the PTS^{Ntr} that exerts regulatory

functions of the PTS^{Ntr} depending on its phosphorylation state (Postma *et al.*, 1993; Pflüger-Grau and Görke, 2010). The phosphorylation state of the EIIA^{Ntr} can be additionally modulated by the activity of the canonical PTS via cross-phosphorylation (Pflüger-Grau and Görke, 2010), as EIIA^{Ntr} is also capable of exchanging phosphoryl groups with HPr of the canonical PTS (Zimmer *et al.*, 2008). The EIIA^{Ntr} is predominantly present in its phosphorylated form in *wild-type E. coli* cells (Lüttmann *et al.*, 2015). In mutant cells lacking EI^{Ntr}, the phosphorylation state of EIIA^{Ntr} is dependent on the utilized carbon source (Jahn *et al.*, 2013; Lüttmann *et al.*, 2015). When *E. coli* cells utilize a PTS-sugar, the phosphoryl groups are used for carbohydrate uptake, thus decreasing the cross-phosphorylation of EIIA^{Ntr} from HPr, resulting in a predominantly dephosphorylated form of EIIA^{Ntr} (Jahn *et al.*, 2013; Lüttmann *et al.*, 2015). In contrast, when *E. coli* cells utilize non PTS-sugars, EIIA^{Ntr} accumulates in its phosphorylated form. Therefore, the canonical PTS likely controls the phosphorylation state of EIIA^{Ntr} through cross-talk under conditions that inhibit the activity of EI^{Ntr} (Lüttmann *et al.*, 2015). Furthermore, Lee *et al.* (2013) have reported that the phosphorylation state of EIIA^{Ntr} is dependent on the glutamine/ α -ketoglutarate (Gln/ α -KG) ratio which indirectly reflects the nitrogen availability in the cell (Lee *et al.*, 2013). High glutamine levels block autophosphorylation of EI^{Ntr} by PEP *in vitro*, while high α -ketoglutarate levels stimulate it, thereby regulating the phosphorylation state of EIIA^{Ntr} (Lee *et al.*, 2013). Since this effect was abolished in the EI^{Ntr} mutant lacking the GAF domain, it has been suggested that nitrogen availability is sensed via the glutamine/ α -ketoglutarate ratio by the GAF domain of the EI^{Ntr} (Lee *et al.*, 2013). Nitrogen starvation is signaled by a low glutamine/ α -ketoglutarate ratio and results in phosphorylation of the EIIA^{Ntr}, while nitrogen sufficiency is indicated by a high glutamine/ α -ketoglutarate ratio and results in a dephosphorylated form of EIIA^{Ntr}. Nevertheless, those observations could not be confirmed in an independent study (Jahn *et al.*, 2013; Lüttmann *et al.*, 2015).

Recently, it was discovered that the phosphorylation state of EIIA^{Ntr} can be additionally modulated by the phosphohistidine phosphatase SixA via dephosphorylation of NPr (Schulte and Goulian, 2018; Schulte *et al.*, 2020). Dephosphorylation of NPr by SixA proceeds through a formation of a transient phosphohistidine intermediate and results in the phosphoryl group transfer from a histidine residue on NPr to a histidine residue on SixA (Schulte *et al.*, 2020). SixA is a part of the diverse histidine phosphatase superfamily (Rigden, 2008; Rigden, 2020) and contains a conserved active-site histidine (His 8) that is essential for catalysis (Hamda *et al.*, 2005). SixA was suggested to be part of the σ^E regulon (Rezuchova *et al.*, 2003).

4.2.2.2. Regulatory targets of PTS^{Ntr}

The PTS^{Ntr} regulates diverse metabolic processes such as potassium uptake, carbon utilization and secondary metabolite production (Pflüger-Grau and Görke, 2010; Jahn *et al.*, 2013; Pflüger-Grau and de Lorenzo, 2014) through protein-protein interaction which are predominantly exerted by the non-phosphorylated form of PtsN (EIIA^{Ntr}). However, recent ligand fishing experiments also suggested numerous interaction partners for the phosphorylated form of EIIA^{Ntr} (Gravina *et al.*, 2021). The PTS^{Ntr} was named 'nitrogen-related' PTS because two of its genes, *ptsN* and *npr*, are localized in the *rpoN* operon together with *rpoN*, which encodes the alternative sigma factor σ^{54} , controlling the expression of many genes involved in nitrogen assimilation (Powell *et al.*, 1995; Reitzer and Schneider, 2001). Since the impact of PTS^{Ntr} on nitrogen metabolism still remains unclear and could be somehow indirect, it is likely not its main target (Pflüger-Grau and Görke, 2010; Reaves and Rabinowitz, 2011). Nevertheless, the PTS^{Ntr} plays a major role in the regulation of potassium (K⁺) homeostasis in *E. coli* by controlling the expression and activity of a high- and a low-affinity K⁺ transporter (Figure 10) (Lee *et al.*, 2007; Lüttmann *et al.*, 2009; Mörk-Mörkenstein *et al.*, 2017). When external or intracellular potassium levels are low, the kinase (KdpD) of the TCS is active, leading to phosphorylation of the cognate response regulator (KdpE), which in turn upregulates expression of the *kdpFABC* locus encoding the components of the high affinity K⁺ transporter to maintain the cellular homeostasis (Heermann and Jung, 2010; Schramke *et al.*, 2016). The dephosphorylated form of PtsN can interact with the kinase KdpD to stimulate the activity of the TCS, likely coordinating another stimulus, such as carbon or nitrogen metabolism, with K⁺ homeostasis (Mörk-Mörkenstein *et al.*, 2017). In *E. coli*, potassium is the crucial and most abundant monovalent intracellular cation that is required for maintenance of important cellular functions like cell turgor, osmolarity, pH homeostasis and it is also important for the enzymatic activity of many proteins (Silver, 1996; Lüttmann *et al.*, 2009). When external potassium concentrations are sufficient, two low affinity K⁺ transporters of the Trk family, the constitutively expressed Trk and Kup, catalyze potassium uptake (Silver, 1996). The Trk transporter is composed of the regulatory NAD-binding peripheral membrane protein TrkA and the integral membrane K⁺-translocating unit TrkH or TrkG (Pflüger-Grau and Görke, 2010). The non-phosphorylated form of EIIA^{Ntr} is able to bind the TrkA subunit and downregulate the activity of transporter to inhibit potassium uptake (Lee *et al.*, 2007). However, when external potassium concentrations are low, the Trk transporters are insufficient and the potassium homeostasis is maintained by the high-affinity ATP-dependent K⁺ transporter KdpFABC (Haupt *et al.*, 2006). The KdpFABC consists of four subunits: the inner membrane subunit KdpA that contains the K⁺ binding site and forms the K⁺ transport channel (Buurman *et al.*, 1995; Silberberg *et al.*, 2021), the transmembrane KdpB subunit that forms a phosphorylated intermediate and provides energy for the K⁺

uptake (Siebers and Altendorf, 1989; Haupt *et al.*, 2006) and the KdpC and KdpF subunits that are necessary for assembly and stabilization of the transport complex (Gassel *et al.*, 1999). The TCS KdpDE controls the expression of the respective transporter in response to potassium availability and changes in medium osmolality (Walderhaug *et al.*, 1992; Heermann *et al.*, 2003). Decreasing potassium concentrations (Laermann *et al.*, 2013) or increase in the osmolality (Heermann *et al.*, 2003) trigger autophosphorylation of the membrane-bound K^+ sensor histidine kinase KdpD that transfers the phosphoryl group to the cytoplasmic response regulator KdpE (Nakashima *et al.*, 1992; Jung *et al.*, 1997), which in turn binds upstream of the *kdpFABC* promoter and activates transcription of the high affinity K^+ transporter KdpFABC (Narayanan *et al.*, 2014). The non-phosphorylated form of EIIA^{Ntr} stimulates autophosphorylation activity of the histidine kinase KdpD which leads to higher levels of phosphorylated response regulator KdpE, inducing expression of the *kdpFABC* operon to increase potassium uptake (Lüttmann *et al.*, 2009; Mörk-Mörkenstein *et al.*, 2017).

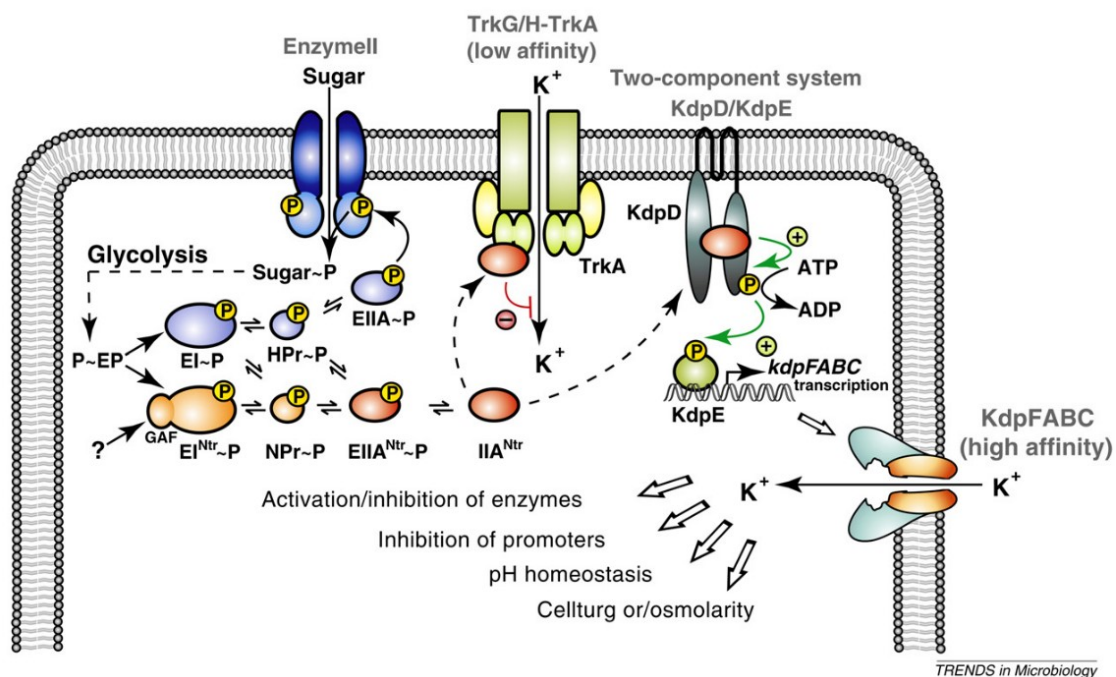


Figure 10. Regulation of potassium uptake by PTS^{Ntr} in *Escherichia coli* (Pflüger-Grau and Görke, 2010).

Additionally, the PTS^{Ntr} plays a role in the regulation of phosphate starvation response by activating the TCS PhoBR (Lamarche *et al.*, 2008; Hsieh and Wanner, 2010). The PhoBR TCS controls expression of a large number of genes for phosphate uptake and assimilation (VanBogelen *et al.*, 1996). The membrane-bound histidine kinase PhoR senses extracellular inorganic phosphate indirectly and when its concentrations are limiting, PhoR phosphorylates and activates the cytoplasmic response regulator and transcription factor PhoB which in turn induces its regulon to cope with phosphate starvation (Hsieh and Wanner, 2010). The non-

phosphorylated form of EIIA^{Ntr} is able to directly bind the histidine kinase PhoR which leads to higher levels of phosphorylated response regulator PhoB, inducing expression of the *phoBR* regulon (Lüttmann *et al.*, 2012). In sum, EIIA^{Ntr} activates the kinases of the TCSs KdpDE and PhoBR, boosting phosphorylation of cognate response regulators, which in turn upregulate their regulons including the high affinity K⁺ transporter KdpFABC and phosphate acquisition genes, respectively (Lüttmann *et al.*, 2009; Lüttmann *et al.*, 2012; Lüttmann *et al.*, 2015; Mörk-Mörkenstein *et al.*, 2017). Furthermore, the K⁺ efflux transporter YcgO and the low affinity K⁺ transporter TrkA are also regulated by EIIA^{Ntr} (Lee *et al.*, 2007; Sharma *et al.*, 2016), suggesting that K⁺ transport is a major target of PTS^{Ntr} in *E. coli* (Deuschle *et al.*, 2015).

4.2.2.3. The role of PTS^{Ntr} on GlmS enzymatic activity

The enzyme GlmS synthesizes glucosamine-6-phosphate (GlcN6P) from fructose-6-phosphate (Fru6P) and L-glutamine (Gln) through glutamine hydrolysis, initiating the amino sugar pathway which generates uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), an essential precursor required for biosynthesis of peptidoglycan and LPS (Milewski, 2002). The multifunctional sRNA-binding protein RapZ post-transcriptionally controls synthesis of GlmS in response to the level of GlcN6P (Göpel *et al.*, 2014). The regulation of GlmS by RapZ will be explained in details in the following chapter. Yoo *et al.* (2016) have reported that the PTS^{Ntr} directly interacts with the GlmS and inhibits its activity *in vitro* in *Salmonella* (Yoo *et al.*, 2016). Remarkably, this regulation is carried out by the phosphorylated form of EIIA^{Ntr} which is rarely observed. When intracellular nitrogen concentrations are excessive, bacteria convert α -ketoglutarate to glutamate (Glu) and further to glutamine (Gln), which is utilized as nitrogen source under nitrogen deficient conditions (Doucette *et al.*, 2011). As previously mentioned, nitrogen starvation is signaled by a low glutamine/ α -ketoglutarate ratio and results in phosphorylation of the EIIA^{Ntr}, while nitrogen sufficiency is indicated by a high glutamine/ α -ketoglutarate ratio and results in dephosphorylation of EIIA^{Ntr}. Yoo *et al.* (2016) carried out an enzymatic assay in which the phosphorylated form of EIIA^{Ntr} exerted an inhibitory effect on GlcN6P synthesis by GlmS (Yoo *et al.*, 2016). Furthermore, in the mutant cells lacking GlmS, the depletion of GlcN6P led to decreased EIIA^{Ntr} levels due to degradation by Lon protease. These results suggest that upon amino sugar depletion, Lon proteases degrade EIIA^{Ntr} in order to release GlmS from the inhibitory complex and supplement the lack of GlcN6P to maintain amino sugar homeostasis (Figure 11) (Yoo *et al.*, 2016). When GlcN6P levels in the cell are sufficient or glutamine is limited, GlmS is not required. Hence, EIIA^{Ntr} is phosphorylated to directly interact with GlmS and inhibit its activity (Yoo *et al.*, 2016). Under these conditions, the GlcN6P degrading enzyme NagB might be activated to produce Fru6P and NH₃ from GlcN6P (Rodionova *et al.*, 2018).

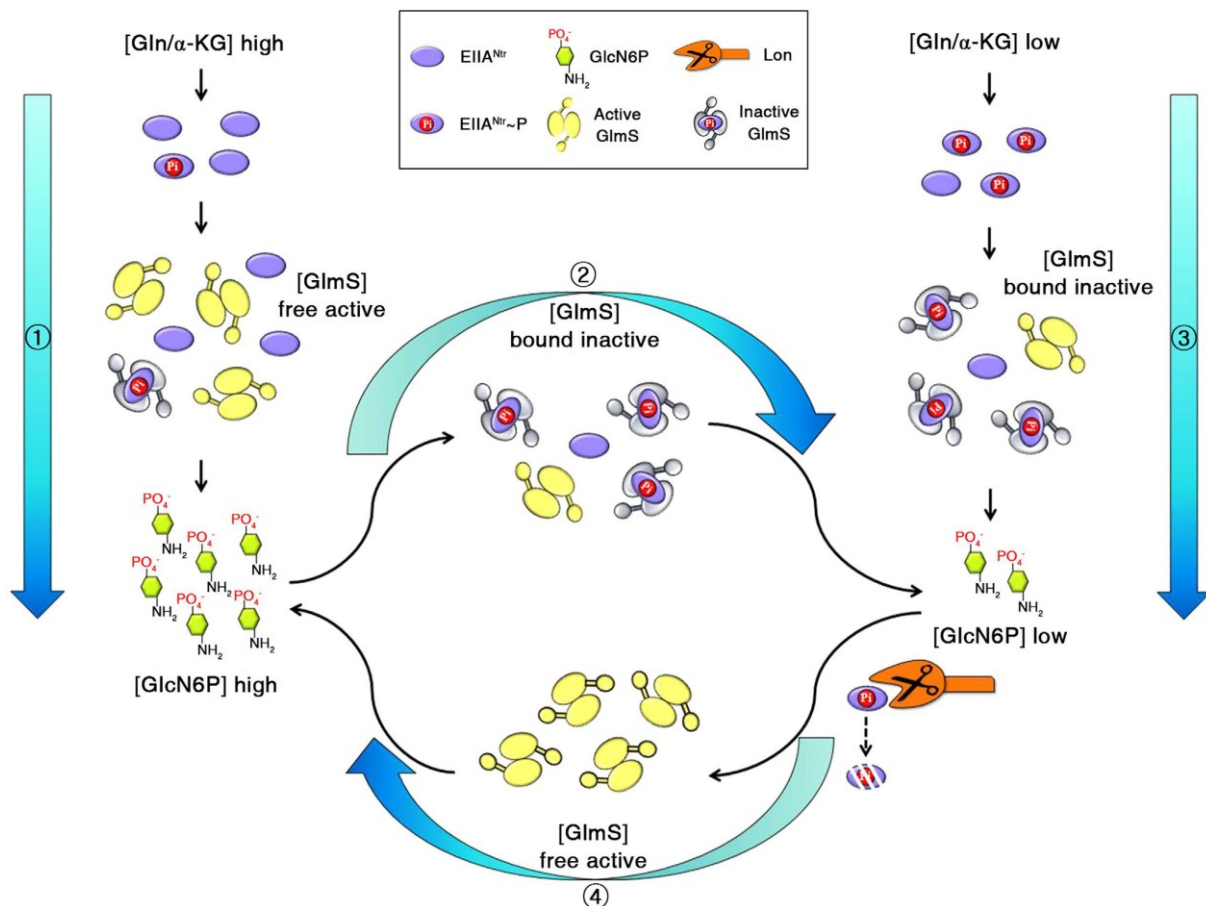


Figure 11. Proposed regulatory model for the control of GlmS activity by EIIA^{Ntr} (Yoo *et al.*, 2016).

Therefore, GlmS activity and thereby GlcN6P levels are likely regulated by the joint activities of EIIA^{Ntr} and RapZ, providing yet another functional connection between the two genes, *ptsN* and *rapZ*, that are localized in the *rpoN* operon, regulating amino sugar homeostasis in response to GlcN6P concentration and nitrogen availability.

4.2.3. RNase adaptor protein RapZ and regulation of GlmS

Protein RapZ is a multifunctional sRNA-binding protein that post-transcriptionally controls the synthesis of glucosamine-6-phosphate synthase GlmS in response to the level of glucosamine-6-phosphate (GlcN6P) (Göpel *et al.*, 2014; Khan and Görke, 2020). The enzyme GlmS synthesizes GlcN6P *de novo*, initiating the amino sugar pathway which generates uridine diphosphate N-acetylglucosamine (UDP-GlcNAc). RapZ regulates *glmS* expression by binding two homologous small RNAs GlmY and GlmZ to achieve GlcN6P homeostasis (Khan and Görke, 2020). In *E. coli*, GlmS is essential when exogenous amino sugars (e.g. GlcN, GlcNAc) which can be directly converted to GlcN6P, are lacking (Kim *et al.*, 2013). However, when *E. coli* cells grow in the presence of exogenous amino sugars that are directly taken up by the sugar transporters (e.g. NagE, ManXYZ) in a PTS-mediated phosphoryl transfer reaction and converted into GlcN6P, the enzymatic activity of GlmS is

dispensable since the *de-novo* GlcN6P biosynthesis is not required (Wu and Wu, 1971; Postma *et al.*, 1993).

4.2.3.1. Two small RNAs GlmY and GlmZ regulate *glmS* expression

In *Enterobacteriaceae*, GlmS is encoded in the *glmUS* operon and expressed as a bi-cistronic mRNA. The bifunctional enzyme GlmU catalyzes the two final reactions of the hexosamine biosynthesis pathway, synthesizing UDP-GlcNAc from GlcN1P (Mengin-Lecreux and Van Heijenoort, 1996). While *glmU* is an essential gene, the mutant cells lacking GlmS are viable if the exogenous amino sugars are supplied to the cells (Plumbridge and Vimr, 1999). The two promoters upstream of the *glmU* gene, which drive expression of the *glmUS* operon, are both controlled by the global transcriptional regulator NagC. NagC modulates expression of genes for the biosynthesis and catabolism of amino sugars by regulating the PTS (Plumbridge, 1991; Plumbridge, 2001; Plumbridge and Pellegrini, 2004; Pennetier *et al.*, 2008). N-acetyl D-glucosamine-6-phosphate (GlcNAc6P) is the product of GlcNAc transport by the PTS and the effector molecule for NagC (Plumbridge, 1991; Plumbridge, 2001). Furthermore, it is also produced from MurNAc6P during the process of cell wall recycling (Park and Uehara, 2008). By binding to the operator sequence upstream of the *glmU* gene, NagC upregulates expression of the *glmUS* operon (Plumbridge *et al.*, 1993). Nevertheless, interaction of NagC with GlcNAc6P releases NagC from the operator sequence and results in decreased expression of the *glmUS* operon, when enough GlcNAc6P is present in the cells which can be converted by enzyme NagA to GlcN6P (Plumbridge *et al.*, 1993). Once synthesized, the *glmUS* transcript is subsequently cleaved by the endoribonuclease RNase E, resulting in two independent *glmU* and *glmS* transcripts which are normally rapidly degraded (Plumbridge, 1995; Kalamorz *et al.*, 2007).

To ensure GlcN6P homeostasis, two small RNAs GlmY and GlmZ regulate *glmS* expression at the post-transcriptional level (Figure 12) (Göpel *et al.*, 2014). The sRNA GlmZ base-pairs with the 5'-UTR of the *glmS* mRNA in an Hfq-dependent manner, disrupting an inhibitory stem loop structure in the *glmS* mRNA, which sequesters the ribosomal binding site. As a result, GlmZ stabilizes the *glmS* mRNA and activates *glmS* translation (Kalamorz *et al.*, 2007; Urban and Vogel, 2008; Göpel *et al.*, 2013). RapZ is an RNase E adaptor protein that controls the stability of GlmZ (Islam *et al.*, 2023). When GlcN6P levels in the cell are sufficient, GlmS is not required. Hence, RapZ binds GlmZ and recruits endoribonuclease RNase E by protein-protein interaction to inactivate GlmZ by processing, which removes the base-pairing nucleotides and results in decreased *glmS* translation (Göpel *et al.*, 2013; Durica-Mitic *et al.*, 2020). Decreasing GlcN6P concentrations cause up-regulation of another

homologous small RNA GlmY (Urban *et al.*, 2007), which mimics GlmZ and is also bound by RapZ (Göpel *et al.*, 2013). GlmY accumulates and inhibits GlmZ processing by binding and sequestering RapZ. As a result, GlmZ remains unprocessed and is able to base-pair with the *glmS* mRNA which results in increased *glmS* translation and in turn GlcN6P synthesis (Göpel *et al.*, 2013; Göpel *et al.*, 2014). Thus, the GlmYZ circuit feedback-regulates *glmS* expression at the post-transcriptional level in order to maintain GlcN6P homeostasis. Furthermore, the sRNA GlmZ is never completely processed. While the processed form of GlmZ can no longer base-pair with the *glmS* mRNA, it is still able to bind and sequester RapZ from the full-length GlmZ, providing an internal feedback mechanism to inhibit the cleavage of the latter and prevent a complete GlmZ turnover (Durica-Mitic and Görke, 2019). Consequently, a small fraction of GlmZ always remains unprocessed in order to maintain *glmS* translation at a basal level.

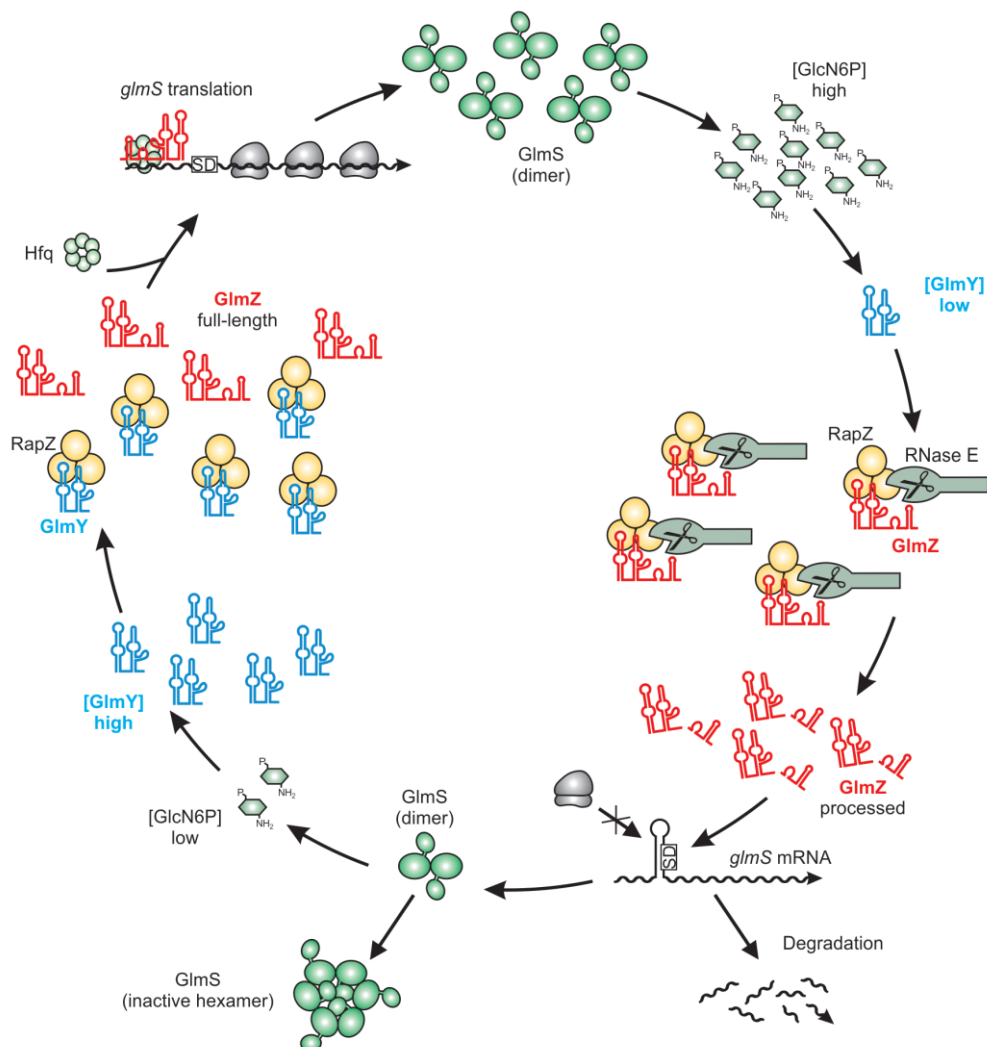


Figure 12. Regulatory GlmY/GlmZ/RapZ circuit maintaining GlcN6P homeostasis in *Escherichia coli* (Göpel *et al.*, 2014).

4.2.3.2. RapZ senses glucosamine-6-phosphate levels

RapZ itself also binds and senses GlcN6P concentrations in the cell (Khan and Görke, 2020). In *E. coli*, two perfectly overlapping σ^{70} - and σ^{54} -dependent promoters control expression of the *glmY* gene (Reichenbach *et al.*, 2009; Göpel *et al.*, 2011). While the constitutively active σ^{70} promoter allows for a basal level of *glmY* expression during the exponential phase, the σ^{54} promoter is controlled by the TCS QseEF and requires activation in a QseF-dependent manner, which can be stimulated by GlcN6P-free RapZ (Khan *et al.*, 2020). The TCS QseEF controls cell envelope related functions (Klein and Raina, 2019) and is involved in maintaining the GlcN6P homeostasis (Khan and Görke, 2020) by activating the expression of sRNA *glmY* and of *rpoE* from σ^{54} -dependent promoters (Reichenbach *et al.*, 2009; Klein *et al.*, 2016). The system consists of the cytoplasmic membrane bound kinase QseE which autophosphorylates and transfers the phosphoryl group to the response regulator QseF. QseF contains a σ^{54} interaction domain and acts as a transcriptional activator that binds far upstream of cognate promoters to form an open promoter complex and trigger σ^{54} -dependent transcription initiation of sRNA *glmY* and of *rpoE* (Reichenbach *et al.*, 2009). Activity of the TCS QseEF depends on the outer membrane lipoprotein QseG which interacts with QseE in the periplasm and is essential for the activation (Göpel and Görke, 2018).

When RapZ is not bound to GlcN6P, it directly interacts with the TCS QseEF, triggering QseE autophosphorylation which in turn activates *glmY* expression from the σ^{54} -dependent promoter in a QseF-dependent manner (Figure 13) (Göpel and Görke, 2018). This interaction depends on the additional component, lipoprotein QseG, which activates QseE by interaction in the periplasm and is essential for the activity of this TCS (Göpel and Görke, 2018). Activation of the latter promoter results in increase of the *glmY* expression which leads to sequestration of RapZ by GlmY into long-lasting complexes and increased *glmS* translation by full-length GlmZ until GlcN6P is replenished (Khan *et al.*, 2020). When RapZ is bound to GlmY, it is unable to interact with the TCS QseEF, providing a negative feedback loop to limit *glmY* expression. Once GlcN6P is replenished, the metabolite again binds RapZ to counteract GlmY binding. Consequently, GlmY is released from RapZ and rapidly degraded (Khan *et al.*, 2020). Taken together, RapZ upregulates its own decoy in order to form long-lasting RapZ/GlmY complexes until GlcN6P levels are replenished (Khan *et al.*, 2020). This mechanism adjusts GlcN6P homeostasis and additionally provides resistance against antibiotics targeting enzymatic activity of GlmS (Khan *et al.*, 2016).

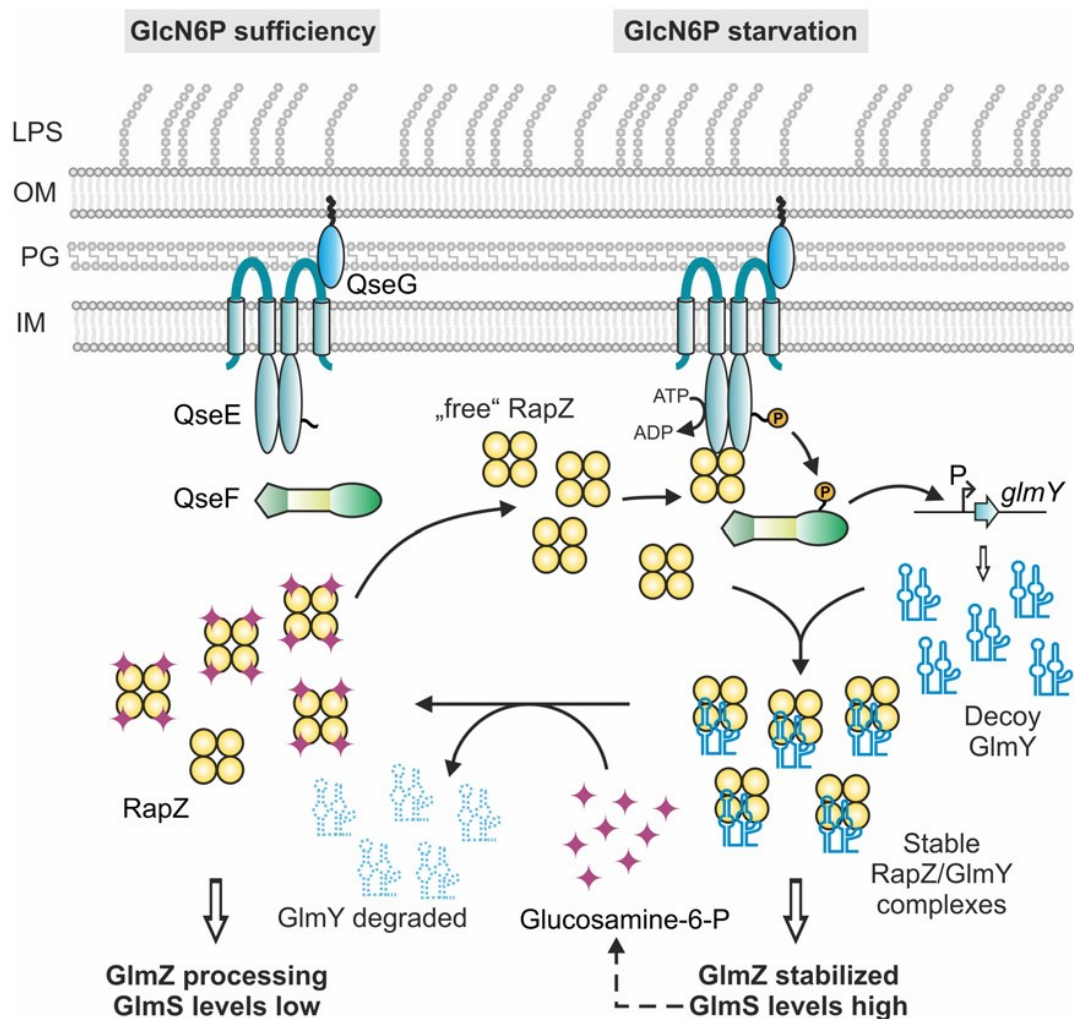


Figure 13. Model for GlcN6P sensing in *Escherichia coli* (Khan *et al.*, 2020).

4.2.3.3. Nva-FMDP targets enzymatic activity of GlmS

The enzyme GlmS synthesizes GlcN6P from fructose-6-phosphate and glutamine, catalyzing the first committed step in the biosynthesis of cell envelope components and, therefore, is a good target for antibiotic treatment. There are several naturally occurring or modified dipeptide antibiotics that selectively inhibit GlmS enzymatic activity, including a synthetic derivative L-norvalyl-N(3)-(4-methoxyfumaroyl)-2,3-diaminopropionic acid (Nva-FMDP) (Figure 14). A peptide transporter such as Dpp is required for the uptake of those antibiotics into the cytoplasm, where they are hydrolyzed and their active moiety is released to impair cell envelope synthesis (Milewski, 2002; Özcengiz and Ögülür, 2015). In case of Nva-FMDP, the active moiety FMDP acts as glutamine analogue and inhibits GlmS by covalently binding to a residue in the glutamine binding pocket, resulting in an irreversible inhibition of GlmS that in turn leads to GlcN6P depletion (Milewski *et al.*, 1986; Kucharczyk *et al.*, 1990). In *Enterobacteriaceae*, including *E. coli*, there is an intrinsic resistance mechanism against antibiotics that inhibit GlmS enzymatic activity. GlcN6P deprivation, caused by Nva-FMDP, leads to upregulation of sRNAs GlmY and GlmZ to achieve increased *glmS* expression in a

dose-dependent manner in order to overcome antibiotic inhibition (Khan *et al.*, 2016). GlcN6P depletion triggers accumulation of sRNA GlmY which in turn sequesters RapZ from processing GlmZ through molecular mimicry. As a result, full-length GlmZ activates translation of *glmS* mRNA in an Hfq-dependent manner, triggering *glmS* expression to replenish intracellular GlcN6P and achieve GlcN6P homeostasis (Khan *et al.*, 2016). In mutant cells lacking GlmY and GlmZ, this response is disabled and the bacteria are highly susceptible to GlmS inhibitors (Khan *et al.*, 2016). However, when exogenous amino sugars which can be directly converted to GlcN6P are present in the environment, the upregulation of GlmS in response to Nva-FMDP does not occur. Under these conditions, the enzymatic activity of GlmS is dispensable and Nva-FMDP becomes ineffective (Khan *et al.*, 2016).

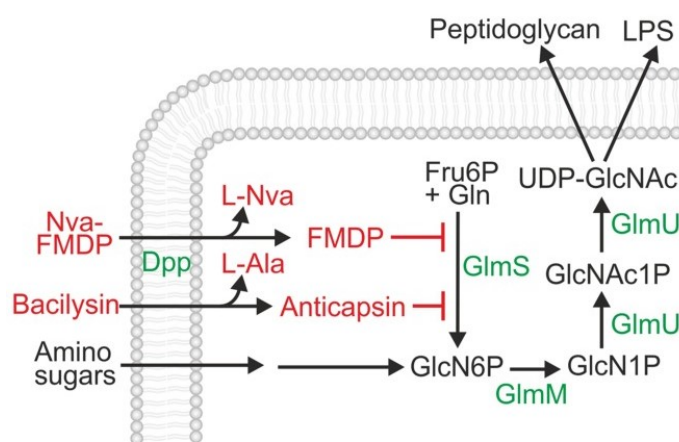


Figure 14. Role, regulation and inhibitors of enzyme GlmS in *Escherichia coli* (Khan *et al.*, 2016).

4.3. Aims of this study

The σ factor RpoE (σ^E) represents the major cell envelope stress response system of Gram-negative bacteria, including *Escherichia coli* (Lima *et al.*, 2013; Klein and Raina, 2019; Mitchell and Silhavy, 2019). RpoE responds to the buildup of unfolded OMPs in the periplasm and to the production of altered forms of LPS (Mitchell and Silhavy, 2019), regulating extracytoplasmic functions of LPS translocation and some of its modifications (Klein *et al.*, 2016). Recently, it was discovered that activation of the σ^E ESR, achieved by ectopic expression of *rpoE* from a plasmid, leads to a burst of *rpoN* operon expression at the transcriptional level (Sikora and Görke, unpublished). This likely results from the read-through transcription of the σ^E -dependent *lptAp1* promoter upstream of the *rpoN* operon (Martorana *et al.*, 2011) and/ or by another σ^E -responsive element within the *rpoN* operon (Sikora and Görke, unpublished). All gene products of the *rpoN* operon (except for *rpoN* itself) exert regulatory functions at the post-transcriptional (RapZ) and post-translational level (Hpf, PTS^{Ntr}). Thus, they might help bacteria to counteract envelope stress by interaction with target proteins or small regulatory RNAs. The RNase E adaptor protein RapZ itself contributes to the cell envelope precursor homeostasis by regulating *glmS* expression in response to the level of glucosamine-6-phosphate (GlcN6P) via sRNAs GlmY and GlmZ (Khan and Görke, 2020).

4.3.1. Revealing the consequences of σ^E -driven upregulation for downstream processes regulated by RapZ

The role of protein RapZ for regulation of *glmS* has so far been studied under the assumption that *rapZ* is constitutively expressed and RapZ amounts do not significantly vary (Göpel *et al.*, 2013; Gonzalez *et al.*, 2017; Durica-Mitic *et al.*, 2020; Khan *et al.*, 2020; Khan and Görke, 2020). However, the activation of σ^E ESR, perhaps mediated by read-through transcription from the *lptAp1* promoter into the *rpoN* operon or by another σ^E -responsive element within the *rpoN* operon, results in increased biosynthesis of RapZ (Sikora and Görke, unpublished). Those higher amounts of RapZ should activate QseE/QseF TCS to upregulate *glmY* expression from the σ^{54} -dependent promoter. In case of RapZ amounts exceeding GlmY amounts, part of the RapZ population is expected to be left un-sequestered and as a consequence, processing of GlmZ by RapZ/RNase E should be reinforced, resulting in even lower *glmS* expression levels. To study the consequences of active σ^E for accumulation of RapZ protein and for the GlmY/GlmZ/*glmS* circuit, σ^E was ectopically expressed either from an arabinose-inducible or from an anhydrotetracycline-inducible promoter on a plasmid. The levels of RapZ and GlmS proteins were addressed by Western Blot analysis and the levels of sRNAs GlmY and GlmZ by Northern Blot analysis. Furthermore, the regulatory elements

responding to envelope stress in the *lptAB* and the *rpoN* operon were dissected in strains carrying different reporter gene fusions (*lptA'-lacZ* and *lptA → rapZ'-lacZ*).

4.3.2. Identification of mutations and compounds that activate the *lptAp1* promoter

Most of the σ^E -dependent expression of the *rpoN* operon comes from the upstream located *lptAp1* promoter (Sikora and Görke, unpublished). The latter promoter was shown to be selectively activated upon depletion of the LPS transport machinery, suggesting that it responds to LPS biogenesis defects and trafficking problems (Martorana *et al.*, 2011; Martorana *et al.*, 2021). However, beyond depletion of the Lpt machinery no signals are known that would lead to activation of the latter promoter. Therefore, a random mutagenesis screen was carried out to search for mutations that cause activation of the *lptAp1* promoter in the strain carrying the *lptA'-lacZ* reporter gene fusion. To further confirm the involvement of the genes, interrupted by the transposon insertions, in the activation of the σ^E -dependent *lptAp1* promoter, clean deletion mutants were established in the *wild-type* strain carrying the *lptA'-lacZ* reporter gene fusion. Additionally, the effect of thanatin on the *lptAp1* promoter and the possible impact on the RapZ/GlmY/GlmZ/*glmS* circuit were addressed as thanatin is one of the few known inhibitors of the LPS transport machinery and could elicit the ESR via the latter promoter.

4.3.3. Identification of mutations that lead to the σ^E -dependent upregulation of *sixA* expression

Genes *ptsN* and *npr* of the *rpoN* operon encode proteins working in the nitrogen-related phosphotransferase system (PTS^{Ntr}). A major role of the PTS^{Ntr} is the regulation of potassium homeostasis (Lee *et al.*, 2007; Lüttmann *et al.*, 2009; Lüttmann *et al.*, 2015; Sharma *et al.*, 2016; Mörk-Mörkenstein *et al.*, 2017). NPr can be dephosphorylated by phosphohistidine phosphatase SixA (Schulte and Goulian, 2018; Schulte *et al.*, 2020) that was suggested to be part of the σ^E regulon (Rezuchova *et al.*, 2003). Therefore, another random mutagenesis screen was carried out in the strain carrying the *sixA'-lacZ* reporter gene fusion to search for genes (mutations) that lead to a σ^E -dependent upregulation of *sixA* expression. These results could bring important insights into the functional connections between PtsN and the regulated potassium transporters, since the physiological conditions leading to dephosphorylation of PtsN are so far not completely clear.

4.3.4. Elucidation of the role of the RapZ protein for expression of cold-shock related transcripts

A recent RNA-seq analysis in the CSH50 (R1279) strain lineage revealed upregulation of a couple of cold-shock related transcripts (*cspI*, *cspH*, *cspB*, *cspF*, *cspG*, *ydfK*, *ynaE*) specifically in a $\Delta glmY \Delta glmZ$ double mutant, but not in a $\Delta rapZ$ single mutant or in a $\Delta rapZ \Delta glmY \Delta glmZ$ triple mutant (Durica-Mitic *et al.*, 2020). To elucidate the role of the RapZ protein for expression of cold-shock related transcripts, the *cspB* and *cspH* mRNA levels in the different mutants were compared by Northern Blot analysis.

5. Materials and Methods

5.1. Materials

5.1.1. Media and plates

E. coli cultures were grown either in liquid LB medium or on LB-agar (LB medium, solidified with 15 g/L agar), supplemented with appropriate antibiotics and/ or inducers when necessary. For selection of phenotypes on the plates, either LB-agar or MacConkey-agar (Carl Roth® and Difco) with lactose was used. MacConkey-lactose plates were prepared from corresponding pre-supplemented agar base that was mixed with dH₂O (50 g base/L).

Table 1. Composition of LB medium.

LB
5 g NaCl (Carl Roth®)
5 g Yeast Extract (Carl Roth®)
10 g Trypton (Carl Roth®)
ad 1L with distilled H ₂ O, autoclave

Depending on the strain to be cultivated, various antibiotics and supplements were added after autoclavation, indicated in Table 2. To select for the presence of plasmids or alleles, tagged with antibiotic resistance markers, different antibiotics were used. 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) is an artificial chromogenic substrate for the enzyme β-galactosidase that can be added to agar plates, allowing phenotypic monitoring of strains carrying *lacZ* fused to a gene of interest. L-arabinose or anhydrotetracycline was used to induce expression of genes that are controlled by a corresponding inducible promoter on a plasmid.

Table 2. List of supplements and antibiotics used in this study.

Antibiotic/Supplement	Manufacturer	Stock	Final concentration
Ampicillin	Carl Roth®	50 mg/mL in 70% ethanol, stored at -20°C	100 µg/mL
Chloramphenicol	PanReac AppliChem	15 mg/mL in 70% ethanol, stored at -20°C	15 µg/mL
Kanamycin	PanReac AppliChem	30 mg/mL in dH ₂ O, stored at 4°C	30 µg/mL
Nva-FMDP	Synthesized and obtained from Dr. Ryszard Andruszkiewicz (Gdańsk University of Technology)	1 mg/mL in dH ₂ O, stored at -20°C	60 µg/mL

Polymyxin B	Sigma-Aldrich	10 mg/mL in dH ₂ O, stored at 4°C	1 µg/mL
Spectinomycin	Sigma-Aldrich	50 mg/mL in dH ₂ O, stored at 4°C	50 µg/mL
Thanatin	Obtained from Dr. Alessandra Polissi (University of Milano)	2,26 mg/mL in dH ₂ O, stored at 4°C	5,25 µg/mL
Anhydrotetracycline (hydrochloride)	Sigma-Aldrich	2 mg/mL in ddH ₂ O, stored at -20°C	1 ng/mL
L-(+)-arabinose	Sigma-Aldrich	20 % (w/v) / 1 M in dH ₂ O, stored at RT	0,2 % (w/v) / 10 mM
X-Gal	Carl Roth®	40 mg/mL in Dimethylformamide, stored at -20°C	40 µg/mL

5.1.2. *Escherichia coli*-K12 strains

All strains used in this study are listed in Table 3.

Table 3. List of *Escherichia coli* strains used in this study.

Strain	Genotype	Reference
FL905	AM604 $\Phi(kan\ araC\ araBp-lptC)1$	(Sperandeo <i>et al.</i> , 2008)
FL907	AM604 $\Phi(kan\ araC\ araBp-lptA)1$	(Sperandeo <i>et al.</i> , 2008)
JW1549	F ⁻ , $\Delta(araD-araB)567$, $\Delta lacZ4787(::rrnB-3)$, λ^- , $\Delta cspB779::kan$, $rph-1$, $\Delta(rhaD-rhaB)568$, $hsdR514$	Keio collection (Baba <i>et al.</i> , 2006)
JW2337	F ⁻ , $\Delta(araD-araB)567$, $\Delta lacZ4787(::rrnB-3)$, λ^- , $\Delta sixA748::kan$, $rph-1$, $\Delta(rhaD-rhaB)568$, $hsdR514$	Keio collection (Baba <i>et al.</i> , 2006)
JW2556	F ⁻ , $\Delta(araD-araB)567$, $\Delta lacZ4787(::rrnB-3)$, λ^- , $\Delta rseA741::kan$, $rph-1$, $\Delta(rhaD-rhaB)568$, $hsdR514$	Keio collection (Baba <i>et al.</i> , 2006)
JW3606	F ⁻ , $\Delta(araD-araB)567$, $\Delta lacZ4787(::rrnB-3)$, λ^- , $\Delta rfaG742::kan$, $rph-1$, $\Delta(rhaD-rhaB)568$, $hsdR514$	Keio collection (Baba <i>et al.</i> , 2006)
JW3607	F ⁻ , $\Delta(araD-araB)567$, $\Delta lacZ4787(::rrnB-3)$, λ^- , $\Delta rfaQ743::kan$, $rph-1$, $\Delta(rhaD-rhaB)568$, $hsdR514$	Keio collection (Baba <i>et al.</i> , 2006)
JW4130	F ⁻ , $\Delta(araD-araB)567$, $\Delta lacZ4787(::rrnB-3)$, λ^- , $\Delta hfq-722::kan$, $rph-1$, $\Delta(rhaD-rhaB)568$, $hsdR514$	Keio collection (Baba <i>et al.</i> , 2006)
JW5134	F ⁻ , $\Delta(araD-araB)567$, $\Delta lacZ4787(::rrnB-3)$, λ^- , $\Delta cspH732::kan$, $rph-1$, $\Delta(rhaD-rhaB)568$, $hsdR514$	Keio collection (Baba <i>et al.</i> , 2006)
R1279	CSH50 $\Delta(pho-bgl)201\ \Delta(lac-pro)\ ara\ thi$	(Schnetzer <i>et al.</i> , 1996; Görke and Rak, 1999)
S4197	MG1655 $rph^+ ilvG^+ \Delta lacZ$	(Venkatesh <i>et al.</i> , 2010)

	(wild-type ancestor strain of mutants needed in this study)	
XL1-Blue	<i>recA1 endA1 gyrA96 thi-I hsdR17 supE44 relAI lac [F' proAB lacI^q ZΔM15Tn10 (Tet^r)]</i>	(Bullock, 1987)
Z37	R1279 <i>ΔrapZ</i>	(Kalamorz <i>et al.</i> , 2007)
Z95	R1279 <i>glmY::cat</i>	(Reichenbach <i>et al.</i> , 2009)
Z106	R1279 <i>ΔglmY ΔglmZ</i>	(Göpel <i>et al.</i> , 2013)
Z367	S4197 <i>attB::kdpFA'-lacZ</i>	(Lüttmann <i>et al.</i> , 2015)
Z404	S4197 <i>ΔptsP</i>	(Lüttmann <i>et al.</i> , 2012)
Z405	S4197 <i>ΔptsN</i>	(Lüttmann <i>et al.</i> , 2012)
Z406	S4197 <i>ΔptsN attB::kdpFA'-lacZ</i>	(Lüttmann <i>et al.</i> , 2015)
Z424	S4197 <i>ptsN-3xFLAG</i>	(Lüttmann <i>et al.</i> , 2015)
Z741	S4197 <i>attB::[glmY-5'-lacZ (glmY-5' (-238 to +22))]</i>	(Khan <i>et al.</i> , 2020)
Z833	S4197 <i>attB::[glmY-5'-lacZ (glmY-5' (-238 to +22))], ΔrapZ</i>	Laboratory collection (constructed by Yvonne Göpel, unpublished)
Z834	S4197 <i>glmY::cat</i>	(Khan <i>et al.</i> , 2016)
Z838	S4197 <i>ΔglmY</i>	(Khan <i>et al.</i> , 2016)
Z839	S4197 <i>ΔglmZ</i>	(Khan <i>et al.</i> , 2016)
Z840	S4197 <i>ΔrapZ</i>	Laboratory collection (constructed by Muna A. Khan, unpublished)
Z854	S4197 <i>attB::[glmS-5'-lacZ (glmS -311 to +129) aadA (spec^R)]</i>	(Khan <i>et al.</i> , 2016)
Z857	S4197 <i>ΔrapZ, attB::[glmS-5'-lacZ (glmS -311 to +129) aadA (spec^R)]</i>	(Khan <i>et al.</i> , 2020)
Z864	R1279 <i>ΔglmZ ΔglmY ΔrapZ</i>	(Göpel <i>et al.</i> , 2016)
Z956	S4197 <i>ΔrapZ ΔglmY ΔglmZ</i>	Laboratory collection (constructed by Yvonne Göpel, unpublished)
Z1040	S4197 <i>ptsN-3xFLAG glmZ::kan</i>	Laboratory collection (constructed by Florian Sikora, unpublished)
Z1072	S4197 <i>ptsN-3xFLAG attB::[yhbG(+100)→ptsN(+51)'-lacZ, aadA (spec^R)]</i>	Laboratory collection (constructed by Florian Sikora, unpublished)
Z1272	S4197 <i>[attB::sixA(-300 to +51)'-lacZ] ptsN-3xFLAG</i>	Laboratory collection (constructed by Florian Sikora, unpublished)
Z1297	S4197 <i>ptsN-3xFLAG [attB::lptA(-152 to +78)'-lacZ]</i>	Laboratory collection (constructed by Florian Sikora, unpublished)
Z1298	S4197 <i>glmZ::kan</i>	This study, <i>glmZ::kan</i> transduced with T4-GT7 from Z1040 to S4197
Z1299	S4197 <i>glmZ::kan glmY::cat</i>	This study, <i>glmY::cat</i> transduced with T4-GT7 from Z95 to Z1298
Z1300	S4197 <i>ΔglmZ ΔglmY</i>	This study, Z1299 cured from <i>kan</i> and <i>cat</i> with pCP20 (according to Datsenko and Wanner, 2000)
Z1301	S4197 <i>ΔglmZ</i>	This study, Z1298 cured from <i>kan</i> with pCP20 (according to Datsenko and Wanner, 2000)
Z1303	S4197 <i>[attB::lptA(-152 to +78)'-lacZ]</i>	This study, <i>lptA'-lacZ</i> transduced

		with <i>T4-GT7</i> from Z1297 to S4197
Z1307	S4197 [<i>attB::lptA(-152 to +78)'</i> - <i>lacZ</i>], <i>hfq::kan</i>	This study, <i>hfq::kan</i> transduced with <i>T4-GT7</i> from JW4130 to Z1303
Z1308	S4197 <i>sixA::kan</i>	This study, <i>sixA::kan</i> transduced with <i>T4-GT7</i> from JW2337 to S4197
Z1315	S4197 [<i>attB::sixA(-300 to +51)'</i> - <i>lacZ</i>]	This study, <i>sixA'-lacZ</i> transduced with <i>T4-GT7</i> from Z1272 to S4197
Z1323	S4197 <i>rseA::kan</i>	This study, <i>rseA::kan</i> transduced with <i>T4-GT7</i> from JW2556 to S4197
Z1324	S4197 [<i>attB::lptA(-152 to +78)'</i> - <i>lacZ</i>], <i>rseA::kan</i>	This study, <i>rseA::kan</i> transduced with <i>T4-GT7</i> from JW2556 to Z1303
Z1325	S4197 [<i>attB::lptA(-152 to +78)'</i> - <i>lacZ</i>], <i>waaG::kan</i>	This study, <i>waaG::kan</i> transduced with <i>T4-GT7</i> from JW3606 to Z1303
Z1326	S4197 [<i>attB::lptA(-152 to +78)'</i> - <i>lacZ</i>], <i>waaQ::kan</i>	This study, <i>waaQ::kan</i> transduced with <i>T4-GT7</i> from JW3607 to Z1303
Z1348	S4197 $\Phi(kan\ araC\ araBp-lptC)1$	This study, $\Phi(kan\ araC\ araBp-lptC)1$ transduced with <i>T4-GT7</i> from FL905 to S4197
Z1349	S4197 $\Phi(kan\ araC\ araBp-lptA)1$	This study, $\Phi(kan\ araC\ araBp-lptA)1$ transduced with <i>T4-GT7</i> from FL907 to S4197
Z1350	S4197 [<i>attB::lptA(-152 to +78)'</i> - <i>lacZ</i>], Δhfq	This study, Z1307 cured from <i>kan</i> with pCP20 (according to Datsenko and Wanner, 2000)
Z1351	S4197 [<i>attB::lptA(-152 to +78)'</i> - <i>lacZ</i>], $\Delta rseA$	This study, Z1324 cured from <i>kan</i> with pCP20 (according to Datsenko and Wanner, 2000)
Z1352	S4197 [<i>attB::lptA(-152 to +78)'</i> - <i>lacZ</i>], $\Delta waaG$	This study, Z1325 cured from <i>kan</i> with pCP20 (according to Datsenko and Wanner, 2000)
Z1353	S4197 [<i>attB::lptA(-152 to +78)'</i> - <i>lacZ</i>], $\Delta waaQ$	This study, Z1326 cured from <i>kan</i> with pCP20 (according to Datsenko and Wanner, 2000)
Z1375	S4197 $\Delta sixA$	This study, Z1308 cured from <i>kan</i> with pCP20 (according to Datsenko and Wanner, 2000)
Z1377	S4197 <i>ptsN-3xFLAG [attB::lptA(-152)→rapZ(+78)'</i> - <i>lacZ</i>]	This study, <i>lptA(-152)→rapZ(+78)'</i> - <i>lacZ</i> from pFS35 integrated in Z424 with pLDR8 (according to Diederich <i>et al.</i> , 1992)
Z1378	S4197 [<i>attB::lptA(-152)→rapZ(+78)'</i> - <i>lacZ</i>]	This study, <i>lptA(-152)→rapZ(+78)'</i> - <i>lacZ</i> transduced with <i>T4-GT7</i> from Z1377 to S4197

5.1.3. Plasmids

All plasmids used in this study are listed in Table 4.

Table 4. List of plasmids used in this study.

Plasmid	Description	Reference
pAraX	Derivative of pMW218 carrying the <i>araC</i> P_{ara} cassette, pSC101-ori, <i>kan</i>	(Otaka <i>et al.</i> , 2011) (obtained from T. Morita)
pBAD33	expression plasmid; P_{ara} , MCS 2, pACYC-ori, <i>cat</i> ; induce expression with 0,2% arabinose	(Guzman <i>et al.</i> , 1995)
pBGG61	<i>rapZ</i> with its authentic SD under P_{ara} control in pBAD33; induce expression with 0,2% arabinose	(Göpel <i>et al.</i> , 2013)
pCP20	expresses FLP recombinase upon temperature shift, pSC101 ^{repTS} -ori, <i>bla</i> , <i>cat</i>	(Datsenko and Wanner, 2000)
pKEM20	p15A-ori, <i>neo</i> , <i>lattP</i> , <i>aadA</i> , <i>T4gene32</i> -terminator, P_{proV} - <i>lacZ</i> (vector for translational <i>lacZ</i> fusions)	(Nagarajavel <i>et al.</i> , 2007) Laboratory collection (obtained from K. Schnetz, Institute of Genetics, Cologne)
pFS5	translational fusion <i>lptB</i> (+100)→ <i>rapZ</i> (+78)- <i>lacZ</i> in pKEM20, with mutated BamHI site in <i>rpoN</i> for integration; p15A-ori, <i>attP</i> , <i>aadA</i> , <i>T4gene32</i> -terminator, <i>neo</i>	Laboratory collection (constructed by Florian Sikora, unpublished)
pFS28	expression plasmid; <i>rpoE</i> with <i>B. subtilis</i> <i>sacB</i> RBS under P_{ara} control in pBAD33; induce expression with 0,2% arabinose	Laboratory collection (constructed by Florian Sikora, unpublished)
pFS32	translational fusion <i>lptA</i> (-152)→ <i>ptsN</i> (+51)- <i>lacZ</i> in pKEM20, with mutated BamHI site in <i>rpoN</i> for integration; p15A-ori, <i>attP</i> , <i>aadA</i> , <i>T4gene32</i> -terminator, <i>neo</i>	Laboratory collection (constructed by Florian Sikora, unpublished)
pFS35	translational fusion <i>lptA</i> (-152)→ <i>rapZ</i> (+78)- <i>lacZ</i> in pKEM20, with mutated BamHI site in <i>rpoN</i> for integration; p15A-ori, <i>attP</i> , <i>aadA</i> , <i>T4gene32</i> -terminator, <i>neo</i>	This study, 2393 bp long fragment from pFS5 (Sall/XbaI), cloning into pFS32 via Sall/XbaI
pFS36	expression plasmid; pBAD33 vector in which <i>araC</i> P_{ara} cassette was replaced with <i>Tet^R</i> P_{tet} cassette from pKESL307; MCS 2, pACYC-ori, <i>cat</i> ; induce expression with anhydrotetracycline	This study, PCR of pKESL307 with BG2122/1963, cloning into pBAD33 via ClaI/SacI
pFS37	expression plasmid; <i>rpoE</i> with <i>B. subtilis</i> <i>sacB</i> RBS under P_{tet} control in pFS36; induce expression with anhydrotetracycline	This study, PCR of pKESL307 with BG2122/1963, cloning into pFS28 via ClaI/SacI
pFS38	<i>surA</i> with its authentic SD under P_{ara} control in pAraX; <i>araC</i> -pBAD, pSC101-ori, <i>kan</i> ; induce expression with 0,2% arabinose	This study, PCR of <i>surA</i> gene with BG2128/2129, cloning into pAraX via XbaI/HindIII

pKESK18	Tn10-transposase under λP_R -control, λcl_{857} , mTn10:: <i>cat</i> , pSC101-rep ^{TS} , <i>neo</i>	(Madhusudan <i>et al.</i> , 2005)
pKESL307	<i>Tet^R</i> <i>P_{tet}</i> cassette, <i>mNeonGreen</i> , <i>repA</i> , <i>Cas9LAA</i> , p15A-ori, <i>cat</i> ; induce expression with anhydrotetracycline	(Qi <i>et al.</i> , 2013) (Wiktor <i>et al.</i> , 2016) Laboratory collection (obtained from K. Schnetz, Institute of Genetics, Cologne)
pLDR8	Helper plasmid for integration of DNA-fragments into the λ -attB site; λ -integrase under control of λP_R promoter, λcl_{857} repressor gene, pSC101-rep ^{TS} , <i>kan</i>	(Diederich <i>et al.</i> , 1992)

5.1.4. Oligonucleotides

All oligonucleotides used in this study for PCR reactions or sequencing are listed in Table 5. Oligonucleotides were manufactured by Eurofins Genomics.

Table 5. List of oligonucleotides used in this study (for sequencing or PCR).

Oligo	Sequence (5' → 3')	T _m	Description
BG25	GTGCTGCAAGGCGAT TAAGTTGG	70°C	Reverse sequencing primer for <i>lacZ</i> promoter fusions, primes in <i>lacZ</i> (+79 to +57)
BG146	AAGTAATCGCAACATC CGCATTA	64°C	Forward sequencing primer for Sall/XbaI inserts in pKES20, primes in pKES20 (+8442 to +8464 bp)
BG161	GGATACCGAAGGTAC TCCGG	59°C	Forward check primer for <i>rapZ</i> , primes in <i>rapZ</i> (+4062 to +4082)
BG188	GTGTAGGATCAAGCT CAGG	58°C	Forward check primer for <i>glmZ</i> , primes in <i>glmZ</i> (-131 to -112)
BG230	GTAGATGCTCATTCCA TCTC	58°C	Forward primer for anti-GlmZ RNA probe synthesis, primes in <i>glmZ</i> (+1 to +20)
BG231	<u>CTAATACGACTCACTA</u> <u>TAGGGAGAAAACAGG</u> TCTGTATGACAAC	58°C	Reverse primer for anti-GlmZ RNA probe synthesis, primes in <i>glmZ</i> (+172 to +152), contains <u>T7 promoter</u>
BG232	TGATTTAATCTGTATC AGGCTG	60°C	Reverse sequencing primer for inserts in pBAD33 and other pBAD plasmids, primes in pBAD33 (+1413 to +1392 bp)
BG233	CGGACGCCTACGATT ACGC	62°C	Reverse check primer for <i>glmZ</i> , primes in <i>glmZ</i> (+376 to +358)
BG250	GGTGTTACTCTCGTCA GACGCG	70°C	Reverse check primer for <i>glmY</i> , primes in <i>glmY</i> (+242 to +221)
BG251	GTCTCTTTTtagcgac ACAGTGGC	72°C	Forward check primer for <i>glmY</i> , primes in <i>glmY</i> (-133 to -110)
BG260	AGTGGCTCATTACC GAC	56°C	Forward primer for anti-GlmY RNA probe synthesis, primes in <i>glmY</i> (+1 to +18)
BG261	<u>CTAATACGACTCACTA</u> <u>TAGGGAGATAAGGCG</u> GTGCCTAACTC	56°C	Reverse primer for anti-GlmY RNA probe synthesis, primes in <i>glmY</i> (+151 to +132), contains <u>T7 promoter</u>

BG275	GGCTCTAGAAAAACA GGTCTGTATGACAAC AAGTGG	76°C	Reverse primer for integration of <i>glmZ</i> into pBAD30, primes in <i>glmZ</i> (+172 to +146), contains <u><i>XbaI</i> restriction site</u>
BG287	TGCCTGGCGGCCGTA G	56°C	Forward primer for anti-5S rRNA probe synthesis, primes in <i>rrfD</i> (+1 to +16)
BG288	CTAATACGACTCACTA TAGGGAGAGCCTGGC AGTTCCCTAC	54°C	Reverse primer for anti-5S rRNA probe synthesis, primes in <i>rrfD</i> (+118 to +102), contains <u><i>T7</i> promoter</u>
BG400	GCCCTCGAGGGTCCG TAAAAGCAGATAAAGC C	63°C	Forward sequencing primer for <i>lptA</i> (-152)→ <i>rpoN</i> (+78) translational fusion in pKEM20, contains <u><i>XhoI</i> restriction site</u>
BG401	GCCTCTAGACAACAG ACGAATTGCCT	50°C	Reverse sequencing primer for <i>lptA</i> (-152)→ <i>rpoN</i> (+78) translational fusion in pKEM20, primes in <i>rpoN</i> (+78 to +61), contains <u><i>XbaI</i> restriction site</u>
BG445	AAAAAACGCCTGCT CTTATTACGGAGC	75°C	Reverse check primer for <i>glmZ</i> (full-length), primes in <i>glmZ</i> (+207 to +179)
BG447	AACAAAGCCGGAAT TACCCGGC	72°C	Reverse check primer for <i>glmY</i> (full-length), primes in <i>glmY</i> (+184 to +161)
BG506	GGCTGCTAGCATGAC AAATAATGATACAACT CTAC	60°C	Forward sequencing primer for strep-tag- <i>ptsN</i> in pBGG164 backbone, contains <u><i>NheI</i> restriction site</u>
BG621	GCGTCTAGAGTTGGT GTAGATGGGCG	54°C	Reverse sequencing primer for <i>lacZ</i> promoter fusions, primes in <i>lacZ</i> (+309 to +292), contains <u><i>XbaI</i> restriction site</u>
BG862	GCATCAGAGCAGCCG ATTG	60°C	Reverse check primer for plasmids carrying <i>neo</i> , primes in <i>neo</i> (+101 to +82) of pKD4
BG916	CATACGATGAAATGGT TTATCG	60°C	Forward check primer for <i>hfq</i> , primes in <i>hfq</i> (-249 to -228)
BG917	CAAAATACTTTGGGTG CGGC	62°C	Reverse check primer for <i>hfq</i> , primes in <i>hfq</i> (+250 to +231)
BG921	GGCTGCTAGCATGGC TAAGGGGCAATCTTTA C	62°C	Forward sequencing primer for strep-tag- <i>hfq</i> in pBGG164 backbone, contains <u><i>NheI</i> restriction site</u>
BG922	GGCTCTAGATTATTCG GTTTCTTCGCTGTC	62°C	Reverse sequencing primer for strep-tag- <i>hfq</i> in pBGG164 backbone, contains <u><i>XbaI</i> restriction site</u>
BG972	CTAATACGACTCACTA TAGGGAGAGGTAGAT GCTCATTCCATCTCTT ATG	70°C	Forward primer for <i>GlmZ</i> <i>in vitro</i> transcription with an additional G for effective 5' end labeling, primes in <i>glmZ</i> (+1 to +25), contains <u><i>T7</i> promoter</u>
BG1278	CTAATACGACTCACTA TAGGGAGAAACAAAG CCGGGAATTACC	56°C	Reverse primer for anti-full-length <i>GlmY</i> RNA probe synthesis, primes in <i>glmY</i> (+184 to +166), contains <u><i>T7</i> promoter</u>
BG1361	CGCGAATTCCTGCAG CTATTTATCGTCGTCA TCTTTG	60°C	Reverse check primer for 3xFLAG-tagged genes, primes in FLAG-tag, contains <u><i>EcoRI</i> and <i>PstI</i> restriction sites</u>
BG1386	GCCTCTAGATTCCCT GTAAAGAACTG	56°C	Reverse sequencing primer for <i>lptB</i> (+100)→ <i>ptsN</i> (+51) translational fusion in

			pKEM20, primes in <i>ptsN</i> (+51 to +33), contains <u><i>XbaI</i> restriction site</u>
BG1780	ATGATCATTTATTCTG CCTCCCA	64°C	Nested primer annealing 100 nt from the end of the mTn10 transposon insertion site (from pKESK18). Used as an alternative to S359 for ST-PCR II, together with semi-random primer S361, to amplify the LEFT flank of the transposon. Template for the second round of the ST-PCR is the product of ST-PCR I obtained with primers S357 and S360.
BG1781	ACGGGGTGGTGCCTA ACGG	64°C	Nested primer annealing 100 nt from the end of the mTn10 transposon insertion site (from pKESK18). Used as an alternative to S359 for ST-PCR II, together with semi-random primer S361, to amplify the RIGHT flank of the transposon. Template for the second round of the ST-PCR is the product of ST-PCR I obtained with primers S358 and S360.
BG1805	ATGCAAGTTTTTATCA TGCCTCAC	66°C	Forward check primer for <i>sixA</i> , primes in <i>sixA</i> (+1 to +24)
BG1806	TCAGATAGCTTTTGCC ATCTTCA	64°C	Reverse check primer for <i>sixA</i> , primes in <i>sixA</i> (+486 to +463)
BG1807	AAGGTCAAAGCTATAT GAATCCG	64°C	Forward check primer for <i>sixA</i> , primes in <i>sixA</i> (-202 to -179)
BG1808	TCAAGATACGCGCAG AAGAATAC	66°C	Reverse check primer for <i>sixA</i> , primes in <i>sixA</i> (+645 to +622)
BG1813	AATGTTGTCAGAAGAA CTGAGACA	66°C	Forward check primer for <i>rseA</i> , primes in <i>rseA</i> (-246 to -222)
BG1814	TTGCAACAATTGTGCA AGAGGAC	66°C	Reverse check primer for <i>sixA</i> , primes in <i>sixA</i> (+875 to +852)
BG1896	GCCTCTAGATTATCAA CGCCTGATAAGCGGT TG	64°C	Reverse sequencing primer for <i>rpoE</i> cloned in pBAD33 plasmid via <i>XbaI</i> , contains <u><i>XbaI</i> restriction site</u>
BG1927	AGTGCAGCGCAGTAT TTTCAGT	64°C	Forward primer for anti-CspH RNA probe synthesis, primes in <i>cspH</i> (-34 to -12)
BG1928	CTAATACGACTCACTA <u>TAGGGAGACATTTGC</u> CGCTGTTGGTCCTC	66°C	Reverse primer for anti-CspH RNA probe synthesis, primes in <i>cspH</i> (+199 to +179), contains <u><i>T7</i> promoter</u>
BG1942	GCCGAAAGGCTCAAG TTAAGG	64°C	Forward primer for anti-CspB RNA probe synthesis, primes in <i>cspB</i> (-29 to -9)
BG1943	CTAATACGACTCACTA <u>TAGGGAGAGCACCAC</u> TCTCTATAGAGAAGG	66°C	Reverse primer for anti-CspB RNA probe synthesis, primes in <i>cspB</i> (+176 to +155), contains <u><i>T7</i> promoter</u>
BG1963	GG <u>ACTAGTCCATGGT</u> <u>CTAGAGGTACCCTGC</u> <u>AGGAGCTCGAATTCT</u> TTTCTCTATCACTGAT AG	68°C	Reverse primer to amplify <i>P_{tet}</i> promoter cassette from pKESL30, primes in pKESL307 (+712 to +688 bp), contains internal <i>EcoI</i> site and <u><i>SpeI</i>, <i>NcoI</i>, <i>XbaI</i>, <i>KpnI</i>, <i>PstI</i>, <i>SacI</i> sites</u>
BG1969	GCCCTCGAGGTTTGC	66°C	Forward sequencing primer for <i>rpoN</i> (-

	GACGTTTTAGCAGGA G		37)→ <i>ptsN'</i> - <i>lacZ</i> translational fusion in pKEM20, primes in <i>rpoN</i> (-37 to -16), contains <u><i>XhoI</i> restriction site</u>
BG2047	GCCTCTAGAAAATGC CGGAATGCTGGCGG	64°C	Reverse sequencing primer for <i>lptA'</i> - <i>lacZ</i> translational fusion in pKEM20, primes in <i>lptA</i> (+78 to +58), contains <u><i>XbaI</i> restriction site</u>
BG2086	ATGCGATATCATGGG GATATGTT	64°C	Forward check primer for <i>waaQ</i> , primes in <i>waaQ</i> (+1 to +23)
BG2087	TCATAATTGTGCATTC CTGTCAG	64°C	Reverse check primer for <i>waaQ</i> , primes in <i>waaQ</i> (+1035 to +1013)
BG2088	TCGGGTCGGATTGCT GCGAA	64°C	Forward check primer for <i>waaQ</i> , primes in <i>waaQ</i> (-180 to -160)
BG2089	CGCGATCTGCGGGAT GCTC	64°C	Reverse check primer for <i>waaQ</i> , primes in <i>waaQ</i> (+1281 to +1263)
BG2090	ATGATCGTGGCGTTTT GTTTATAT	64°C	Forward check primer for <i>waaG</i> , primes in <i>waaG</i> (+1 to +24)
BG2091	TCAACCATCTAAACCA CCTGTAA	64°C	Reverse check primer for <i>waaG</i> , primes in <i>waaG</i> (+1125 to +1103)
BG2092	CGGGACATATTGCAG CGGCA	64°C	Forward check primer for <i>waaG</i> , primes in <i>waaG</i> (-250 to -231)
BG2093	TCACTCAGGCGATGA ATAGCG	64°C	Reverse check primer for <i>waaG</i> , primes in <i>waaG</i> (+1374 to +1354)
BG2094	TCGGCGATGATCTCA TCGACT	64°C	Forward check primer for <i>araBp/lptC</i> insertion, primes in <i>lptC</i> (-196 to -175)
BG2095	GTGGTAAGTACCGGC TGCGT	64°C	Forward check primer for <i>araBp/lptC</i> insertion, primes in <i>lptC</i> (+254 to +234)
BG2122	CCGCGATCGATCTTA AGACCCACTTTTACAT TTAAG	68°C	Forward primer to amplify <i>P_{tet}</i> promoter cassette from pKESL307, primes in pKESL307 (+7 to +30 bp), contains <u><i>ClaI</i> site</u>
BG2128	GCCTCTAGACGCAGT GCGGTAAATTGAAATG	64°C	Forward primer to amplify <i>surA</i> with its authentic SD sequence, primes in <i>surA</i> (-30 to -9), contains <u><i>XbaI</i> site</u>
BG2129	CCGCAAGCTTTTAGTT GCTCAGGATTTTAACG TA	64°C	Reverse primer to amplify <i>surA</i> , primes in <i>surA</i> (+1287 to +1264), contains <u><i>HindIII</i> site</u>
BG2130	CGACTTTATCGACTAC CTGGG	64°C	Reverse sequencing primer for <i>surA</i> , primes in <i>surA</i> (+85 to +65)
BG2131	AGGCGACATTGTTGG CCCGA	64°C	Forward sequencing primer for <i>surA</i> , primes in <i>surA</i> (+756 to +775)
S93	CCGGGCCGACAACAA AGTA	60°C	Primer for amplification of junction at <i>attB'</i> , primes at 17 min. region
S95	CATATGGGGATTGGT GGCGA	62°C	Primer for amplification of <i>lacZ</i> -fusion insertions at <i>attB'</i> , primes in <i>lacZ</i>
S96	CACTCTGCCAGATGG CGCAA	64°C	Primer for amplification of junction at <i>attB'</i> , primes at 17 min. region
S164	GAGCAGGGGAATTGA TCCGGTGGA	76°C	Primer for amplification of <i>attB::spec</i> -fragment insertions, primes in <i>spec^R</i>
S357	GGCAGGGTCGTAAA TAGCCGCTTATGT	64.7 °C	Primer annealing to the mTn10 transposon insertion from the plasmid pKESK18

			(Madhusudan <i>et al.</i> , 2005). Used for ST-PCR I, together with semi-random primer S360, to amplify LEFT flank of the transposon.
S358	CGGTATCAACAGGGA CACCAGGATTTATTTA TTCT	65.8 °C	Primer annealing to the mTn10 transposon insertion from the plasmid pKESK18 (Madhusudan <i>et al.</i> , 2005). Used for ST-PCR I, together with semi-random primer S360, to amplify RIGHT flank of the transposon.
S360	GGCCACGCGTCGACT AGTACNNNNNNNNNN GATC	54.7 °C	Semi-random primer (Madhusudan <i>et al.</i> , 2005). Used for ST-PCR I, together with miniTn10 specific primer S357 (for amplification of left flank) or S358 (for amplification of right flank).
S361	GCTCTAGAGGCCACG CGTCGACTAGTAC	64.2 °C	Primer that matches to the semi-random primer S360 (Madhusudan <i>et al.</i> , 2005). Used for ST-PCR II, together with nested primer BG1780 (for amplification of left flank) or BG1781 (for amplification of right flank).

5.2. Methods

5.2.1. Cultivation of *E. coli*

5.2.1.1. General growth conditions of *E. coli*

E. coli strains were first streaked out from a cryo culture on a (selective) LB-agar plate and incubated at 37°C overnight to ensure that only single colonies were used for the experiment. Subsequent overnight cultures were then grown in 5 mL LB with required antibiotics at 37°C and shaking at 165 rpm. The next morning, fresh cultures were inoculated from the overnight cultures to a final OD₆₀₀ of 0.1 (if not stated otherwise) and grown at 37°C and shaking at 165 rpm for experimental purposes.

5.2.1.2. Determination of optical density

The growth of liquid cultures was monitored by measuring the light absorbance at the wavelength of 600 nm in a photometer (Biochrom, Libra S22), to follow growth of liquid cultures. For this purpose, plastic cuvettes with a path length of 10 mm were used. The cultures were appropriately diluted in LB when the optical density was close or above the value of 1 because the linear range of the photometer is between 0,1 and 1.

5.2.1.3. Strain storage / Preparation of cryo cultures

Cryo-cultures were prepared for long-time storage of *E. coli* strains by mixing 1 mL of a dense overnight culture with 80 µL dimethyl-sulfoxide (DMSO). Afterwards, the cells were transferred to the -80°C freezer.

5.2.1.4. Plate spotting assay

The spotting assay was used to evaluate the growth-defect phenotypes of *E. coli* strains carrying the desired expression plasmid. Overnight cultures were adjusted to $1 \text{ OD}_{600} = 10^9$ cells/mL. Afterwards, ten-fold serial dilutions were prepared in fresh LB medium, ranging from 10^8 to 10^4 , and 2 μL of each serial dilution was spotted onto LB plates containing the required antibiotic and inducer. The plates were then incubated overnight at 37°C and photographed on the next day.

5.2.2. Construction of *E. coli*-K12 strains

5.2.2.1. General transduction of genetic markers using phage T4-GT7

For transduction of genetic markers, a derivative of the T4 bacteriophage (T4-GT7) is used. This phage is able to infect bacterial cells and to transduce chromosomal parts with a high frequency of 1 transductant per 10^4 infections (Wilson *et al.*, 1979). The phage was used to introduce reporter gene fusions or chromosomal deletions tagged with an antibiotic resistance marker and move them between *E. coli* strains. First, a phage lysate of the appropriate donor strain was prepared and later used to infect the recipient strain for transduction.

5.2.2.1.1. Preparation of T4-GT7 phage lysates

The donor strain carrying the desired deletion cassette to be transduced was first grown overnight in 5 mL LB with required antibiotics. Different dilutions (10^{-2} to 10^{-6}) of another T4-GT7 phage lysate (carrying a different selective marker) were prepared in a total volume of 100 μL LB. Dilutions were then mixed with 100 μL of an overnight culture of the donor strain and incubated at room temperature for 20 min. After incubation, 300 μL LB and 3 mL T4 top agar (heated to 44°C) were added to each dilution and the mixtures were then plated on LB-agar plates and incubated overnight at 37°C . The next day, the plate displaying congruent lysis, i.e. plaques and living cells in a ratio of 1:1, was used for further production of the phage lysate. The top agar was loosened with a spatula and carefully transferred into a 15 mL falcon tube. Subsequently, plate was washed with 3 mL LB to collect all the remaining parts of top agar. After adding 1 mL of chloroform, the mixture was homogenized using a glass rod and centrifuged (4.000 rpm, 20 min, RT). Finally, the supernatant was transferred into a fresh falcon tube and mixed with 1 mL of chloroform. Phage lysates were stored at 4°C until further use.

5.2.2.1.2. Transduction of *E. coli* using phage T4-GT7

The recipient strain was first grown overnight in 5 mL LB with required antibiotics. Different dilutions (10^{-1} to 10^{-4}) of the desired T4-GT7 phage lysate were prepared in a total volume of

100 µL LB. Dilutions were then mixed with 500 µL of an overnight culture of the recipient strain and incubated at room temperature for 20 min. After incubation, 300 µL and 100 µL of each dilution were plated on selective LB-agar plates, containing the required antibiotics that allow selection for the presence of the desired allele. The plates were then incubated overnight at 37°C for one or two days. Afterwards, four colonies showing the characteristic star-shaped morphology were re-streaked on selective LB-agar plates and incubated overnight at 37°C. The re-streaking was repeated at least three times to remove all the bacteriophages and establish the marker deletion or mutation. Finally, the individual colonies were checked via colony PCR (as described in 5.2.4.3.2.) for the specific transduced region using flanking and/or internal primers for the gene of interest.

Table 6. Composition of T4 top agar for the preparation of phage lysates.

T4 top agar
10 g tryptone
8 g NaCl
3 g glucose
2 g sodium citrate
6 g agar
ad 1L with distilled H ₂ O

5.2.2.2. Removal of antibiotic resistance markers from *E. coli*-K12 strains (Datsenko and Wanner, 2000)

In order to acquire strains with clean deletion to avoid polar effects on the downstream genes, newly constructed strains were cured from acquired antibiotic resistance cassettes using the FLP-FRT recombination system, adapted from Datsenko and Wanner (Datsenko and Wanner, 2000). The system is based on the recombination between the flippase recognition target (FRT) and the flippase recombination enzyme (FLP). FRT sites are located up- and downstream of the antibiotic resistance cassette. When FLP is expressed in cells carrying FRT sites, it cleaves these specific sequences which allows for the recombination between the two sites and results in removal of the antibiotic resistance cassette that was located in between. There is an 82 to 85 nucleotides long scar sequence left after the recombination event in the cured strains.

Plasmid pCP20 encodes the flippase recombination enzyme (FLP) under the control of an arabinose-inducible promoter and possesses the temperature sensitive origin of replication (ori). The plasmid is only able to replicate at 28°C and the temperature shift to 42°C leads to loss of the plasmid. The strains to be cured were first grown overnight in 5 mL LB with required antibiotics and the next day, competent cells were prepared (as described in 5.2.4.1.) and transformed with plasmid pCP20. Since pCP20 is a temperature-sensitive

plasmid, the cells were incubated at 28°C during and after the transformation. The next day, three transformants were inoculated in 3 mL LB containing 0,2% arabinose and grown at 37°C for several h during the day. Afterwards, 2 µL of each culture was plated on LB-agar without antibiotics and the plates were incubated overnight at 42°C. This procedure was repeated at least three times to ensure removal of the helper plasmid pCP20. Finally, the individual colonies were checked via colony PCR (as described in 5.2.4.3.2.) for the removal of the antibiotic resistance cassette using flanking primers for the gene of interest and by re-streaking colonies on LB-agar and on selective LB-agar plates; one supplemented with ampicillin for the selection of pCP20 and the other supplemented with antibiotic that allows for the selection of the resistance cassette.

5.2.2.3. Integration of DNA fragments into the chromosomal λ -attachment site *attB* of *E. coli*

The integration of *lacZ* reporter constructs into the chromosomal lambda attachment site was conducted according to the protocol published by Diederich (Diederich *et al.*, 1992). Reporter constructs to be integrated are based on the vector pKEM20, carrying the λ -*attP*-site, spectinomycin resistance cassette (encoded by *aadA*) and the respective reporter gene fusion. Prior to integration, the *E. coli* strain in which DNA fragments should be integrated, was transformed with the helper plasmid pLDR8. Plasmid pLDR8 encodes the λ -integrase, kanamycin resistance cassette and possesses a temperature sensitive origin of replication (*ori*). The plasmid is only able to replicate at 28°C and the temperature shift to 42°C leads to loss of the plasmid, therefore transformants carrying this plasmid were cultured exclusively at 28°C.

In order to generate *ori*-less DNA, the fragments to be integrated were obtained by first incubating 2 to 5 µg of the respective plasmid with 10 U of BamHI in 50 µL reactions. The fragment of interest was gel purified (1% agarose gel electrophoresis). Finally, 10 ng of the obtained DNA were ligated with 1,25 U of T4 DNA-ligase in 20 µL reactions, creating *ori*-less DNA circles to be integrated.

The pLDR8 transformant was first grown overnight in 5 mL LB with required antibiotics. The next day, the overnight culture was diluted 1:50 in 25 mL LB supplemented with kanamycin and grown at 37°C for exactly 90 min. Competent cells were prepared (as described in 5.2.4.1.) and 100 µL of the competent cells were mixed with half of the ligation reaction that was taken up in TEN buffer to a final volume of 40 µL. After the transformation, the cells were shaking in glass culture tubes at 37°C for 1 h and were then plated on LB-agar plates supplemented with spectinomycin and X-Gal to select for integrants. The plates were incubated overnight at 42°C to lose the helper plasmid pLDR8. Upon temperature shift to

42°C, the λ -integrase gene is expressed while replication of the plasmid stops, resulting in a transient burst of λ -integrase production. The desired reporter-constructs are then integrated into the λ -attachment site *attB* on the chromosome. The resulting colonies were first tested for loss of the helper plasmid by re-streaking them on LB-agar plates supplemented with kanamycin. Lastly, colonies were checked via extensive colony PCR (as described in 5.2.4.3.2.) to assess the success of the integration. Primer pairs that were used include S93/S164 (specific to the λ -*attB*/*P'*-side), S95/S96 (specific to the λ *attB*/*P*-side) and flanking primers specific to the reporter-gene fusion itself. Additionally, primer pair S95/S164 was used to exclude the integration of dimers that might form during the ligation reaction.

5.2.2.3.1. The ectopic integration of the *lptA'*-*lacZ* reporter construct into the chromosomal λ -*attB*-site

The ectopic integration of the *lptA*(-152 to +78)-*lacZ* reporter construct into the chromosomal λ -*attB*-site was carried out according to the protocol published by Diederich (Diederich *et al.*, 1992). To this end, the pre-constructed plasmid backbone from the laboratory collection, containing the DNA cassette with the latter reporter construct was used to generate the *ori*-less circular DNA fragments which were integrated into the chromosome of the target *E. coli* K12 *wild-type* strain S4197 (as described in 5.2.2.3.). Prior to integration, the target *E. coli* MG1655 (S4197) strains, the *wild-type* strain and the strain carrying the *ptsN*-3xFLAG allele respectively, were transformed with the temperature-sensitive integrase expression plasmid pLDR8 (Diederich *et al.*, 1992). The integration of *lptA'*-*lacZ* reporter construct into the chromosomal lambda attachment site was successful only in the MG1655 strain carrying a FLAG-tagged *ptsN* allele (*ptsN*-3xFLAG), generating strain Z1297.

5.2.3. Transposon mutagenesis screen using a miniTn10 delivery system

Transposon mutagenesis screen was performed in strains carrying the desired *lacZ* reporter gene fusion on the chromosome by using the plasmid pKESK18 (Madhusudan *et al.*, 2005), encoding a miniTn10-Cam^r transposon.

The low-copy plasmid pKESK18 carries the phage lambda λ *CI*₈₅₇ allele (encoding the temperature sensitive lambda repressor CI), the Tn10 transposase gene under control of the phage lambda promoter (*P_R*) and the miniTn10 transposon with a chloramphenicol resistance cassette (miniTn10-Cam^r). It is a temperature sensitive (rep_{ts}) plasmid that is only able to replicate at 28°C and the temperature shift to 42°C leads to loss of the plasmid (Figure 15) (Madhusudan *et al.*, 2005).

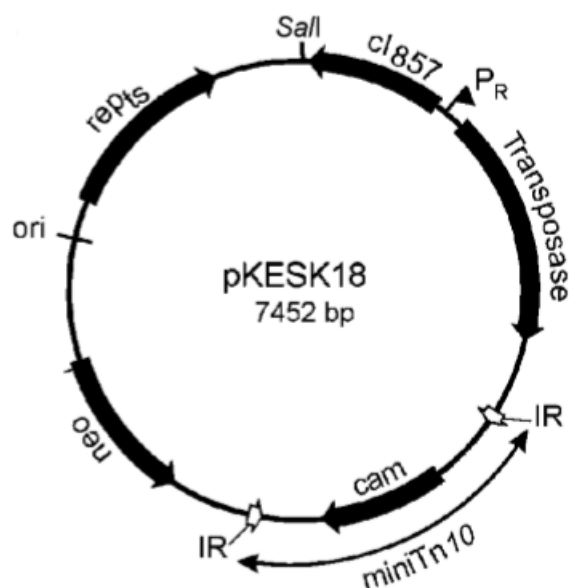


Figure 15. Plasmid pKESK18 (Madhusudan *et al.*, 2005). The plasmid pKESK18 is replication temperature sensitive (rep_{ts}) plasmid, carrying the lambda cl_{857} gene, the Tn10 transposase gene that is under control of the lambda P_R promoter, the mini-Tn10 transposon with the chloramphenicol resistance cassette (miniTn10-Cam^r) and the kanamycin resistance cassette (*neo*) (Madhusudan *et al.*, 2005).

In this plasmid, replication and expression of the transposase gene is temperature sensitive. At 28°C, the plasmid is able to replicate and expression of the transposase is repressed by the temperature sensitive cl_{857} . Upon temperature shift to 42°C, replication of the plasmid stops as well as repression of the transposase and thus transposition of the miniTn10 element is induced temporarily. Ideally, this results in a single insertion of the mini-Tn10 transposon carrying the chloramphenicol resistance cassette into the chromosome, allowing the selection of transposon insertion mutants on chloramphenicol plates. Transposition takes place in approximately 1 to 5% of the cells with this system and the miniTn10 transposon insertions are occurring randomly within the genome due to a relaxed specificity of the Tn10 transposase.

The strain carrying the desired *lacZ* reporter gene fusion on the chromosome was first grown overnight in 5 mL LB with required antibiotics. The next day, competent cells were prepared (as described in 5.2.4.1.) and transformed with plasmid pKESK18. Since pKESK18 is a temperature-sensitive plasmid, transformants carrying this plasmid were cultured exclusively at 28°C. First, a single transformant was inoculated in 100 mL LB supplemented with kanamycin and grown overnight. The next morning, overnight culture was diluted to an appropriate factor (10^{-3} and 10^{-4} -fold) and several LB-agar plates supplemented with X-Gal or MacConkey-lactose agar plates in parallel were inoculated each with 100 μ L of the diluted culture. In each case, the plates were supplemented with chloramphenicol to select for

colonies carrying a transposon insertion. Plates were then incubated overnight at 42°C to induce transposition and mediate loss of the plasmid. To test the efficiency of transposition, 100 µL of the 10⁻³ and 10⁻⁴-fold diluted overnight culture was also plated on LB-agar supplemented with kanamycin and incubated overnight at 28°C. The next day, total cell count and the fraction of transposon mutants were calculated and colonies were screened for mutants exhibiting a strong blue colored or lactose⁺ phenotype which were subsequently re-streaked to re-evaluate the phenotypes and further investigated by quantitative β-galactosidase activity assays (as described in 5.2.7.1.). Afterwards, the genome positions of the miniTn10 transposon insertions were determined for mutants exhibiting increased expression of the *lacZ* reporter fusion. To this end, semi-random two-step PCR was performed (as described in 5.2.4.3.3.).

5.2.4. Methods for working with recombinant DNA

5.2.4.1. Preparation of competent *E. coli* cells

Preparation of competent cells was conducted according to the protocol published by Lederberg and Cohen (Lederberg and Cohen, 1974). The strain to make competent was first grown overnight in 5 mL LB with required antibiotics. On the next day, the culture was diluted 1:100 in 25 mL LB and grown to an OD₆₀₀ of 0.3 to 0.4. Subsequently, cells were harvested by centrifugation (3.900 rpm, 15 min, 4°C), resuspended in 10 mL 0,1 M cold CaCl₂ and incubated on ice for 20 min. After the incubation, cells were centrifuged again (as before), resuspended in 1 mL 0,1 M cold CaCl₂ and kept on ice until the transformation.

5.2.4.2. Transformation of competent cells with plasmid DNA

Transformation of competent cells was conducted according to the protocol published by Lederberg and Cohen (Lederberg and Cohen, 1974). 100 µL of competent cells were mixed with circular plasmid DNA (10 to 100 ng) that was taken up in TEN buffer to a final volume of 50 µL and incubated on ice for 20 min. After the incubation, cells were heat-shocked at 42°C for 2,5 min and placed back on ice for additional 10 to 15 min. Subsequently, 1 mL of LB was added and cells were incubated at 37°C for 45 min and shaking at 165 rpm for expression of the antibiotic resistance gene. Finally, 100 µL of the cell suspensions were plated on selective LB-agar supplemented with antibiotics that are selecting for the presence of the plasmid and incubated overnight at 37°C. For working with temperature sensitive plasmids, the respective steps were performed at 28°C instead of 37°C, and in this case, the recovery phase after adding LB was prolonged to 90-120 min.

5.2.4.3. Polymerase chain reaction (PCR)

Polymerase chain reactions were used to amplify DNA regions of interest *in vitro*. DNA to be amplified was mixed in a buffer containing MgCl₂ with either *PfuS*- or *Taq*-polymerase, primer pairs flanking the region of interest and dNTPs. PCRs were carried out in a thermocycler (Labcycler, SensoQuest) in a total reaction volume of 25 or 50 µL, depending on the purpose of the experiment. PCR programs were adjusted to annealing temperatures (T_A) of the primers (T_m minus 5°C) and elongation times to the size of the amplified fragment. The melting temperature of the primers (T_m) was calculated with the following formula:

$$T_m = (n \times A + n \times T) \times 2 + (n \times G + n \times C) \times 4$$

n = number

5.2.4.3.1. DNA amplification with *PfuS* (for cloning)

PfuS-polymerase carrying a proofreading function was used to amplify DNA fragments for cloning with high fidelity in order to avoid undesired mutations during amplification. As DNA template either 0,4 µL of an overnight *E. coli* culture or plasmid DNA (10 to 100 ng) was used. PCR reactions were carried out in a total volume of 50 µL, using HF Buffer (NEB) containing MgCl₂ to provide the necessary ions for the reaction.

Table 7. PCR reaction mixture for DNA amplification with *PfuS*.

Component	Amount	Final concentration	Stock concentration
Buffer Hf (NEB)	10 µL	1x	5x
dNTPs (Thermo)	5 µL	0,2 mM	2 mM
Forward primer	4 µL	0,4 pmol/µL	5 pmol/µL working concentration prepared from 100 pmol/µL oligo solution
Reverse primer	4 µL	0,4 pmol/µL	5 pmol/µL
<i>PfuS</i> polymerase (NEB)	1 µL	5 U	2,5 U/µL
DNA template	1 µL	~ 2 ng/µL	~ 100 ng
dH ₂ O	25 µL		

Table 8. PCR program for DNA amplification with *PfuS*.

Steps	Temperature	Time	Number of cycles
1. Initial denaturation	98.5°C	5 min	
2. Denaturation	98.5°C	45 s	35 cycles
3. Annealing	T_A	15 s	35 cycles
4. Elongation	72°C	30 s/kb	35 cycles
5. Final elongation	72°C	3 min	
6. Hold	4°C	∞	

5.2.4.3.2. DNA amplification with *Taq* (colony PCR)

Taq-polymerase was used for PCR to amplify specific DNA sequences in order to check genotypes of strains or transformants, using whole *E. coli* cells as template. DNA fragments for subsequent sequencing were also amplified using *Taq*-polymerase. As DNA template either 0,4 µL of an overnight culture or 1 µL of a colony that was picked directly from the plate and diluted in 100 µL of LB or ddH₂O was used. PCR reactions were carried out in a total volume of 25 µL, using ThermoPol® Buffer (NEB).

Table 9. PCR reaction mixture for DNA amplification with *Taq*.

Component	Amount	Final concentration	Stock concentration
ThermoPol® Buffer (NEB)	2,5 µL	1x	10x
dNTPs (Thermo)	2,5 µL	0,2 mM	2 mM
Forward primer	2 µL	0,4 pmol/µL	5 pmol/µL working concentration prepared from 100 pmol/µL oligo solution
Reverse primer	2 µL	0,4 pmol/µL	5 pmol/µL
<i>Taq</i> polymerase (NEB)	0,125 µL	0,625 U	5 U/µL
Colony suspension	1 µL		
dH ₂ O	14,875 µL		

Table 10. PCR program for DNA amplification with *Taq*.

Steps	Temperature	Time	Number of cycles
1. Initial denaturation	94°C	5 min	
2. Denaturation	94°C	40-45 s	31-34 cycles
3. Annealing	T _A	40-45 s	31-34 cycles
4. Elongation	68°C	1 min/kb	31-34 cycles
5. Final elongation	68°C	10 min	
6. Hold	4°C	∞	

5.2.4.3.3. Semi-random two-step PCR

The insertion position of the miniTn10 transposon was determined for transposon mutants exhibiting increased expression of the *lacZ* reporter fusion (4.2.2.3.). A PCR fragment comprising the insertion site (left or right flank of the transposon) was obtained via semi-random, two-step PCR, as described by Chun et al. (Chun *et al.*, 1997), with some modifications (Kalamorz *et al.*, 2007). 0,4 µL of an overnight culture was used as DNA template. PCR reactions were carried out in a total volume of 20 µL (ST-PCR I) and 50 µL (ST-PCR II), using ThermoPol® Buffer (NEB) and *Taq*-polymerase.

In the first reaction (ST-PCR I), a semi-random primer S360 was used together with a miniTn10-specific primer S357 or S358, depending on the flank of the transposon to be

amplified. S357 was used to amplify the left flank of the transposon and S358 to amplify the right flank of the transposon (Figure 16). The annealing temperature of the first PCR starts with 42°C and is decreased by 1°C for each subsequent cycle of the first five cycles to achieve an efficient hybridization of the semi-random primer S360. The amplification products of the first PCR were diluted 1:10 in dH₂O and used as a template for the second PCR. In the second PCR, they were re-amplified using primer S361 (annealing to the sequence added by the semi-random primer S360) together with the nested mTn10-specific primer BG1780 or BG1781, depending on the flank of the transposon to be amplified. BG1780 was used to amplify the left flank of the transposon and BG1781 to amplify the right flank of the transposon (Figure 16). The amplification products of the second PCR were gel purified (1% agarose gel electrophoresis) and sequenced with primers BG1780 and BG1781 to identify the exact insertion site of the transposon.

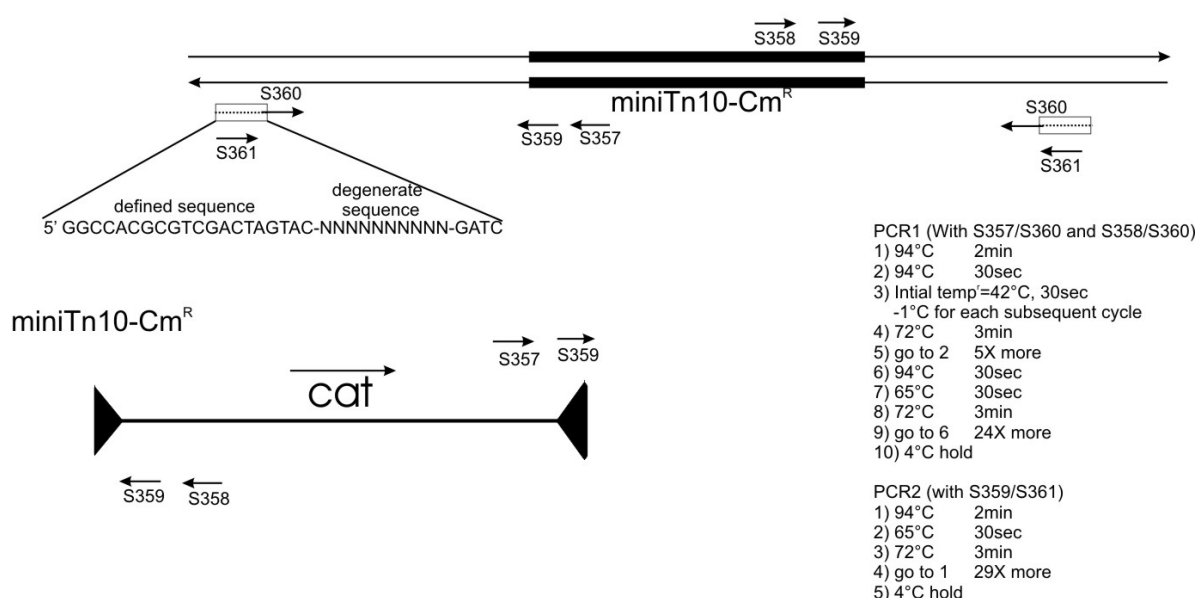


Figure 16. Summary of ST-PCR (Figure provided by Görke B., unpublished). Semi-random two step PCR with the required primers and their hybridization sequences as well as description of the first and the second PCR program.

Table 11. Reaction mixture for DNA amplification in the ST-PCR I.

Component	Amount	Final concentration	Stock concentration
ThermoPol® Buffer (NEB)	2 µL	1x	10x
dNTPs (Thermo)	2 µL	0,2 mM	2 mM
Semi-random primer (S360)	4 µL	1 pmol/µL	5 pmol/µL working concentration prepared from 100 pmol/µL oligo solution
Nested primer (S357/S358)	4 µL	1 pmol/µL	5 pmol/µL
Taq polymerase (NEB)	0,1 µL	0,625 U	5 U/µL
Colony suspension	0,4 µL		
dH₂O	7,5 µL		

Table 12. ST-PCR I program.

Steps	Temperature	Time	Number of cycles
1. Initial denaturation	94°C	10 min	
2. Denaturation	94°C	45 s	6 cycles
3. Annealing	Starts with 42°C, -1°C for each subsequent cycle	45 s	6 cycles
4. Elongation	68°C	3 min	6 cycles
5. Denaturation	94°C	45 s	25 cycles
6. Annealing	65°C	45 s	25 cycles
7. Elongation	68°C	3 min	25 cycles
8. Final elongation	68°C	10 min	
9. Hold	4°C	∞	

Table 13. Reaction mixture for DNA amplification in the ST-PCR II.

Component	Amount	Final concentration	Stock concentration
ThermoPol [®] Buffer (NEB)	5 µL	1x	10x
dNTPs (Thermo)	5 µL	0,2 mM	2 mM
Semi-random primer (S361)	4 µL	0,4 pmol/µL	5 pmol/µL working concentration prepared from 100 pmol/µL oligo solution
Nested primer (BG1780/BG1781)	4 µL	0,4 pmol/µL	5 pmol/µL
Taq polymerase (NEB)	0,25 µL	0,625 U	5 U/µL
DNA template	2 µL	(1:10 dilution of ST- PCR I in dH ₂ O)	
dH ₂ O	29,75 µL		

Table 14. ST-PCR II program.

Steps	Temperature	Time	Number of cycles
1. Initial denaturation	94°C	10 min	
2. Denaturatuon	94°C	45 s	34 cycles
3. Annealing	58°C	45 s	34 cycles
4. Elongation	68°C	3 min	34 cycles
5. Final elongation	68°C	10 min	
6. Hold	4°C	∞	

5.2.4.4. Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate DNA fragments by size. Depending on the expected size of the fragment, 0,8 to 1% agarose gels were pre-casted by boiling agarose in 1x TAE buffer. Ethidium bromide was added to a final concentration of 0,5 µg/mL to stain the

gels. Separation of nucleic acids was performed at 100 to 135V in 1x TAE running buffer for 30 to 90 min, depending on the purpose of the experiment. Samples were mixed with 10x DNA loading dye to a final concentration of 1x before loading the DNA into pre-cast wells. The 1 kb Plus DNA ladder (NEB) was loaded onto each gel as a DNA size marker. Subsequently, gels were visualized in the BioRad GelDoc system upon exposure to UV light at 365 nm for preparative gel electrophoresis or at 305 nm for analytic gel electrophoresis.

Table 15. Composition of 50x TAE and 10x loading dye for DNA agarose gel electrophoresis.

50x TAE	10x DNA loading dye
242 g Tris	10 mM Tris-HCl (pH 8,0)
57,1 mL acetic acid (glacial)	1 mM EDTA (pH 8,0)
18,61 g EDTA	30% (w/v) glycerol
ad 1 L with distilled H ₂ O	0,25% (w/v) bromophenol blue

5.2.4.5. Extraction of DNA from agarose gels

After agarose gel electrophoresis, the desired bands were cut out of the gel upon UV-light exposure at 365 nm on a UV-trans-illuminator. For preparative gel electrophoresis exposure at 365 nm is used due to a less intense energy of the light at longer wavelengths, in order to avoid formation of thymine dimers in DNA during the exposure. The NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel) was used to extract the DNA from agarose gels, according to manufacturer's instruction. The DNA was eluted from the silica membrane with sterile ddH₂O.

5.2.4.6. DNA clean-up following PCR or restriction digests

DNA was purified following enzymatic reactions (e.g. after PCR, restriction digestion or vector de-phosphorylation). The NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel) was used to purify the DNA following the manufacturer's instruction. The DNA was eluted from the silica membrane with sterile ddH₂O.

5.2.4.7. Molecular cloning

5.2.4.7.1. Restriction of DNA

The restriction digestions of DNA were carried out in 50 µL reactions, containing 10 U of the corresponding restriction endonucleases and 3 to 5 µg of plasmid DNA or 500 to 1000 ng of insert DNA (amplified PCR fragments). Restriction reactions were mixed with the appropriate 10x restriction buffer (CutSmart Buffer, NEB) to a final concentration of 1x and 1 µL of each restriction enzyme. The length and temperature of the reaction were according to manufacturer's instructions. After the restriction reaction, the DNA was subjected to dephosphorylation with alkaline phosphatase. In parallel, PCR products were purified after

endonuclease cleavage to prepare for ligation. Analytic DNA cleavage was carried out in smaller reaction volumes, containing 200 to 500 ng of DNA and lower amounts of restriction enzymes.

Table 16. List of restriction enzymes used in this study (for cloning).

Restriction enzyme	Concentration	Buffer	Temperature	Manufacturer
BamHI	20 U/μL	CutSmart®	37°C	NEB
Clal	20 U/μL	CutSmart®	37°C	NEB
HindIII-HF	20 U/μL	CutSmart®	37°C	NEB
SacI-HF	20 U/μL	CutSmart®	37°C	NEB
Sall-HF	20 U/μL	CutSmart®	37°C	NEB
XbaI	20U/μL	CutSmart®	37°C	NEB

5.2.4.7.2. Dephosphorylation of 5'-ends of DNA vectors

The alkaline phosphatase treatment of vectors used for cloning is necessary to prevent self-ligation of complementary overhangs produced by restriction enzymes. The 5'-ends of DNA fragments were de-phosphorylated in 40 μL reactions by treatment with *Fast Alkaline Phosphatase* (FastAP). Reactions were mixed with the appropriate 10x Fast-AP buffer to a final concentration of 1x and 1 μL (corresponding to 1 U) of FastAP and incubated at 37°C for 1 h. Subsequently, vectors were purified before ligation.

5.2.4.7.3. Ligation of DNA fragments

For cloning an insert into a vector, 50 to 100 ng of the digested dephosphorylated vector and a 3x molar excess of the digested insert were ligated in 20 μL reactions. Ligation reactions were mixed with 10x T4 ligase buffer (NEB) to a final concentration of 1x and 1,25 U of T4 ligase (NEB) and incubated at room temperature for at least 2 h. Subsequently, reactions were transformed into competent XL1-blue *E. coli* strain. Recombinant plasmids of single colonies were checked via colony PCR (as described in 5.2.4.3.2.) and correct clones were sequenced after plasmid purification.

Ligation: $\text{ng (fragment)} = 3 \times (\text{ng vector} / \text{bp vector}) \times \text{bp fragment}$

5.2.4.8. Isolation of plasmid DNA

The transformant carrying the desired plasmid was first grown overnight in 5 mL (for isolation of high copy plasmids) or 10 mL (for isolation of low copy plasmids) LB with required antibiotics to select for the presence of the plasmid. The next day, plasmid DNA was extracted from the culture using “NucleoSpin® Plasmid” kit (Macherey-Nagel), according to manufacturer's instruction. Plasmid DNA was eluted from the silica membrane with sterile ddH₂O or 1x TE buffer and sent for sequencing. Plasmids eluted in ddH₂O were stored at -

20°C in order to avoid hydrolyzation and plasmids eluted in 1x TE were stored at 4°C. 1x TE (10 mM Tris-HCl (pH 8) and 1 mM EDTA (pH 8) contains cations in the form of salt in the solution that stabilize DNA molecules.

5.2.4.9. DNA sequencing (according to Sanger)

Sequencing of plasmids or PCR-amplified DNA fragments was performed by Microsynth AG, using the sequencing method published by Sanger. The DNA fragment (18 ng/ 100 bp) or plasmid DNA (100 to 700 ng) to be sequenced was mixed with 15 pmol of the appropriate sequencing primer and ddH₂O in a total volume of 15 µL. Obtained DNA sequences were analyzed using the alignment tool in VectorNTI or NCBI Blast database. The chromatogram files generated by sequencing were analyzed using the software Chromas Lite.

5.2.4.10. Construction of plasmid pFS35

For integration of the long *lptA*(-152)→*rapZ*(+78)'-*lacZ* reporter construct into the chromosomal λ -*attB*-site of the *E. coli*-K12 *wild-type* strain S4197, a plasmid backbone carrying the latter fusion was initially constructed. To this end, the pre-constructed plasmid backbones pFS5 and pFS32 from the laboratory collection, carrying the translational *lptB*(+100)→*rapZ*(+78)'-*lacZ* fusion or the translational *lptA*(-152)→*ptsN*(+51)'-*lacZ* fusion respectively, were used (Figure 17). Both plasmid backbones are based on the original vector pKEM20, carrying the λ -*attP*-site and the spectinomycin resistance cassette (encoded by *aadA*), which allows the integration of the translational fusion into the chromosomal λ -*attB*-site of the *E. coli*. Both plasmid backbones were initially treated with the restriction endonucleases *Sall*/*XbaI*. Subsequently, the 2393 bp long fragment from pFS5, carrying the *rpoN*(+79)-*hpf*-*ptsN*-*rapZ*(+78) gene cluster was cloned into digested pFS32 backbone via the restriction sites *Sall* and *XbaI*, creating the long *lptA*(-152)→*rapZ*(+78)'-*lacZ* reporter fusion in plasmid backbone pFS35 (Figure 17). The desired fusion was confirmed by sequencing and subsequently, the ectopic integration of the reporter construct into the chromosomal λ -*attB*-site was carried out according to the protocol published by Diederich (Diederich *et al.*, 1992). The plasmid backbone 35 was used to generate the *ori*-less circular DNA fragments which were integrated into the chromosome of the target *E. coli*-K12 *wild-type* strain S4197 (as described in 5.2.2.3.). Prior to integration, the target *E. coli* MG1655 (S4197) strains, the *wild-type* strain and the strain carrying the *ptsN*-3xFLAG allele respectively, were transformed with the temperature-sensitive integrase expression plasmid pLDR8 (Diederich *et al.*, 1992). The integration of the reporter construct into the chromosomal lambda attachment site was successful only in the MG1655 strain carrying a FLAG-tagged *ptsN* allele (*ptsN*-3xFLAG), generating strain Z1377.

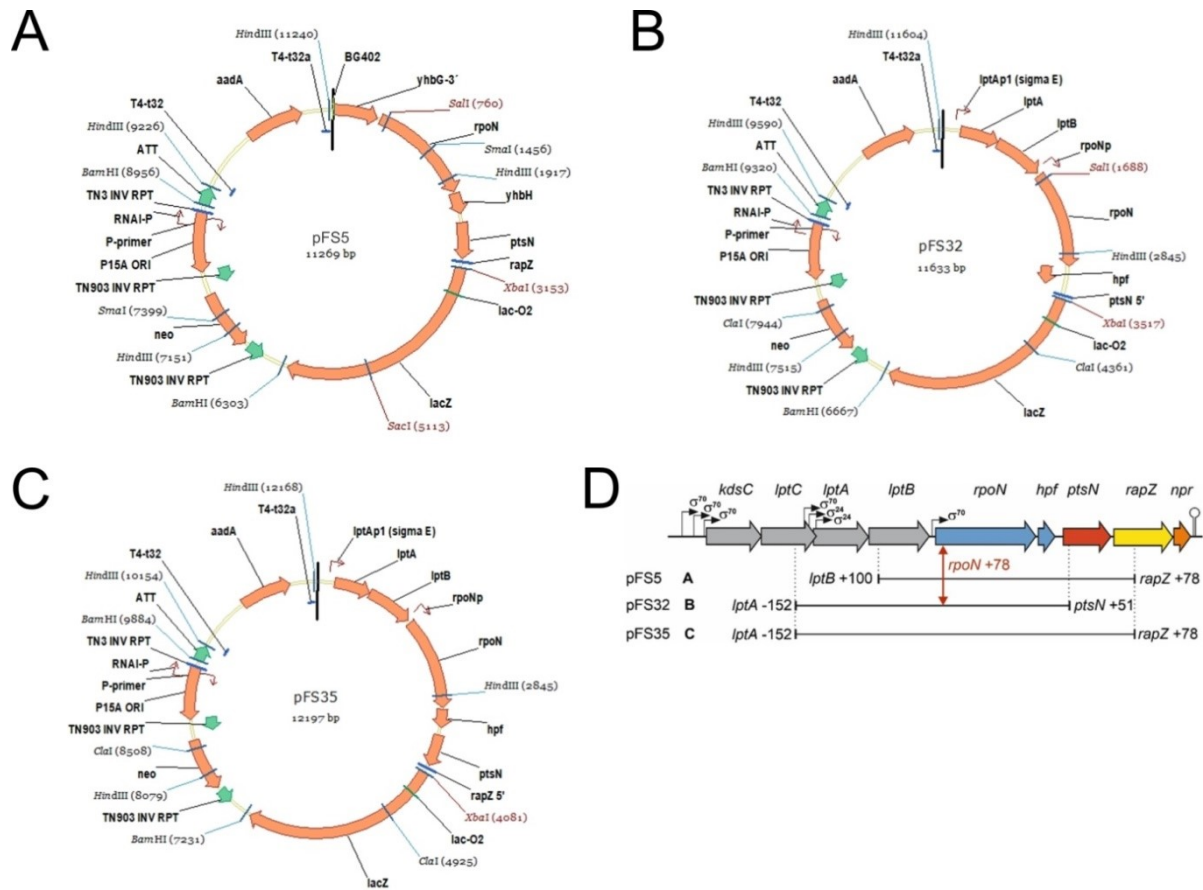


Figure 17. The construction of the translational *lptA(-152)→rapZ(+78)-lacZ* reporter gene fusion. **A.** The plasmid backbone pFS5, carrying the translational fusion *lptB(+100)→rapZ(+78)-lacZ* in pKEM20. **B.** The plasmid backbone pFS32, carrying the translational fusion *lptA(-152)→ptsN(+51)-lacZ* in pKEM20. Plasmids pFS5 and pFS32 were digested with *Sall/XbaI*. The digested 2393 bp long fragment from pFS5 was cloned into digested pFS32 backbone via *Sall / XbaI* restriction sites. **C.** The generated plasmid backbone pFS35, carrying the translational fusion *lptA(-152)→rapZ(+78)-lacZ* in pKEM20. **D.** Depiction of the translational *lacZ* reporter gene fusions in plasmids pFS5, pFS32 and pFS35. The red arrow indicates the position of ligation. Plasmid descriptions are also listed in Table 4.

5.2.5. RNA methods

5.2.5.1. RNA extraction from bacterial cells for Northern Blot

Total RNA was extracted from *E. coli* cultures using the ReliaPrep™ RNA Miniprep kit from Promega. At desired time points or optical densities, a 2 mL aliquote of the culture was harvested and immediately centrifuged (13.000 rpm, 5 min, 4°C). The supernatant was removed and the pellets were immediately shock-frozen in liquid nitrogen. Subsequently, the pellets were lysed in 100 µL 1x TE buffer containing lysozyme (final concentration of 400 µg/mL) and used for RNA isolation according to manufacturer's instruction immediately or stored at -80°C until further use.

Table 17. Composition of solutions required for RNA extraction.

1x TE with lysozyme
10 mM Tris-HCl (pH 8)
1 mM EDTA (pH 8)
400 µg/mL lysozyme (added freshly)

5.2.5.2. *In vitro* transcription to generate DIG-labeled probes for Northern Blot

To create appropriate template DNA for *in vitro* transcription, PCR reactions were carried out with primer pairs annealing to the gene of interest (GlmZ: BG230 / BG231, GlmY: BG260 / BG261, 5S: BG287 / BG288, CspH: BG1927 / BG1928, CspB: BG1942 / BG1943). Each reverse primer additionally contains the sequence of the *T7* RNA polymerase promoter at its 5'-end. PCR reactions were carried out in a total volume of 50 µL, using ThermoPol® Buffer (NEB) and *Taq*-polymerase. As DNA template 1 µL of liquid overnight culture of *E. coli* S4197 was used. Subsequently, PCR reactions were checked for the desired product on a 1% agarose gel and purified.

In vitro transcription was carried out in 20 µL reactions, containing ~ 1 µg of the purified PCR product, 25 U of *T7* RNA polymerase (NEB) and 1 µL of RNase inhibitor (Thermo). The reaction was mixed with 10X transcription buffer (NEB) and 10x DIG-RNA labeling mix (Roche) to a final concentration of 1x and incubated overnight at 37°C. In this labeling mix, the small organic molecule digoxigenin (DIG) is bound to UTP, which is incorporated into transcribed RNA by RNA polymerase, generating single-stranded DIG-labeled RNA probes. This method finally allows to visualize Northern Blots, as DIG can be detected by an anti-DIG alkaline-phosphatase conjugated antibody.

Table 18. *In vitro* transcription reaction mixture.

Component	Amount	Final concentration	Stock concentration
Purified PCR product	1 µg (at least 100 ng)		
DIG labeling mix (Roche)	2 µL	1x	10x
Transcription buffer (NEB)	2 µL	1x	10x
T7 RNA polymerase (NEB)	0,5 µL	1,25 U/µL	50 U/µL
RNase inhibitor (Thermo)	1 µL	2 U/µL	40 U/µL
ddH₂O	-		

After incubating the *in vitro* transcription overnight, the reaction was stopped by addition of 1 µL 0,5 M EDTA (pH 8.0). RNA probes were then precipitated at -80°C for approximately 2 h by addition of 3 volumes 96% EtOH and 1/10 volume sterile 4M LiCl. Samples were centrifuged (14.000 rpm, 30 min, 4°C), supernatant was removed and RNA was washed with

500 μ L 70% EtOH and centrifuged again (as before). Finally, the supernatant was removed again, the RNA was air-dried at RT and dissolved in 50 μ L RNase free water by shaking at 65°C for 5 min. RNA probes were stored at -20°C until further use.

To determine the labelling efficiency of the synthesized RNA probes, ten-fold serial dilutions were prepared in 10 μ L ddH₂O, ranging from 10⁻¹ to 10⁻⁶, and spotted onto a nylon membrane (GE Healthcare). The membrane was subsequently detected (as described in 5.2.5.4).

5.2.5.3. Denaturing urea-PAA gel electrophoresis

Denaturing urea polyacrylamide gel electrophoresis was used to separate small RNA molecules according to their size. For this purpose, equal amounts (ranging from 3 to 4 μ g) of total RNA of each sample was mixed with 2x RNA loading dye to a final concentration of 1x in a total volume of 7 to 10 μ L. RNA was denatured by incubation at 65°C for 15 min, incubated on ice for 2 to 3 min and briefly centrifuged (11.000 rpm, 30 s, 4°C). Subsequently, samples were loaded on an 8% denaturing urea gel and separation of nucleic acids was performed in a BioRad electrophoresis chamber at 100 V in 0,5x TBE running buffer for approximately 2 h, until the bromophenol blue (BPB) dye entered the buffer chamber.

Table 19. Composition of denaturing urea polyacrylamide gels and required solutions.

Denaturing urea PAA gels	10x TBE	2x RNA loading dye
7 M Urea	890 mM Tris	95% formamide
8% AA/bis (40% stock)	890 mM boric acid	0,025% SDS
1x TBE (10x stock)	20 mM EDTA	0,025% bromophenol blue
0,1% APS (10% stock)	adjust to pH 8 with HCl	0,025% xylene cyanol FF
0,02% TEMED		0,5 mM EDTA

5.2.5.4. Analysis of specific RNA species by Northern blotting

RNA molecules were transferred to a nylon membrane by Northern blotting after being separated by size via gel electrophoresis. Blots were assembled in a semi-dry blotting apparatus (BioRad Trans-Blot® Turbo™ Transfer system) in the following order (Figure 18): (cathode) and 3 sheets of Whatman paper, nylon membrane (GE Healthcare), urea-PAA gel, 3 sheets of Whatman paper (anode). The Whatman papers were soaked in transfer buffer prior to blot assembly. Northern blotting was performed at 120 mA & 15V in 0,5x TBE transfer buffer for 1 h. Immediately after blotting, RNA was cross-linked to the membrane by exposure to UV light (254 nm) for 2 to 3 min. The membrane was then placed into a hybridization tube, containing 7 mL pre-warmed pre-hybridization solution, and incubated under rotation in a hybridization oven at 68°C. After 2 h, the DIG-labeled RNA probe (0,5 to 1 μ L in 1 mL pre-hybridization solution) complementary to the RNA of interest was added and hybridization continued at 68°C overnight.

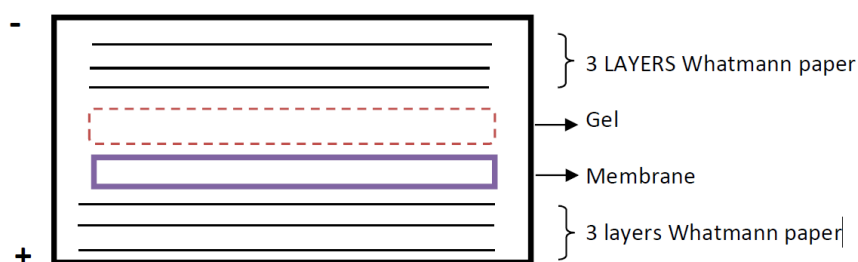


Figure 18. Assembly of the Blots (Figure provided by Görke B., unpublished).

The next day, several washing steps were performed before detection in the following order: membrane was washed with wash buffer I (2x in 10 mL for 5 min at RT), wash buffer II (2x in 10 mL for 15 min at 68°C), rinsed with dH₂O. For detection, the membrane was incubated in several solutions at RT in the following order: blocking in Buffer II (in 15 mL for 30 min), incubation in Buffer II containing 1:10.000 diluted α -DIG alkaline-phosphatase conjugated antibody from Roche (in 15 mL for 30 min), washing with 1x Buffer I (2x in 15 mL for 15 min), adjusting the pH with Buffer III (in 15 mL for 3 to 5 min). For subsequent detection, the membrane was put between transparent sheets and incubated with 500 μ L CDP-Star solution (Roche, 1:4 diluted in Buffer III) for 5 min in the dark. Afterwards, RNA-RNA hybrids were detected using the BioRad ChemiDoc imaging system.

After detection of a specific RNA species, the RNA probe was removed from the membrane by stripping. The membrane was briefly rinsed with dH₂O, washed with stripping solution (0,2 M NaOH and 0,1% SDS, 2x in 50 mL for 15 min at 37°C, shaking), incubated in 2x SSC (in 50 mL for 5 min at RT, shaking). Afterwards, the membrane was rinsed with dH₂O, placed into a hybridization tube (containing 7 mL pre-warmed pre-hybridization solution) and incubated under rotation in a hybridization oven at 68°C for 2 h before addition of a new RNA probe. Subsequently, hybridization with RNA probe was continued at 68°C overnight.

Table 20. Composition of buffers and solutions for Northern Blots.

Wash-buffer I	Wash-buffer II	20x SSC
2x SSC	0,1x SSC	3 M NaCl
0,1% SDS	0,1% SDS	0,3 M sodium citrate
		adjust to pH 7,0 with HCl
Pre-hybridization solution	10x TBE	Buffer III
100 mL formamide	890 mM Tris	0,1 M Tris-HCl
50 mL 20x SSC	890 mM boric acid	0,1 M NaCl
40 mL Blocking solution (10%)	20 mM EDTA	adjust to pH9,5 with HCl
2 mL N-laurylsarcosine (10%)	adjust to pH 8 with HCl	
0,4 mL SDS (10%)		
ad 200 mL with sterile ddH ₂ O		

Blocking solution (10%)	5x Buffer I	Buffer II
10% Blocking reagent (Merck) in 1x Buffer I	0,5 M malic acid 0,75 M NaCl adjust to pH 7,5 with NaOH	1% Blocking solution in 1x Buffer I

5.2.6. Protein methods

5.2.6.1. Preparation of total cell extracts for SDS-PAGE

The desired strain was first grown overnight in 5 mL LB with required antibiotics. The next day, cells were diluted to an OD₆₀₀ of 0.1 and grown at 37°C for a certain amount of time. At the desired time points or optical densities, a culture volume equivalent to 1 OD₆₀₀ was harvested and immediately put on ice. Samples were centrifuged (13.000 rpm, 5 min, 4°C), supernatant was removed and the pellets were directly resuspended in 200 µL 1x Laemmli buffer for analysis or stored at -20°C until further use.

5.2.6.2. Denaturing polyacrylamide gel electrophoresis (Cleveland *et al.*, 1977)

Denaturing SDS-polyacrylamide gel electrophoresis was used to separate proteins according to their molecular weight. For this purpose, the cell pellets were resuspended in 200 µL 1x Laemmli buffer (described in 5.2.6.1.) and proteins were denatured by incubation at 95°C for 10 min. Subsequently, 7 to 10 µL of samples were loaded to a 12,5% SDS-polyacrylamide gel, next to 3 µL of prestained protein molecular weight marker (Product #26612, Thermo Scientific). Separation of proteins was performed in a BioRad electrophoresis chamber in 1x SDS running buffer, first at 70 V until the loading dye reached the separating gel. Upon entry into the separating gel, the voltage was increased to 120 V and continued until the bromophenol blue (BPB) dye entered the buffer chamber. Afterwards, the gel was stained with Coomassie staining solution in order to visualize the protein bands.

Table 21. Composition of buffers for SDS-PAGE.

Laemmli buffer	1x SDS running buffer
62,5 mM Tris-HCl (pH 6,8)	25 mM Tris
5% (v/v) β-mercaptoethanol	184 mM glycine
2% (w/v) SDS	0,1% (w/v) SDS
0,05% (w/v) bromophenol-blue	
10% (v/v) glycerol	

Table 22. Gel composition for denaturing SDS-PAA gels.

Stacking gel (5%)	Separating gel (12,5%)
120 mM Tris-HCl (pH 6,8) (1M stock)	375 mM Tris-HCl (pH 8,7) (1,5 M stock)
5% AA/bis 29:1 (40% stock)	15% AA/Bis 29:1 (40% stock)
0,1% SDS (10% stock)	0,1% SDS (10% stock)
0,1% APS (10% stock)	0,1% APS (10% stock)
0,1% TEMED	0,1% TEMED

5.2.6.3. Coomassie staining of SDS-PAA gels

After gel electrophoresis or Western blotting, the gels were stained with Coomassie staining solution overnight under agitation at RT. The staining allows visualization of proteins as blue bands on bright background. This is necessary to serve as a loading control for Western blots, providing an overview of the total protein amounts loaded. Moreover, it allows to identify protein bands that are strongly changing in abundance between the different samples. The next day, the gel was briefly washed with dH₂O and the excess dye was removed with destaining solution. The gels were destained under agitation at RT until the protein bands were clearly visible (2 to 3x for appr. 30 min). Once the gel was destained, it was visualized in a BioRad ChemiDoc imaging system.

Table 23. Solutions for Coomassie staining and destaining.

Coomassie staining solution	Destaining solution
40% (v/v) ethanol	40% (v/v) ethanol
10% (v/v) acetic acid	10% (v/v) acetic acid
0,4% (w/v) Coomassie brilliant blue R-250	0,1% TEMED

5.2.6.4. Analysis of proteins by Western blotting

Proteins were transferred to a PVDF-membrane by Western blotting after being separated via PAA gel electrophoresis. Blots were assembled in a semi-dry blotting apparatus (BioRad Trans-Blot® Turbo™ Transfer system) in the following order (Figure 18): (cathode) and 3 sheets of Whatman paper, PVDF-membrane (GE Healthcare), SDS-PAA gel, 3 sheets of Whatman paper (anode). The Whatman papers were soaked in transfer buffer prior to blot assembly. The PVDF-membrane was activated by a short drench in 100% MeOH and incubated for a few min in transfer buffer. Western blotting was performed at 1,0 A/ 15V for 1 h.

After blotting, unspecific binding sites on the membrane were blocked by incubation in 15 mL 5% Blotto under agitation at 4°C for at least 3 h or overnight. After blocking, the membrane was briefly rinsed with dH₂O and incubated in TBS-T +5% BSA for 30 min at 4°C. Subsequently, several washing and detection steps were performed in the following order:

membrane was incubated with a specific primary antibody (Table 25), diluted in TBS-T +5% BSA (in 15 mL for at least 3 h or overnight at 4°C), washed with 5% Blotto (3x in 15 mL for 30 min at RT), incubated with a secondary antibody (α -rabbit coupled to alkaline phosphatase from Promega, Table 25), diluted 1:100.000 in 5% Blotto (in 15 mL for 1 h), washed with 5% Blotto (3x in 15 mL for 30 min at RT), rinsed with dH₂O, pH was adjusted with Buffer III (in 15 mL for 5 to 10 min). For subsequent detection, the membrane was put between transparent sheets and incubated with 500 μ L CDP-Star solution (Roche, 1:2 diluted in Buffer III) for 5 min in the dark. Afterwards, antibodies bound to the protein of interest were detected using the BioRad ChemiDoc imaging system.

After detection of a specific protein of interest, the antibodies bound to it were removed from the membrane by stripping. The membrane was briefly rinsed with dH₂O and stripped by three subsequent cycles of boiling in 50 mL 1% SDS in a microwave and cooling down under agitation at RT. Afterwards, the membrane was rinsed with dH₂O and incubated in 15 mL 5% Blotto under agitation at 4°C for at least 3 h or overnight before incubation with another specific primary antibody.

Table 24. Buffers and solution for Western Blots.

Transfer buffer	Blotto (5%)	Buffer III
3,02 g Tris	100 mL 10x TBS	0,1 M Tris-HCl
14,42 g glycine	50 g milk powder	0,1 M NaCl
150 mL 100% methanol	1 μ L Tween 20	adjust to pH9,5 with HCl
ad 1L with dH ₂ O	ad 1L with dH ₂ O	ad 1L with distilled H ₂ O
10x TBS	TBS-T +5% BSA	
60 g Tris	5 mL 10x TBS	
90 g NaCl	2,5 g BSA (Sigma-Aldrich, 5% final conc.)	
adjust to pH 7,6 with HCl	50 mL Tween 20	
ad 1L with distilled H ₂ O	ad 50 mL with ddH ₂ O	

Table 25. Antibody or antiserum dilutions used for Western Blots in this study.

Antibody	Dilution
Polyclonal antiserum directed against RapZ	1:3.000 in 15 mL TBS-T/BSA (5%)
Anti-GlmS	1:10.000 in 15 mL TBS-T/BSA (5%)
Polyclonal antiserum directed against PtsN (EIIA^{Ntr})	1:20.000 in 15 mL TBS-T/BSA (5%)
Anti-S2	1:5.000 in 15 mL TBS-T/BSA (5%)
Anti-Rabbit IgG AP Conjugate (Promega)	1:100.000 in 15 mL Blotto (5%)

5.2.7. Reporter gene assays

5.2.7.1. Quantitative β -galactosidase activity assay (Miller, 1972)

The activity of the enzyme β -galactosidase was determined according to the protocol published by Miller (Miller, 1972). ONPG (ortho-nitrophenol- β -D-galactopyranoside) is an artificial β -galactosidase substrate that is cleaved into galactose and ortho-nitrophenol. Within a certain time, the amount of produced yellow ortho-nitrophenol can be measured spectrophotometrically at 420 nm and used as a direct measurement of β -galactosidase activity.

The strain carrying the desired *lacZ* reporter gene fusion was first grown overnight in 5 mL LB with required antibiotics. The next day, cells were diluted to an OD₆₀₀ of 0.1 and grown at 37°C for a certain amount of time while 1 mL samples were taken at the desired time points or optical densities. Samples were centrifuged (8.000 rpm, 5 min, 4°C), supernatant was removed and the pellets were directly resuspended in 1 mL Z-buffer for analysis or stored at -20°C until further use. Subsequently, three appropriate dilutions were prepared in 1 mL Z-buffer for each sample and later the cells were permeabilized by addition of 20 μ L 0,1 % SDS and 40 μ L chloroform and vortexed for 15 s. To ensure optimal assay conditions, samples were pre-incubated at 28°C for 5 min and then reactions were started by addition of 200 μ L ONPG-solution (4mg/ml). Standard reaction time is 20 to 40 min, if no yellow coloration was observed after that time, the samples were incubated longer. When samples became adequately yellow, the reactions were stopped by addition of 500 μ L 1M Na₂CO₃. To pellet chloroform and cell debris, samples were first centrifuged (13.000 rpm, 10 min, RT) before transferring the supernatants to plastic cuvettes and measuring the absorbance at 420 nm. Finally, the β -galactosidase activities in Miller units were calculated with the following formula:

$$\text{Miller Units} = (\text{OD}_{420} \times V \times 1000) / (t \times \text{OD}_{600})$$

V = factor of dilution

t = reaction time in min

Table 26. Composition of Z-buffer and ONPG solution.

Z-buffer	ONPG solution
60 mM Na ₂ HPO ₄	60 mM Na ₂ HPO ₄
40 mM NaH ₂ PO ₄	40 mM NaH ₂ PO ₄
10 mM KCl	4 mg/mL ONPG
1 mM MgSO ₄	

5.2.8. Kits

All commercially available kits used in this study are listed in Table 27. Kits were used according to manufacturer's instruction.

Table 27. List of kits used in this study.

Kit name	Manufacturer	Usage
Macherey-Nagel NucleoSpin® Plasmid	Macherey-Nagel, Duren	plasmid isolation
Macherey-Nagel NucleoSpin® Gel and PCR Clean-up	Macherey-Nagel, Duren	purification of DNA fragments from agarose gels and purification of PCR reactions and restriction digestions
ReliaPrep™ RNA Miniprep Systems, Qiagen RNeasy Mini Kit	Qiagen, Hilden	analysis of DNA sequences

5.2.9. Used softwares and websites

All softwares and websites used in this study are listed in Table 28.

Table 28. List of softwares and websites used in this study.

Name	Publisher	Usage
Chromas Lite	Technelysium Pty Ltd	analysis of chromatogram files generated by sequencing
CorelDRAW Graphics Suite 2019 64-Bit	Corel Corporation	graphic editing
EcoCyc	www.ecocyc.org	encyclopedia reference on genes, pathways, and regulation
Mendeley	Elsevier	citation management
GelQuant.NET	http://biochemlabsolutions.com/GelQuantNET.html	image analysis and quantification program
Microsoft Office	Microsoft Corporation	text and graphic editing
NCBI	www.ncbi.nlm.nih.gov	analysis of molecular and genomic data
vectorNTI	Invitrogen	analysis of DNA sequences

5.2.10. Size markers

All size markers used in this study are listed in Figure 19.

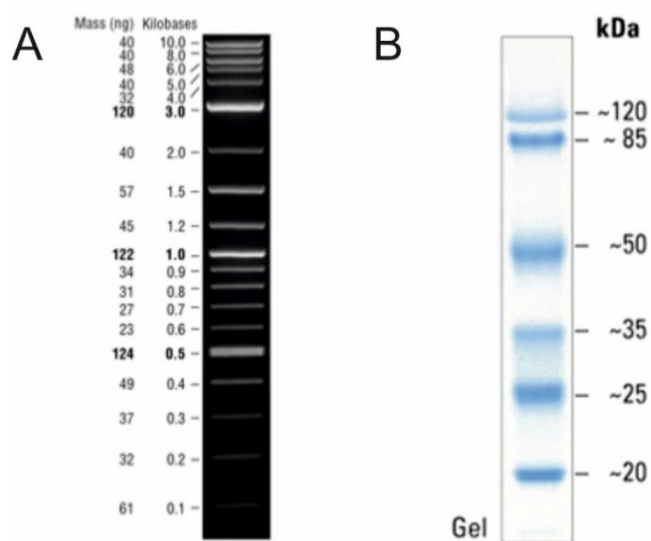


Figure 19. Size markers used in this study. A. 1 kb Plus DNA ladder, NEB (Product #N3200SVIAL). **B.** Pierce™ Prestained Protein Molecular Weight Marker, Thermo Fisher Scientific (Product #26612).

6. Results

6.1. Role of GlmY/GlmZ and RapZ for the expression of cold-shock related transcripts

The protein RapZ regulates *glmS* expression in response to the level of glucosamine-6-phosphate (GlcN6P) by binding two homologous small RNAs GlmY and GlmZ in *Escherichia coli* (Khan and Görke, 2020). Acting as an RNase E adaptor protein, it controls the stability of sRNA GlmZ by guiding the processing of GlmZ by RNase E. The sRNA GlmY inhibits GlmZ processing by binding and sequestering RapZ (Göpel *et al.*, 2013).

A recent RNA-seq analysis in the CSH50 (R1279) strain lineage to explore other RNAs that might be regulated by RapZ revealed upregulation of a couple of cold-shock related transcripts (*cspI*, *cspH*, *cspB*, *cspF*, *cspG*, *ydfK*, *ynaE*) specifically in a $\Delta glmY \Delta glmZ$ double mutant, but not in a $\Delta rapZ$ single mutant or in a $\Delta rapZ \Delta glmY \Delta glmZ$ triple mutant (Durica-Mitic *et al.*, 2020). To validate this result, the accumulation of *cspB* and *cspH* mRNAs in the $\Delta glmY \Delta glmZ$ double mutant was also confirmed by Northern blotting (Durica-Mitic *et al.*, 2020). This suggests that RapZ may only affect these transcripts when GlmZ and GlmY, its primary binding partners, are absent or present at low levels that are insufficient to sequester RapZ.

Bacteria transiently synthesize cold-shock proteins (Csp) upon rapid temperature downshift to help counteract harmful effects of the cold shock. Csps typically act as nucleic acid chaperones that bind to mRNA to prevent formation of detrimental secondary structures and facilitate translation at low temperatures (Keto-Timonen *et al.*, 2016). CspB and CspH are both members of the CspA family of cold-shock proteins; the *cspB* transcript is induced by cold (Etchegaray *et al.*, 1996). However, the *cspH* transcript is only expressed at very low level and the function of the protein is still unknown (Czapski and Trun, 2014). The cold-shock transcripts are regulated by a highly conserved 11 nt long “cold box” in the 5' UTR (Fang *et al.*, 1998), but little is known beyond. So far, a common regulator acting *in trans*, controlling all these cold-shock genes is unknown. Perhaps, RapZ has an additional function in regulation of cold-shock transcripts and may somehow affect these transcripts by interfering with the “cold box”. However, it would be unable to do so in a *wild-type* strain because it is likely bound to GlmY and GlmZ and cannot bind to anything else. It is also possible that RapZ is able to bind other RNAs, including the cold shock transcripts, when present at such high levels that are exceeding those of GlmY and GlmZ.

6.1.1. A possible role of RapZ for abundance of cold-shock related transcripts *cspB* and *cspH*

The results from the CSH50 (R1279) strain lineage (Durica-Mitic *et al.*, 2020), indicating the accumulation of *cspB* and *cspH* mRNAs in the $\Delta glmY \Delta glmZ$ double mutant, were evaluated in the MG1655 (S4197) strain lineage through Northern blot analysis of *cspB* and *cspH*. Confirming the observations in both *wild-type* strain derivatives would ensure that the obtained results are valid for a wider range of *E. coli*-K12 strains. The *E. coli*-K12 *wild-type* strain S4197 is a derivative of the widely used MG1655 strain lineage (Blattner *et al.*, 1997). It was used to test whether the deletion of *glmY* and *glmZ* influences expression of the *cspB* and *cspH* transcripts as observed in the CSH50 strain lineage (Durica-Mitic *et al.*, 2020). The previously used *E. coli*-K12 *wild-type* strain R1279 is a derivative of the CSH50 strain lineage (Miller, 1972).

To this end, a $\Delta glmY \Delta glmZ$ double mutant of strain S4197 was constructed (Z1300) by phage transduction (as described in 5.2.2.1.). The *wild-type* strain, the $\Delta rapZ$ single mutant (Z840) and the $\Delta rapZ \Delta glmY \Delta glmZ$ triple mutant (Z956) were employed as controls.

For the purpose of this experiment, pre-cultures were first grown overnight in 5 mL LB at 37°C. The next morning, fresh cultures were inoculated from the pre-cultures in 10 mL LB to an OD₆₀₀ of 0.1 and grown at 37°C until the exponential phase (OD₆₀₀ ~ 0.3). At this point, 1 mL of each culture was transferred to a pre-cooled microcentrifuge tube and immediately put on ice. Subsequently, total RNA was extracted from the cultures and subjected to Northern blot analysis, using probes specific for *cspB* and *cspH* transcripts. This allows for direct comparison of the *cspB* and *cspH* mRNA levels between the different mutants. Additionally, deletion strains of $\Delta cspB::kan$ and $\Delta cspH::kan$ were included to evaluate specificity of the RNA probes.

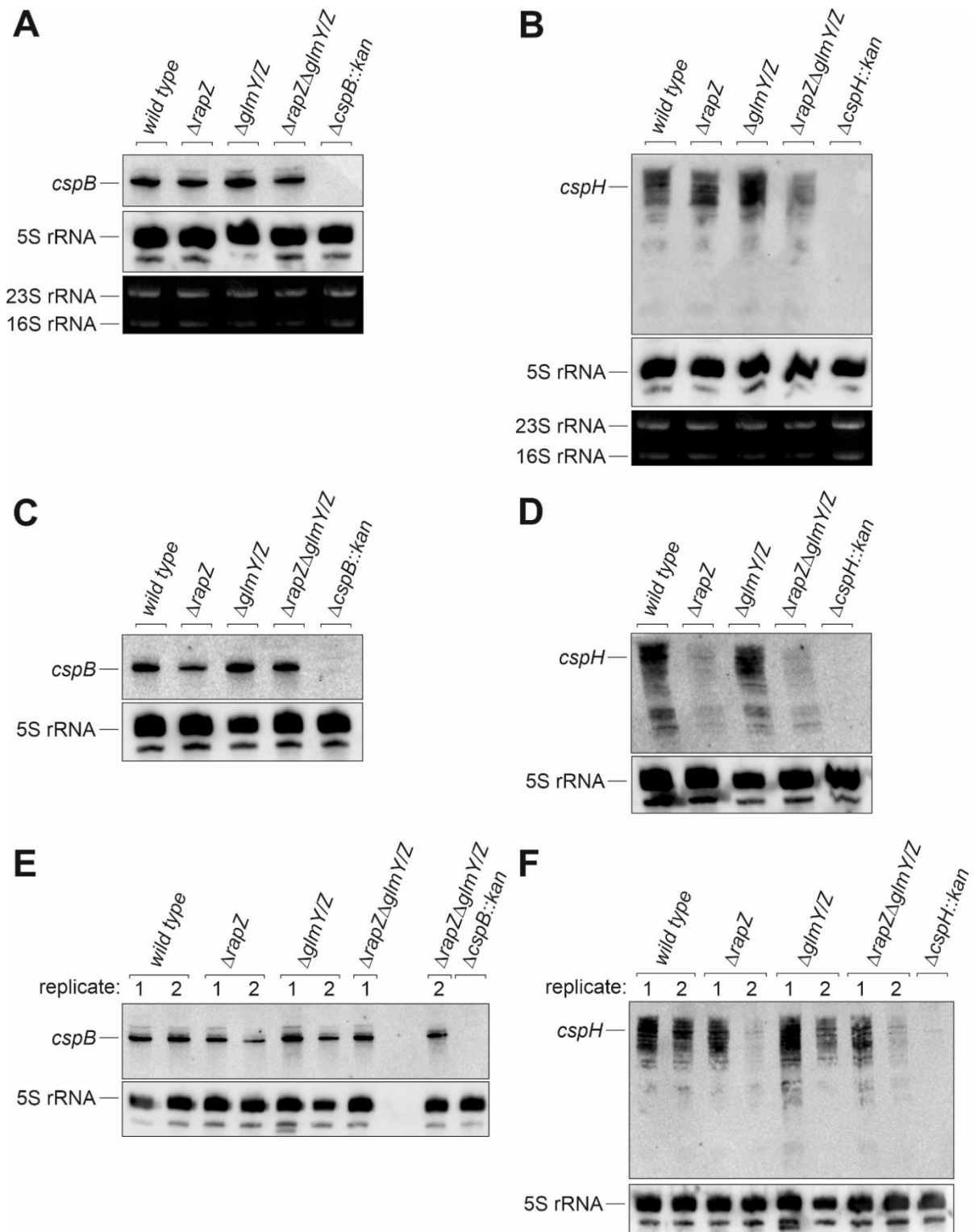


Figure 20. Northern blots analyzing a possible role of protein RapZ for abundance of cold-shock related transcripts *cspB* and *cspH*. Strains S4197 (wild-type), Z840 ($\Delta rapZ$), Z1300 ($\Delta glmY \Delta glmZ$) and Z956 ($\Delta rapZ \Delta glmY \Delta glmZ$) were grown to an $OD_{600} \sim 0.3$. Total RNA was extracted and evaluated by Northern blotting using probes specific for *cspB* (**A**, **C**) and *cspH* (**B**, **D**). To evaluate specificity of the probes, deletion strains (JW1549 ($\Delta cspB::kan$) and JW5134 ($\Delta cspH::kan$)) were included. For all the samples 3 μ g of total RNA was loaded. Loading controls are provided by 5S rRNA or/ and 23/16S rRNA signals. Figures A, B and C, D represent two independent biological replicates, respectively. Figures E and F combine two independent biological replicates. Figure E, lane 8 from the left represents an empty lane.

Transcripts of *cspB* and *cspH* were detectable in all strains, unless the corresponding deletion strains, whereas in the work of Durica-Mitic et al. (2020), *cspB* and *cspH* transcripts could just be observed in the $\Delta glmY \Delta glmZ$ double mutant. Hence, the result of the first replicate (Figure 20 A, B) deviated very much from previous observations. The upregulation of the transcripts was observed in all four employed strains. To clarify this issue, a replicate experiment was performed. The result of the first replicate could be reproduced in an independent experiment for the *cspB* transcript but not for *cspH* (Figure 20C, D). Surprisingly, in case of the *cspH* transcript, a completely different pattern was obtained. To exclude the possibility that the observed differences are due to a blotting artefact or loading unequal amounts of RNA, the RNA samples from the first and the second replicate experiment were blotted next to each other on the same membrane (Figure 20E, F). The direct comparison further demonstrated high fluctuations in the levels of the *cspB* and *cspH* transcripts from the first and the second replicate experiment. Taken together, these data indicate that the results obtained in the previous study (Durica-Mitic *et al.*, 2020) are not reproducible in the MG1655 strain lineage.

The conflicting results obtained from the first and the second replicate experiment raised the question of the reproducibility of the harvesting procedure and how strongly fluctuations in temperature might impact the expression of cold-shock related transcripts. The observed differences could be due to the minor temperature fluctuations while harvesting the samples that can hardly be avoided. Hence, the influence of the temperature during harvest was tested in the MG1655 (S4197) strain lineage through the Northern blot analysis of *cspB* and *cspH*. For the purpose of this experiment, the same strains were grown as previously described. When reaching the exponential phase ($OD_{600} \sim 0.3$), two 1 mL samples of each culture were harvested by centrifugation; one was immediately put on ice and incubated for 5 min and the other was incubated at RT prior to RNA isolation.

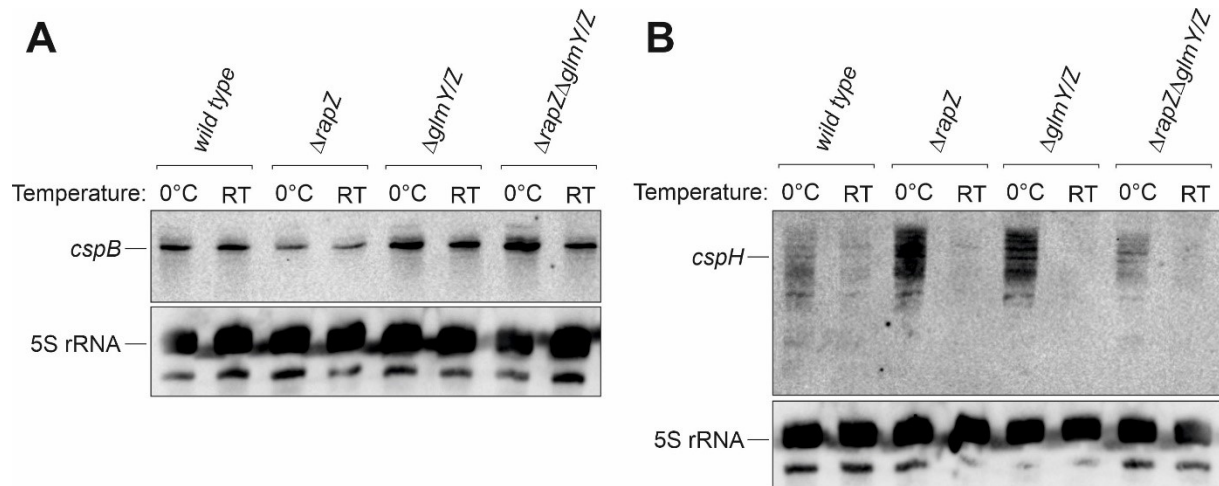


Figure 21. Northern blots analyzing the impact of the temperature on the expression of cold-shock related transcripts *cspB* and *cspH*. Strains S4197 (wild-type), Z840 ($\Delta rapZ$), Z1300 ($\Delta glmY \Delta glmZ$) and Z956 ($\Delta rapZ \Delta glmY \Delta glmZ$) were grown to an $OD_{600} \sim 0.3$. Subsequently, two samples were taken; one was incubated on ice for 5 min and the other at RT before being subjected to RNA isolation. Total RNA was extracted and evaluated by Northern blotting using probes specific for *cspB* (A) and *cspH* (B). For all the samples 3 μ g of total RNA was loaded. Loading controls are provided by 5S rRNA signals.

Testing the harvesting procedure revealed that transcripts become upregulated when cells are incubated on ice prior to RNA isolation (Figure 21A, B). While it does not seem that the harvesting temperature has much impact on the levels of *cspB* transcript (Figure 21A), it strongly influences the levels of *cspH* transcript which are highly elevated in all the samples that were incubated on ice prior to RNA isolation (Figure 21B).

The previous observation that *cspB* and *cspH* transcripts are specifically upregulated in a $\Delta glmY \Delta glmZ$ double mutant of the CSH50 (R1279) strain lineage (Durica-Mitic *et al.*, 2020) could not be reproduced for MG1655 (S4197) derivatives. The previous result in CSH50 could be explained by temperature variation during the harvesting procedure and is most likely not due to absence of GlmY and GlmZ. Perhaps, the $\Delta glmY \Delta glmZ$ double mutant of strain CSH50 also received a spontaneous mutation elsewhere in the chromosome, which was causing the phenotype observed previously by RNA-seq and Northern blot analysis.

6.2. Impact of σ^E on the adaptor protein RapZ and expression of *glmS*

The cell envelope of Gram-negative bacteria is a complex structure that protects the cell from the environment, where they encounter changing conditions and toxic molecules. To survive in these sometimes hostile circumstances, bacteria possess envelope stress response (ESR) systems that maintain cellular homeostasis, helping to limit and repair the damages. The sigma factor RpoE (σ^E) regulates the major cell envelope stress response system of Gram-negative bacteria, including *Escherichia coli*. It responds to the buildup of misfolded OMPs in the periplasm and to the production of altered forms of LPS (Mitchell and Silhavy, 2019) or depletion of the Lpt machinery (Klein and Raina, 2019), regulating extracytoplasmic functions of LPS translocation and some of its modifications (Klein *et al.*, 2016).

Recently, it was discovered that activation of the σ^E ESR, achieved by ectopic expression of *rpoE* from a plasmid, leads to a burst of *rpoN* operon expression at the transcriptional level (Sikora and Görke, unpublished). Data from our lab show that all proteins, encoded in the *rpoN* operon, accumulate enormously (Sikora and Görke, unpublished). The σ^{70} promoter upstream of *rpoN* is constitutive and not affected by σ^E . The first σ^E -responsive element in genetic proximity is the σ^E -dependent *lptAp1* upstream of gene *lptA* that is activated by specific stress conditions, which induce modifications in LPS composition and biogenesis (Martorana *et al.*, 2011). The latter promoter is located 54 nucleotides upstream of gene *lptA* and likely mediates a ~ 10 -fold increase of the *rpoN* operon expression via read-through transcription (Figure 22). The second σ^E -responsive element mediating a ~ 2 to 3-fold upregulation of the *ptsN-rapZ-ptsO* gene cluster is located in the region comprising *hpf* and the 118 nt long intergenic region separating *hpf* and *ptsN* and can also be activated by diverse envelope stressors like 5% ethanol, or a combination of SDS and EDTA (Sikora and Görke, unpublished). This dual regulation of the *rpoN* operon by σ^E indicates the *rpoN* operon as a probable member of the σ^E regulon.

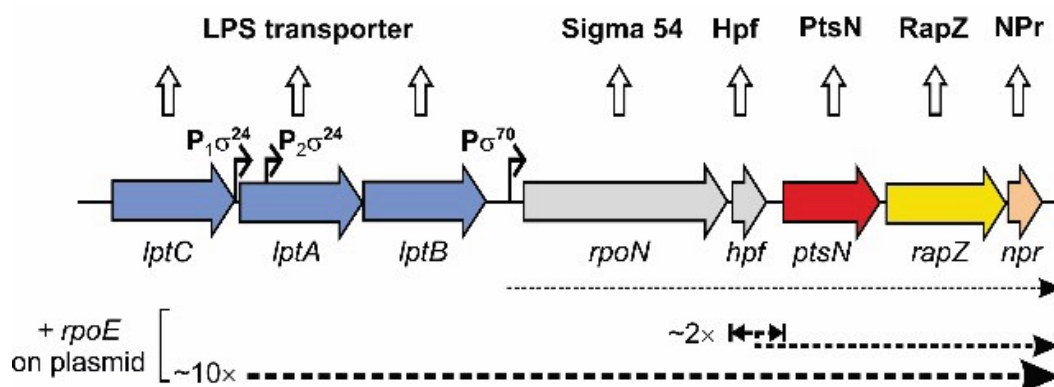


Figure 22. The *E. coli* *rpoN* operon (Figure provided by Görke B., unpublished). The σ^E -responsive regulatory elements, driving σ^E -dependent transcription of the *rpoN* operon. The *rpoN* operon is preceded by *lptA* and *lptB* that are part of the *yrbG-lptB* locus. It consists of *rpoN*, *hpf*, *ptsN*, *rapZ* and *npr*.

All gene products of the *rpoN* operon (except for *rpoN* itself) exert regulatory functions at the post-transcriptional (RapZ) and post-translational level (Hpf, PTS^{Ntr}). Thus, they might help bacteria to counteract envelope stress by interaction with target proteins or small regulatory RNAs. The RNase E adaptor protein RapZ itself contributes to the cell envelope precursor homeostasis by regulating *glmS* expression in response to the level of GlcN6P via sRNAs GlmY and GlmZ (Khan and Görke, 2020). In this study, the impact of σ^E on the adaptor protein RapZ and expression of the enzyme GlmS were addressed through the use of ectopic *rpoE* expression from a plasmid.

6.2.1. Influence of the σ^E overexpression on growth of *E. coli*-K12 under standard laboratory growth conditions

The σ^E ESR is essential in *E. coli* and deletion of *rpoE* is lethal (De Las Penas *et al.*, 1997). Nevertheless, overexpression of *rpoE* causes over-activation of the σ^E ESR, which is in turn harmful for the cell. The σ^E response is achieved through a proteolytic cascade that leads to cleavage of an anti-sigma factor (RseA) which otherwise represses transcriptional activity of *rpoE* (Walsh *et al.*, 2003). Deleterious over-activation of σ^E is exemplified by the toxicity of *rseA* deletion that causes constitutive full activation of the σ^E ESR (Missiakas *et al.*, 1997), leading to sensitivity to chemical compounds (Nicoloff *et al.*, 2017) and cell lysis during the stationary phase (Nitta *et al.*, 2000). This is primarily caused by defects in OM integrity, arising from the σ^E -dependent overexpression of sRNAs MicA and RybB that largely decrease production of OMPs (Nicoloff *et al.*, 2017). Hence, over-activation of σ^E ESR harms the membrane integrity and disrupts the cell growth. To demonstrate the harmful effect of the constitutive full activation of the σ^E ESR on the growth of *E. coli*-K12 *wild-type* strain S4197 under standard laboratory growth conditions, σ^E was ectopically expressed from the plasmid.

For the purpose of this experiment, *wild-type* (S4197) strain was initially transformed with the *rpoE* expression plasmid or the corresponding VC. The latter transformant and untransformed *wild-type* served as negative controls in the experiment. The plasmid used for ectopic *rpoE* expression encodes the *rpoE* gene, provided with the strong *B. subtilis* *sacB* RBS, under the control of the arabinose-inducible *P_{ara}* promoter in plasmid pBAD33, carrying a chloramphenicol resistance cassette. Transformants were grown overnight at 37°C in 5 mL LB supplemented with chloramphenicol. The growth of the transformants was subsequently monitored in two ways: firstly, by growing them in LB medium with and without the appropriate inducer while measuring OD₆₀₀ in time intervals and secondly, by spotting assays at appropriate culture dilutions carried out on LB plates either containing or lacking

arabinose. In order to monitor the growth of liquid cultures, pre-cultures (untransformed *wild-type*, *rpoE* and VC transformants) were inoculated from the overnight cultures to an OD₆₀₀ of 0.1 and subsequently grown at 37°C in 25 mL LB supplemented with chloramphenicol until reaching an OD₆₀₀ ~ 0.3. Next, each pre-culture was split into two 10 mL cultures, one of which was supplemented with 0,2% arabinose to induce expression of *rpoE*, whereas the other culture received dH₂O as control. Arabinose acts as an inducer of the P_{ara} promoter, resulting in ectopic *rpoE* expression. The growth of the cultures was monitored over 2 h by measuring OD₆₀₀ in 30 min intervals following the addition of arabinose. In order to evaluate a possible growth-defect phenotype caused by σ^E overexpression, overnight cultures of *rpoE* and VC transformants were adjusted to 1 OD₆₀₀ (= ~10⁹ cells/mL). Next, ten-fold serial dilutions were prepared in 1 mL LB medium, ranging from 10⁸ to 10⁴ cells/mL. Subsequently, 2 μ L of each serial dilution was spotted onto LB plates supplemented with chloramphenicol and X-Gal and with or without 0,2% arabinose to induce expression of *rpoE*. The plates were then incubated overnight at 37°C and evaluated on the next day.

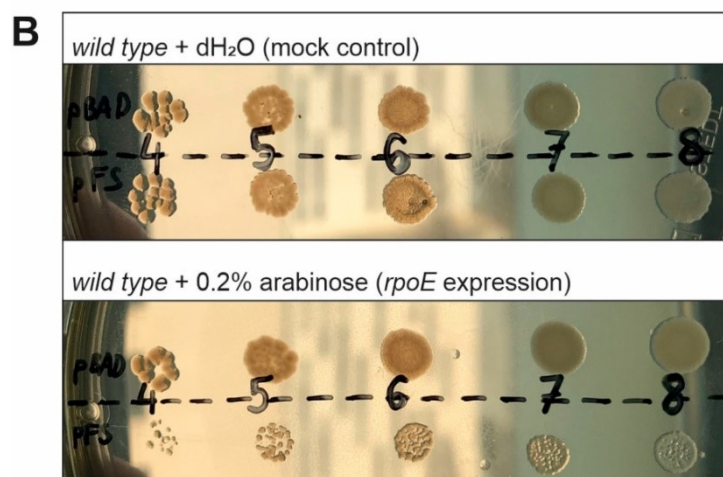
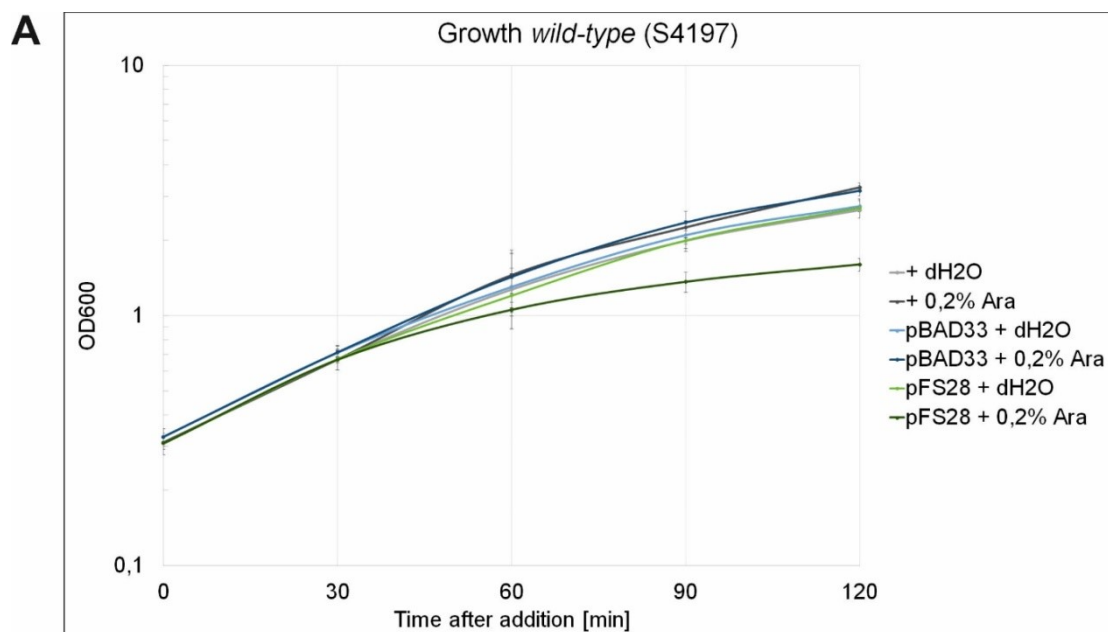


Figure 23. Ectopic *rpoE* expression results in growth retardation of *E. coli*. Strain S4197 (*wild-type*) was transformed with the *rpoE* expression plasmid (pFS28) or the corresponding VC (pBAD33). Transformants were grown in LB supplemented with chloramphenicol +/- 0,2% arabinose. Empty plasmid pBAD33 provided the VC and the untransformed *wild-type* strain was analyzed in parallel for comparison. For induction of P_{ara} promoter, 0,2% arabinose was added. OD₆₀₀ of each culture was measured in 30 min intervals following the addition of arabinose. OD₆₀₀ values are presented as mean $\pm$ SD that derived from five independent measurements (n = 5). **B.** Overnight cultures of *rpoE* (lower row) and VC (upper row) transformants were adjusted to 1 OD₆₀₀ = 10⁹ cells/mL and 10-fold serial dilutions were prepared in 1 mL LB medium, ranging from 10⁸ to 10⁴ cells/mL (spotted from right to left). 2 μ L of each serial dilution were spotted onto LB plates supplemented with chloramphenicol +/- 0,2% arabinose to induce expression of *rpoE*. The plates were incubated overnight at 37°C.

In agreement with previous publications, activation of σ^E achieved by ectopic expression of *rpoE* from the arabinose-inducible promoter on plasmid pFS28 resulted in retardation of growth (Figure 23). An obvious difference in OD₆₀₀ was measured during the whole experiment for the transformant expressing *rpoE* from a plasmid in the presence of arabinose. After 2 h, the latter transformant reached an OD₆₀₀ of $\sim$ 1.6, while the uninduced *rpoE* transformant, induced and uninduced VC transformants as well as the *wild-type* exhibited much higher culture densities (OD₆₀₀ = 2.7 - 3.2; Figure 23A). It is important to mention that the addition of arabinose in the medium increases the growth rate of *E. coli* strains to some minor extent. The presence of arabinose resulted in faster growth of the VC transformant and the *wild-type* strain, reaching a final OD₆₀₀ > 3. In comparison, the *rpoE* transformant, the VC transformant as well as the *wild-type* strain reached an OD₆₀₀ of $\sim$ 2.7 when arabinose was omitted (Figure 23A). An obvious difference between the transformants was observed on the plates as well, demonstrating a growth-defect, i.e. smaller colonies, of the induced *rpoE* transformant while all other cells grew normally (Figure 23B). Hence, ectopic expression of *rpoE* from an arabinose-inducible promoter on a plasmid, resulting in over-activation of the σ^E ESR, impedes the cell growth.

6.2.2. σ^E overexpression triggers accumulation of protein RapZ

The multifunctional sRNA-binding protein RapZ post-transcriptionally controls synthesis of the glucosamine-6-phosphate synthase GlmS (Göpel *et al.*, 2014) in response to the level of GlcN6P. The enzyme GlmS synthesizes GlcN6P, initiating the amino sugar pathway which generates UDP-GlcNAc, a major component of the bacterial cell envelope. RapZ regulates *glmS* expression by binding two homologous small RNAs GlmY and GlmZ to achieve GlcN6P homeostasis (Khan and Görke, 2020).

The role of RapZ for regulation of *glmS* has so far been studied under the assumption that *rapZ* is constitutively expressed and RapZ amounts do not significantly vary (Göpel *et al.*, 2013; Gonzalez *et al.*, 2017; Durica-Mitic *et al.*, 2020; Khan *et al.*, 2020; Khan and Görke, 2020). However, the activation of σ^E ESR, perhaps mediated by read-through transcription from the *lptAp1* promoter into the *rpoN* operon or by another σ^E -responsive element within the *rpoN* operon, possibly results in increased biosynthesis of RapZ.

6.2.2.1. RapZ accumulates upon ectopic *rpoE* expression

To study the consequences of active σ^E for accumulation of RapZ protein and possible downstream effects, σ^E was ectopically expressed from the arabinose-inducible promoter on plasmid pFS28. For the purpose of this experiment, *wild-type* strain S4197 was initially transformed with the *rpoE* expression plasmid (pFS28) or the corresponding VC (pBAD33), respectively. The latter transformant and untransformed *wild-type* served as negative controls in the experiment. Additionally, the *wild-type* strain was transformed with a *rapZ* expression plasmid encoding the *rapZ* gene with its authentic RBS (pBGG61) under P_{ara} promoter control in plasmid pBAD33. A $\Delta rapZ$ mutant strain was included as additional control. The transformants were grown overnight at 37°C in 5 mL LB supplemented with chloramphenicol, with the exception of the untransformed *wild-type* strain that was grown in LB without chloramphenicol during the whole experiment. Next, pre-cultures were inoculated from the overnight cultures to an OD₆₀₀ of 0.1 and grown at 37°C in 25 mL LB until reaching an OD₆₀₀ of ~ 0.3. At this point, each pre-culture was split into two 10 mL cultures, one of which was supplemented with 0,2% arabinose to induce expression of *rpoE* or *rapZ* and the other with dH₂O as mock control. Subsequently, samples were harvested at various times and total protein extracts were prepared (as described in 5.2.6.1.). Of the latter, 10 μ L (cell number ~ 0.05 OD₆₀₀, ~ 2,3 μ g proteins) were separated by SDS-PAGE and analyzed by Western blotting using a polyclonal antiserum directed against RapZ or ribosomal protein S2 (loading control). After blotting, the SDS-PAA gel was stained with Coomassie blue to serve as an additional loading control.

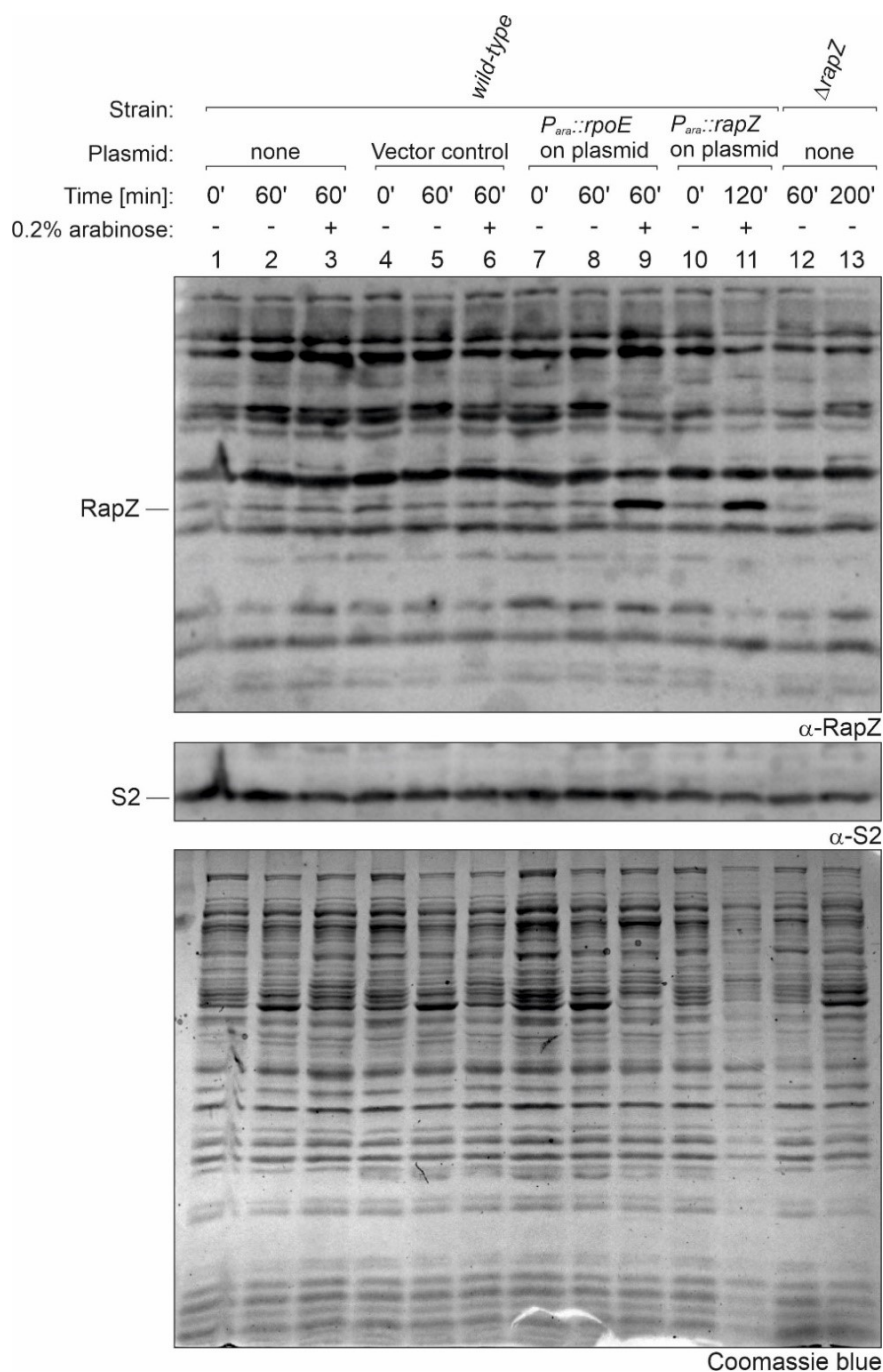


Figure 24. Western blot analysis of RapZ levels upon ectopic *rpoE* expression. Strain S4197 (*wild-type*) was transformed with the *rpoE* expression plasmid (pFS28) or the *rapZ* expression plasmid (pBGG61) or the corresponding VC (pBAD33). Transformants were grown in LB supplemented with chloramphenicol +/- 0,2% arabinose. Empty plasmid pBAD33 provided the VC and the untransformed *wild-type* strain was analyzed in parallel for comparison. Strain Z857 (*ΔrapZ*) was included as additional control. For induction of *P_{ara}* promoter, 0,2% arabinose was added. Samples for preparation of total protein extracts were harvested at the indicated times following the addition of arabinose. 10 μ L of total protein extracts (cell number $\sim$ 0.05 OD₆₀₀, $\sim$ 2,3 μ g proteins) were separated on a 12,5% SDS-PAA gel and analyzed by Western blotting using a polyclonal antiserum directed against RapZ (top panel) or ribosomal protein S2 (middle panel) as a loading control. The SDS-PAA gel was stained with Coomassie blue after blotting to serve as an additional loading control (bottom panel).

Upon ectopic expression of *rpoE* from the arabinose-inducible promoter on plasmid pFS28, mimicking σ^E activation, a strong accumulation of protein RapZ was observed (Figure 24).

The fold-upregulation of RapZ levels, achieved by ectopic expression of either *rpoE* after 60 min (Figure 24, lane 9) or *rapZ* after 120 min (Figure 24, lane 11) appears comparable. In all other samples, basal levels of RapZ protein were detectable (Figure 24, lanes 1-8). Thus, ectopic expression of *rpoE*, resulting in over-activation of the σ^E ESR, triggers accumulation of RapZ.

6.2.2.2. Further dissection of the regulatory elements responding to envelope stress in the *lptAB* and the *rpoN* operon using ectopic *lacZ* fusions

A previously described σ^E -dependent promoter upstream of gene *lptA*, *lptAp1* (Martorana *et al.*, 2011), could potentially explain the observed accumulation of RapZ in response to *rpoE* expression. A second potential σ^E -dependent promoter downstream of *lptAp1*, *lptBp* (Figure 25), is more or less inactive *in vitro* and *in vivo* (Mutalik *et al.*, 2009; Rhodius and Mutalik, 2010) and was shown to not actually respond to σ^E (Martorana *et al.*, 2011). Therefore, it might not play a role in the expression of *lptB* and is not a likely candidate for the element being responsible for the observed accumulation of RapZ in response to *rpoE* expression. Genes *lptA* and *lptB* are located upstream of the *rpoN* operon and are part of the *E. coli* *yrbG-lptB* locus that encodes genes for outer membrane biogenesis (Figure 25). The σ^E -dependent *lptAp1* promoter is not activated by every envelope stress condition that usually induces the σ^E ESR, but it was shown to be selectively activated upon depletion of the LPS transport machinery, suggesting that it responds to LPS biogenesis defects and trafficking problems (Martorana *et al.*, 2011). To determine whether this promoter also contributes to *rapZ* expression when σ^E is active, *E. coli* strains carrying suitable translational *lacZ* reporter gene fusions were tested (Figure 25).

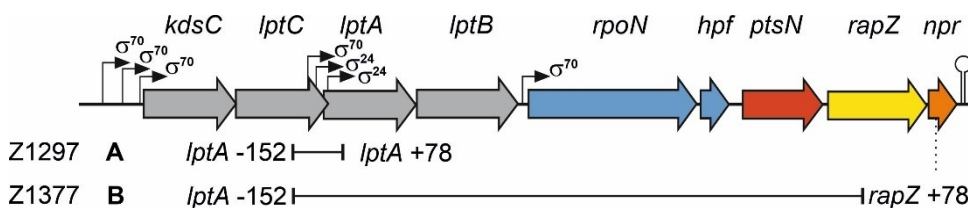


Figure 25. The *E. coli* *rpoN* operon and the translational *lacZ* reporter gene fusions. The *E. coli* *rpoN* operon consists of *rpoN*, *hpf*, *ptsN*, *rapZ* and *npr*. The σ^{70} promoter upstream of *rpoN* is constitutive and not affected by σ^E . The *rpoN* operon is preceded by genes *lptA* and *lptB* that are part of the *E. coli* *yrbG-lptB* locus. **A.** The *lptA*(-152 to +78)-*lacZ* reporter gene fusion, comprising only the *lptAp1* and the *lptB* promoter. **B.** The *lptA*(-152)→*rapZ*(+78)-*lacZ* reporter gene fusion, comprising the *lptAp1* promoter, the *lptB* promoter and the *rpoN* σ^{70} promoter.

The ectopic integration of the *lptA*(-152 to +78)-*lacZ* reporter construct into the chromosomal λ -*attB*-site of the *E. coli*-K12 wild-type strain S4197 is described in the chapters 5.2.2.3.1 and 6.4.1. For integration of the long *lptA*(-152)→*rapZ*(+78)-*lacZ* reporter construct into the chromosomal λ -*attB*-site of the *E. coli*-K12 wild-type strain S4197, a plasmid backbone carrying the latter fusion was initially constructed (as described in 5.2.4.10.).

At first, the strain carrying the chromosomal *lptA*(-152 to +78)'-lacZ fusion (Figure 25) was used to determine whether the *lptAp1* promoter is activated by ectopic *rpoE* expression. Subsequently, the strain carrying the long chromosomal *lptA*(-152) → *rapZ*(+78)'-lacZ fusion (Figure 25) was used to check if the latter activation leads to read-through transcription into the *rapZ* gene encoded in the *rpoN* operon. Possibly, the fusion contains additional σ^E -responsive elements within the *rpoN* operon as well. For the purpose of this experiment, the strain carrying the suitable lacZ reporter fusion was initially transformed with the *rpoE* expression plasmid (pFS28) or the corresponding VC (pBAD33), respectively. Cells were grown as previously described (in 6.2.2.) and samples for β -galactosidase activity measurements were taken from the cultures over 2 h in 30 min intervals following the addition of arabinose.

Induction of the plasmid-encoded *rpoE* under the control of an arabinose-inducible promoter in the strain carrying the *lptA*'-lacZ reporter fusion led to dramatic increase in activity of the reporter fusion (Sikora and Görke, unpublished; Supplementary Figure S1A, dark green columns), demonstrating the activation of the *lptAp1* promoter upon ectopic *rpoE* expression. A maximum induction of the fusion was reached 120 min after the addition of arabinose, resulting in more than 400-fold induction of the β -galactosidase activity. The activities of the controls used in the experiment, the untransformed *wild-type* strain or the VC control grown in the presence or absence of arabinose and the uninduced *rpoE* transformant, are comparable (Supplementary Figure S1A). Together, those data confirm that the σ^E -dependent promoter *lptAp1* is strongly activated in response to *rpoE* expression.

Subsequently, the strain carrying the long chromosomal *lptA*(-152) → *rapZ*(+78)'-lacZ fusion was used to check if strong activation of *lptAp1* also affects the whole *rpoN* operon by read-through transcription.

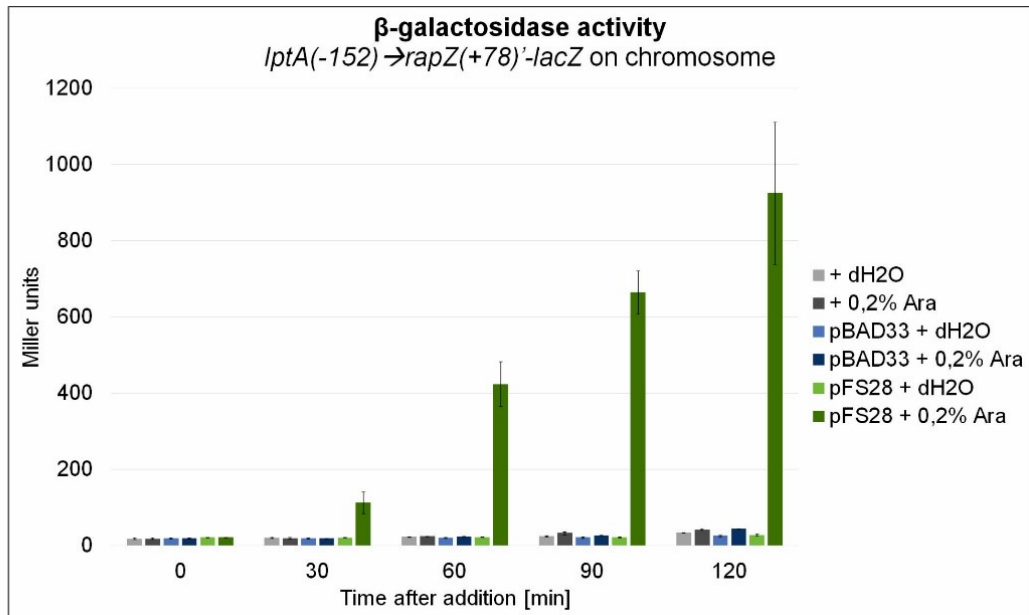
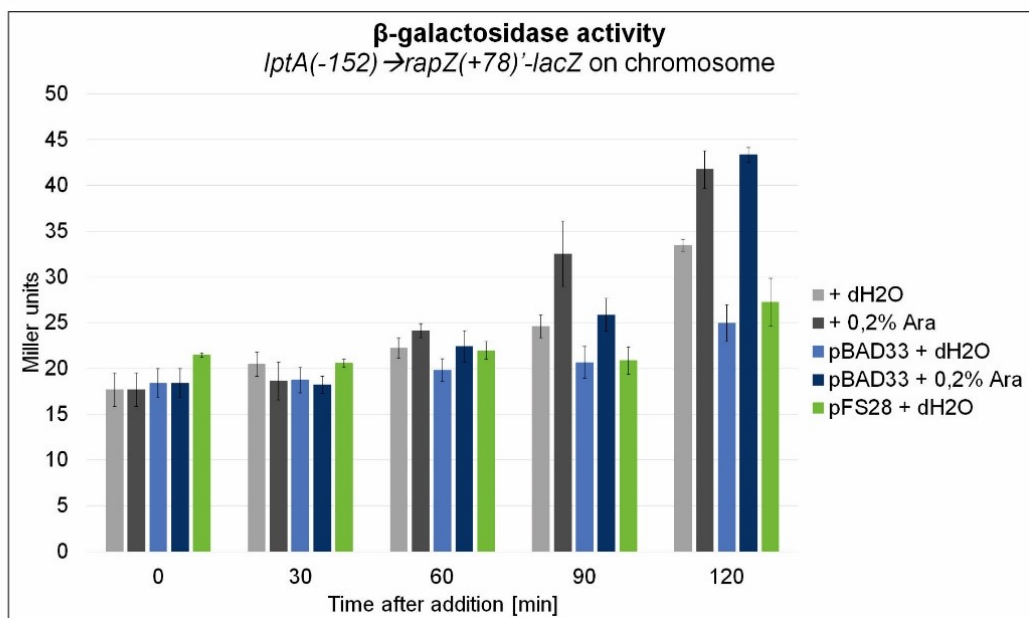
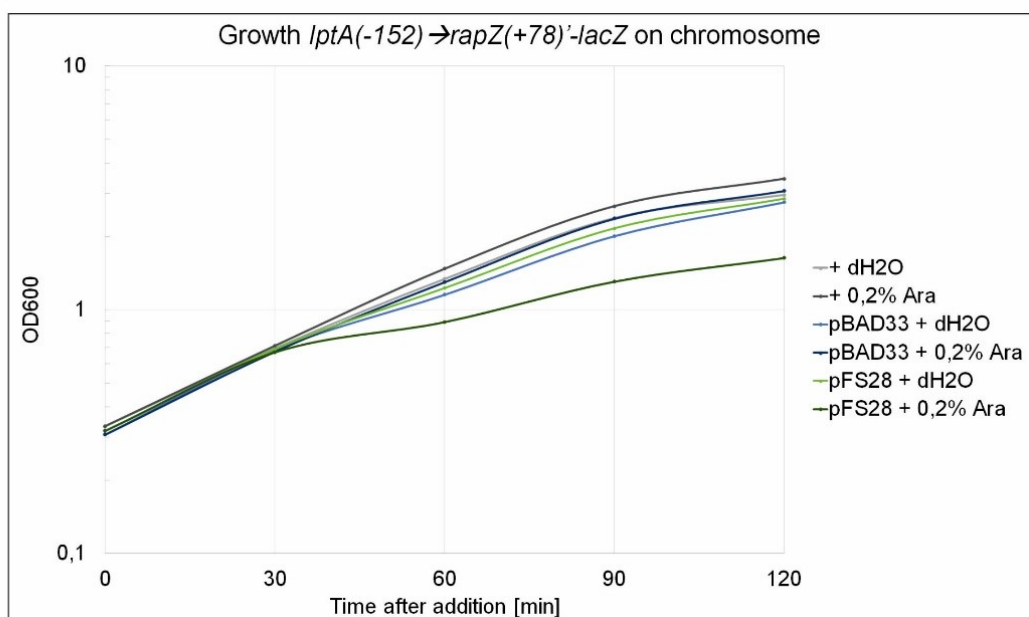
A**B****C**

Figure 26. Ectopic *rpoE* expression leads to upregulation of *lptA*→*rapZ*'-*lacZ*. **A. and B.** β -galactosidase activity assay addressing the expression of the chromosomal *lptA*→*rapZ*'-*lacZ* reporter fusion in *E. coli*. Strain Z1377 (*S4197 ptsN-3xFLAG [attB:: lptA(-152) →rapZ(+78)'*-*lacZ]*) was transformed with the *rpoE* expression plasmid (pFS28) or the corresponding VC (pBAD33). Transformants were grown in LB supplemented with chloramphenicol +/- 0,2% arabinose. Empty plasmid pBAD33 provided the VC and the untransformed *wild-type* strain was analyzed in parallel for comparison. Miller unit values are presented as mean $\pm$ SD that derived from three independent measurements (n = 3). Panel (**B**) shows the same dataset as (A) omitting the activities of the induced *rpoE* transformant for better comparison of the control samples. **C.** The growth of the cultures addressing the expression of the chromosomal *lptA*'-*lacZ* reporter fusion in *E. coli*. OD₆₀₀ values are presented as mean that derived from three independent measurements (n = 3).

Induction of the plasmid-encoded *rpoE* under the control of an arabinose-inducible promoter in the strain carrying the long *lptA*→*rapZ*'-*lacZ* reporter fusion led to a dramatic increase in activity of the reporter fusion as well (Figure 26A, dark green columns). These data confirm that increased transcription from an upstream located promoter, resulting in an increased expression of *rapZ*, is likely responsible for the observed accumulation of protein RapZ. A maximum induction of the fusion was reached 120 min after the addition of arabinose, resulting in almost 35-fold induction of the β -galactosidase activity. The activities of the controls used in the experiment, the untransformed *wild-type* strain or the VC control grown in the presence or absence of arabinose and the uninduced *rpoE* transformant, are comparable 30 and 60 min after the addition (Figure 26B). However, the *wild-type* and VC strains grown in the presence of arabinose later exhibit higher activities that are most apparent 120 min after the addition, resulting in almost 1,5 to 2-fold induction compared to the control strains grown in the absence of arabinose (Figure 26B, dark grey and dark blue columns). The observed activation of the reporter fusion may come from a *cyaA*-dependent element, located upstream of the *ptsN* gene and which could also respond to arabinose (Sikora and Görke, unpublished), resulting in minor elevation of *rapZ* expression. Gene *cyaA* encodes adenylate cyclase CyaA that catalyzes the synthesis of cAMP. cAMP is an important signaling molecule that participates in regulation of transcription of many metabolic genes via binding to the CAP protein. Together, those data confirm that *rapZ* expression increases upon σ^E induction, likely mediated by read-through transcription from the upstream located *lptAp1* promoter.

6.2.2.3. Pilot experiment for determination of an appropriate inducer concentration for the P_{tet} promoter

Using the arabinose-inducible promoter for ectopic *rpoE* expression was not well suited for the further analysis, because the addition of arabinose possibly results in activation of a *cyaA*-dependent element, located upstream of the *ptsN* gene (Sikora and Görke, unpublished; as described in 6.2.2.2.). Furthermore, the latter effect of the inducer on the GlmY/GlmZ/*glmS* system was subsequently reflected by enhanced GlmZ processing in the empty vector control upon Northern blot analysis (as described in 6.2.2.2.). Therefore, the change of inducer and expression plasmid was necessary to overcome this problem. Hence,

the $Tet^R P_{tet}$ cassette was used to replace the *Clal/SacI* fragment in pFS28, which comprises the *araC P_{ara}* module, to achieve ectopic *rpoE* expression in further experiments.

In order to generate the plasmid with *rpoE* under control of an anhydrotetracycline-inducible promoter (P_{tet}) and the corresponding VC, the $Tet^R P_{tet}$ cassette was amplified by PCR and cloned via *Clal/SacI* into the original plasmid pFS28 with *rpoE* under P_{ara} control and the corresponding VC (pBAD33) from which the *araC P_{ara}* cassette was initially removed (Figure 27). The $Tet^R P_{tet}$ cassette carries the *Tet* repressor (Tet^R) and the tetracycline-inducible promoter (P_{tet}), containing the *tet* operator sequence (*tetO*) that is recognized by the TetR repressor protein. In the absence of an inducer, the tetracycline-inducible promoter is tightly repressed by the TetR repressor that binds the *tet* operator sequence in the promoter. When the inducer tetracycline or one of its derivatives like anhydrotetracycline is present, the TetR repressor is released from the *tet* operator, which in turn enables expression of the gene that is encoded downstream of the tetracycline-inducible promoter.

Anhydrotetracycline is a less-toxic derivative of tetracycline and an efficient inducer of the tetracycline promoter (P_{tet}) present in tetracycline-controlled gene expression systems in bacteria. Furthermore, it exhibits a lower antibiotic activity towards *E. coli* (Gossen and Bujard, 1993). The concentration of anhydrotetracycline reported in the literature (Ehrt *et al.*, 2005; Lim *et al.*, 2017; Burke *et al.*, 2020) that is used to induce expression of the desired gene from a tetracycline-inducible promoter on a plasmid ranges from 25 – 200 ng/mL. A pilot experiment was carried out to determine the lowest anhydrotetracycline concentration sufficient for full induction of *rpoE* expression from the newly constructed plasmid through Western blotting to monitor accumulation of RapZ as a consequence of *rpoE* expression as well as through usage of *E. coli* strains carrying suitable *lacZ* reporter gene fusions.

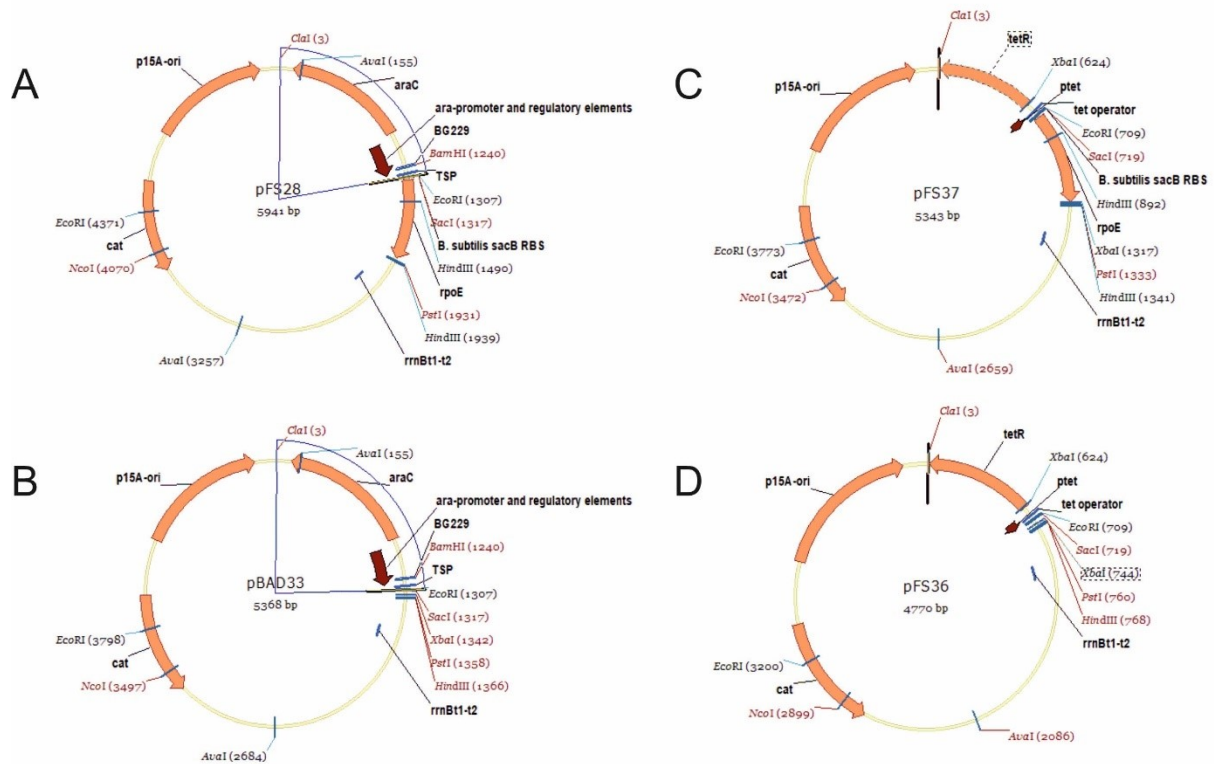


Figure 27. Plasmids used for ectopic *rpoE* expression and the corresponding VCs. The *araC* P_{ara} cassette was initially removed via *Clal* / *SacI* restriction sites from the plasmid pFS28 with *rpoE* under P_{ara} control (A) and the corresponding VC pBAD33 (B). Subsequently, the Tet^R P_{tet} cassette from the plasmid pKESL307 was amplified by PCR and cloned via *Clal* / *SacI* restriction sites into pFS28 and pBAD33 from which the *araC* P_{ara} cassette was initially removed, generating the expression plasmid pFS37 allowing to express *rpoE* upon addition of anhydrotetracycline (C) and the corresponding VC pFS36 (D). **A. pFS28 plasmid** carrying *rpoE* with *B. subtilis* *sacB* RBS under control of an arabinose-inducible promoter (P_{ara}) and the chloramphenicol resistance cassette. **B. pBAD33 plasmid** carrying an arabinose-inducible promoter (P_{ara}) and the chloramphenicol resistance gene (*cat*). **C. pFS37 plasmid** carrying *rpoE* with *B. subtilis* *sacB* RBS under control of an anhydrotetracycline-inducible promoter (P_{tet}) and chloramphenicol resistance gene (*cat*). **D. pFS36 plasmid** carrying an anhydrotetracycline-inducible promoter (P_{tet}) and the chloramphenicol resistance gene (*cat*). Plasmid descriptions are also listed in Table 4.

At first, in order to determine the optimal anhydrotetracycline concentration for induction of the newly constructed *rpoE* expression plasmid pFS37, various concentrations (25 ng/mL, 50 ng/mL, 100 ng/mL and 200 ng/mL) of anhydrotetracycline were tested. For the purpose of this experiment, the *wild-type* (S4197) strain was initially transformed with the *rpoE* expression plasmid (pFS28 or pFS37) or the corresponding VC (pBAD33 or pFS36). The newly constructed plasmid, used for ectopic *rpoE* expression, encodes the *E. coli* *rpoE* coding sequence with *B. subtilis* *sacB* RBS, under the control of the anhydrotetracycline-inducible P_{tet} promoter in plasmid pFS36, harboring a chloramphenicol resistance cassette. Pre-cultures of transformants were inoculated from the overnight cultures to an OD_{600} of 0.1 and subsequently grown at 37°C in 60 mL LB supplemented with chloramphenicol until reaching an OD_{600} of ~ 0.3. Next, each pre-culture was split into two or more 10 mL cultures, one of which was supplemented either with 0,2% arabinose (transformants carrying pBAD33

or pFS28) or with various concentrations (25 ng/mL, 50 ng/mL, 100 ng/mL and 200 ng/mL) of anhydrotetracycline to induce expression of *rpoE* whereas the other culture received dH₂O as control. The growth of the cultures was monitored over 2 h by measuring OD₆₀₀ in 30 min intervals following the addition of an inducer. Additionally, samples were harvested 1 h after the addition of an inducer and total protein extracts were prepared. Of the latter, 7 µL (cell number ~ 0.035 OD₆₀₀) were separated by SDS-PAGE and analyzed by Western blotting using polyclonal antisera directed against RapZ and ribosomal protein S2 (loading control).

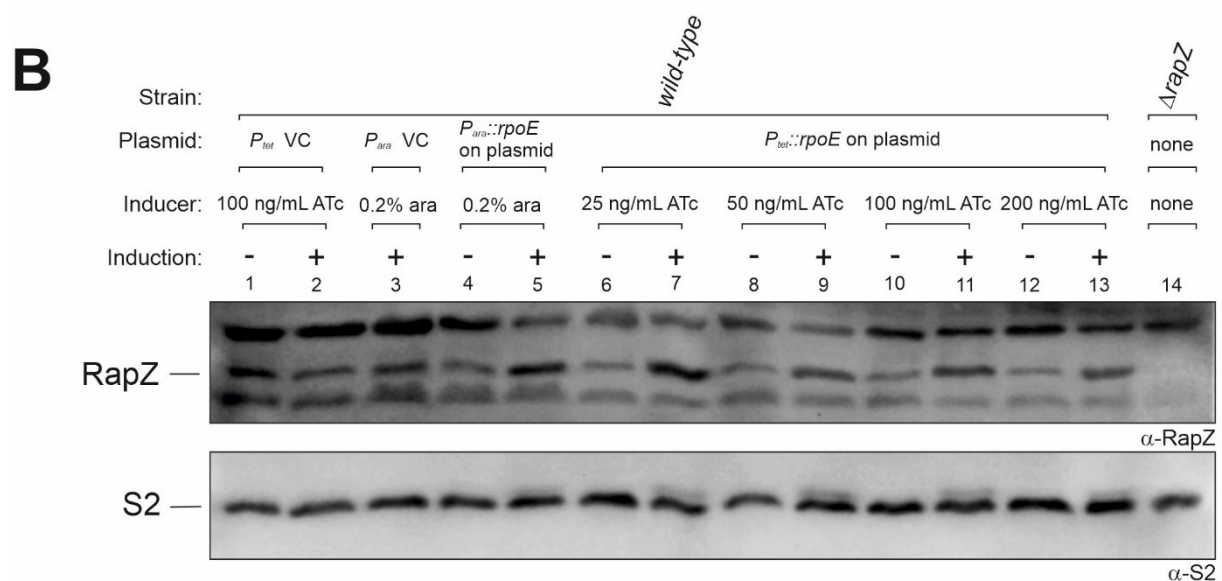
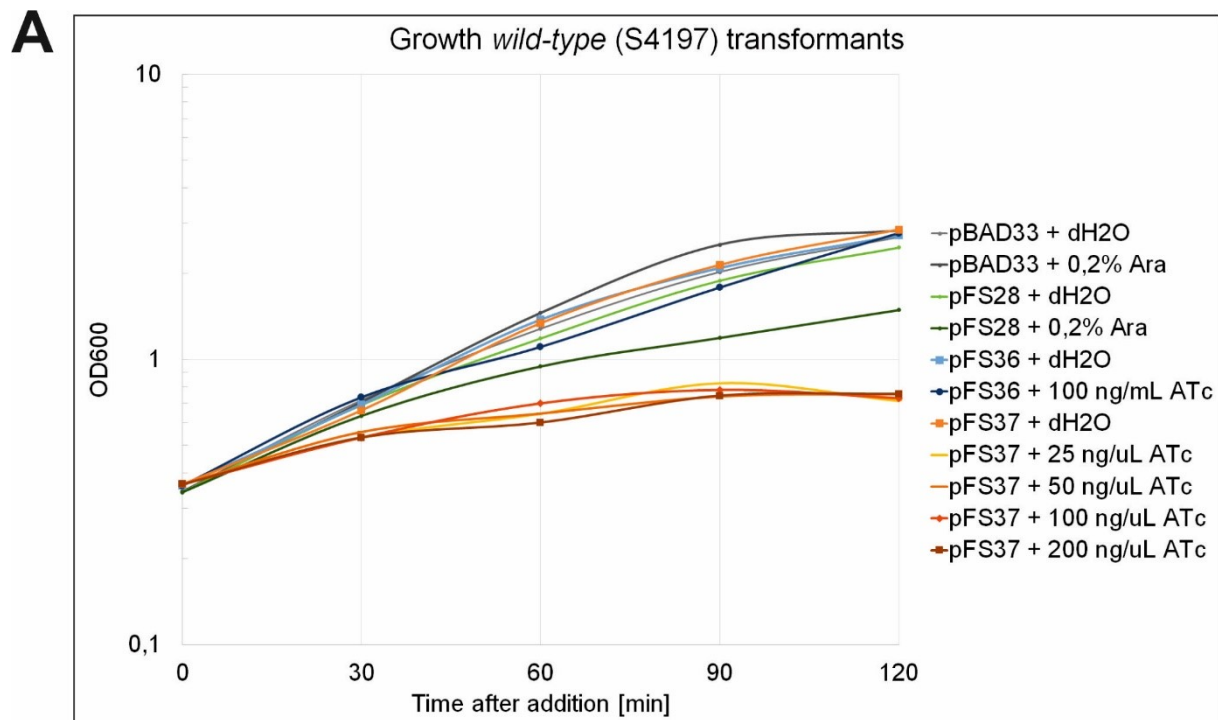


Figure 28. Western blot analysis of RapZ levels upon ectopic *rpoE* expression from different plasmid systems. Strain S4197 (*wild-type*) was transformed either with the *rpoE* expression plasmid under the control of the P_{tet} promoter (pFS37) or the corresponding VC (pFS36) or with the *rpoE* expression plasmid under the control of the P_{ara} promoter (pFS28) or the corresponding VC (pBAD33). Transformants

were grown in LB supplemented with chloramphenicol with or without either 0,2% arabinose or the various concentrations (25 ng/mL, 50 ng/mL, 100 ng/mL and 200 ng/mL) of anhydrotetracycline. Empty plasmids pFS36 and pBAD33 provided the VC and strain Z857 ($\Delta rapZ$) was included as additional control. **A.** The growth of transformants was monitored over 2 h by measuring OD₆₀₀ in 30 min intervals following the addition of an inducer (n = 1). **B.** Samples were harvested 1 h after the addition of an inducer and total protein extracts were prepared. Of the latter, 7 μ L (cell number approximately 0,035 OD₆₀₀) were separated on a 12,5% SDS-PAA gel and analyzed by Western blotting using polyclonal antisera directed against RapZ (top panel) and ribosomal protein S2 (middle panel). Polyclonal antiserum directed against RapZ results in non-specific signals. Loading controls are provided by S2 signals.

Upon ectopic expression of *rpoE* from an arabinose-inducible promoter on a plasmid, mimicking σ^E activation, a strong accumulation of RapZ was observed 1 h after the addition of 0,2% arabinose (Figure 28B, lane 5). Similarly, the upregulation of RapZ, achieved by ectopic expression of *rpoE* from an anhydrotetracycline-inducible promoter on a plasmid 1 h after the addition of various concentrations (25 ng/mL, 50 ng/mL, 100 ng/mL and 200 ng/mL) of anhydrotetracycline appears comparable (Figure 28B, lanes 7, 9, 11 and 13). Hence, various concentrations (25 ng/mL, 50 ng/mL, 100 ng/mL and 200 ng/mL) of anhydrotetracycline that were previously reported in the literature seemingly all equally activate the expression of *rpoE* from the tetracycline-inducible promoter on a plasmid, resulting in comparable accumulation of the protein RapZ. In the control cultures only low levels of RapZ protein were detectable (Figure 28B, lanes 1-4, 6, 8, 10 and 12). Thus, ectopic expression of *rpoE* from either P_{ara} promoter on plasmid pFS28 or P_{tet} promoter on plasmid pFS37, resulting in over-activation of the σ^E ESR, triggers accumulation of protein RapZ to a comparable extent.

However, an obvious difference in respect to growth-defects of the cultures was observed depending on the presence of either arabinose-controlled or anhydrotetracycline-controlled *rpoE* expression plasmid during the whole experiment (Figure 28A). After 2 h of σ^E overexpression, the $P_{ara}::rpoE$ transformant reached an OD₆₀₀ of ~ 1.5 , while the $P_{tet}::rpoE$ transformants induced with various concentrations (25 ng/mL, 50 ng/mL, 100 ng/mL and 200 ng/mL) of anhydrotetracycline were in an OD₆₀₀ range of ~ 0.75 independent of the anhydrotetracycline concentration (Figure 28A). Generally, the addition of arabinose increases the growth rate of *E. coli* strains to a minor extent and could partially be responsible for the growth advantage that the $P_{ara}::rpoE$ transformant has over $P_{tet}::rpoE$ transformants. The growth rate of either the P_{ara} or P_{tet} VC controls grown in the presence or absence of the inducer appears comparable; after 2 h, all these strains reached an OD₆₀₀ of ~ 2.7 to 3.0 (Figure 28A). Hence, the growth disadvantage of the $P_{tet}::rpoE$ transformants is not due to the presence of the inducer anhydrotetracycline, but due to ectopic expression of *rpoE* from the tetracycline-inducible promoter on plasmid pFS37, resulting in over-activation of the σ^E ESR. For the future experiments, an even lower concentration of

anhydrotetracycline that results in a somehow similar growth rate of the $P_{tet}::rpoE$ transformant compared to the $P_{ara}::rpoE$ transformant had to be determined.

Another pilot experiment was carried out to determine the optimal anhydrotetracycline concentration for induction of *rpoE* expression from the plasmid through the use of *E. coli* strains carrying suitable *lacZ* reporter gene fusions. The general transduction by phage T4-GT7 (Wilson *et al.*, 1979) was carried out to introduce the spectinomycin resistance tagged *lptA*(-152 to +78)'-*lacZ* fusion from the strain Z1297, carrying a FLAG-tagged *ptsN* allele, into the *wild-type* strain S4197 (as described in 5.2.2.1.), generating strain Z1303. The same procedure was carried out to introduce the spectinomycin resistance tagged *lptA*(-152)→*rapZ*(+78)'-*lacZ* fusion from the strain Z1377, carrying a FLAG-tagged *ptsN* allele, into the *wild-type* strain S4197 (as described in 5.2.2.1.), generating strain Z1378. The reporter gene fusions are depicted in detail in Figure 25 (in 6.2.2.2.).

At first, strain Z1303 carrying the chromosomal *lptA*(-152 to +78)'-*lacZ* fusion was used to determine to which extent the *lptAp1* promoter is activated upon ectopic *rpoE* expression from P_{tet} on the newly constructed plasmid pFS37. For the purpose of this experiment, strain Z1303 carrying the *lptA*'-*lacZ* reporter fusion was transformed with the *rpoE* expression plasmid (pFS37) or the corresponding VC (pFS36), respectively. Cells were grown as previously described (in 6.2.2.) and instead of 0,2% arabinose, 25 ng/mL anhydrotetracycline was added to induce expression of *rpoE*. Subsequently, samples for β -galactosidase activity measurements were taken from the cultures over 2 h in 30 min intervals following the addition of anhydrotetracycline.

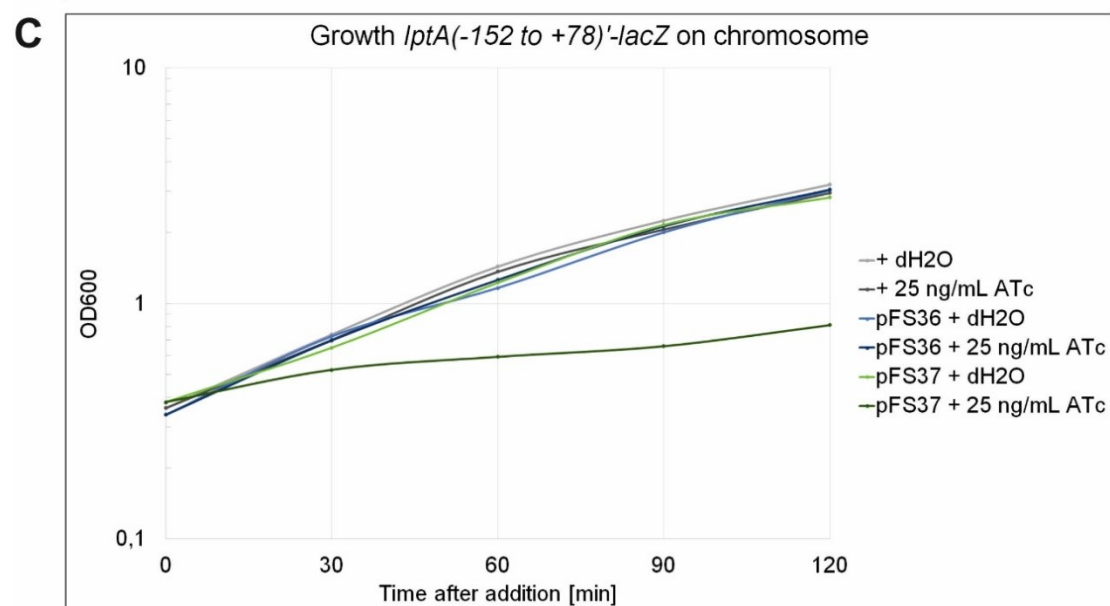
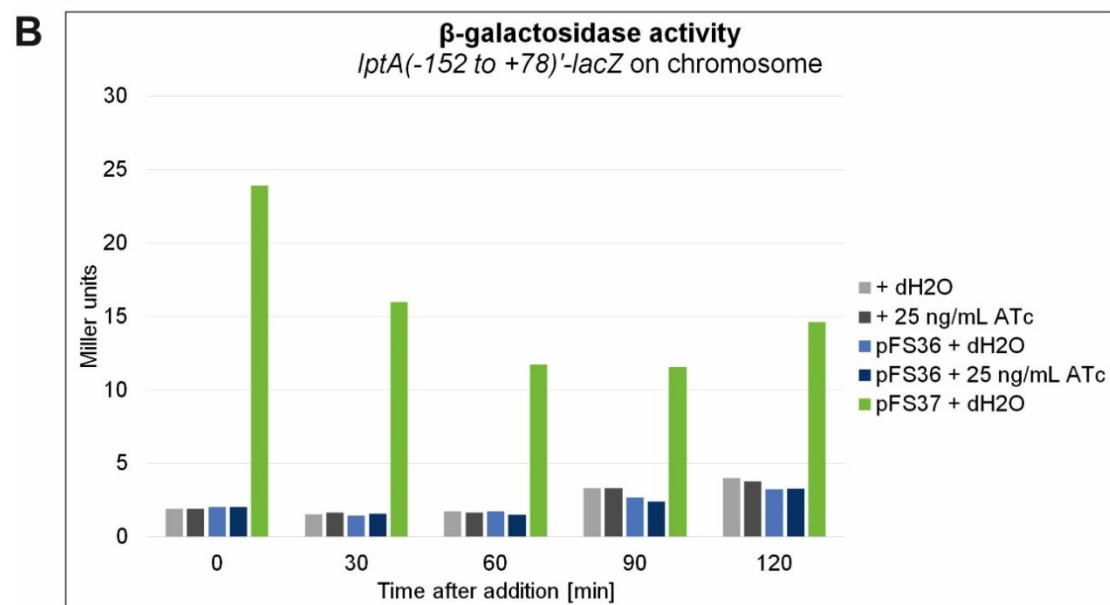
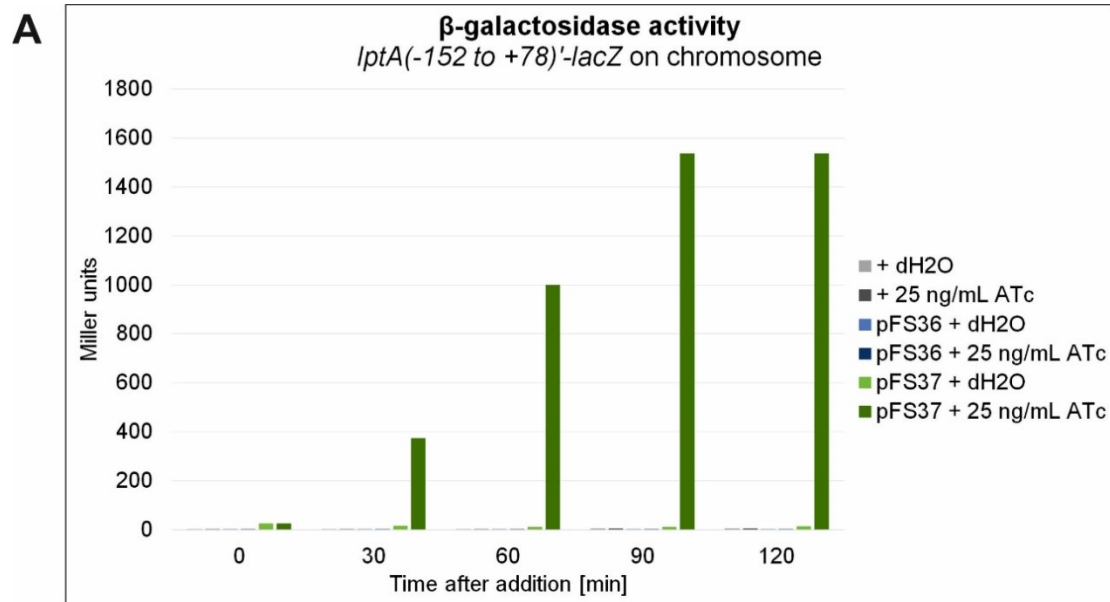


Figure 29. Ectopic *rpoE* expression from P_{tet} on the plasmid activates *lptAp1* promoter. A and B. β -galactosidase activity assay addressing the expression of the chromosomal *lptA'-lacZ* reporter fusion in *E. coli*. Strain Z1303 (*S4197 [attB::lptA(-152 to +78)-lacZ]*) was transformed with the *rpoE* expression plasmid (pFS37) or the corresponding VC (pFS36). Transformants were grown in LB supplemented with chloramphenicol +/- 25 ng/mL anhydrotetracycline. Empty plasmid pFS36 provided the VC and strain Z857 ($\Delta rapZ$) was included as additional control. Miller unit values derived from one measurement (n = 1). In (B) the activities of the induced *rpoE* transformant are not included. C. The growth of the cultures addressing the expression of the chromosomal *lptA'-lacZ* reporter fusion in *E. coli* that derived from one independent measurement.

Induction of plasmid-encoded *rpoE* under the control of the anhydrotetracycline-inducible promoter in the strain carrying the *lptA'-lacZ* reporter fusion led to a dramatic increase in activity of the reporter fusion (Figure 29A, dark green columns), confirming the activation of *lptAp1* promoter upon σ^E expression. A maximum induction of the fusion was reached 90 min after the addition of anhydrotetracycline, resulting in 465-fold induction of the β -galactosidase activity. Hence, a similar fold-induction of the fusion (more than 400-fold) was achieved previously (in 6.2.2.2.) using the plasmid-encoded *rpoE* under the control of an arabinose-inducible promoter 120 min after the addition of 0,2% arabinose (Supplementary Figure S1A, dark green columns). The activities of the controls used in the experiment, i.e. the untransformed *wild-type* strain or the VC control grown in the presence or absence of anhydrotetracycline, are comparable (Figure 29B). However, the uninduced *rpoE* transformant indicated 3- to 8-fold induction of β -galactosidase activities (Figure 29B, green columns). The observed minor activation of the *lptA'-lacZ* reporter fusion likely comes from the leaky expression of *rpoE* from the plasmid in the absence of an inducer. However, the activation of the *lptA* expression due to leaky expression of *rpoE* is negligibly small compared to the activation achieved upon induction of the plasmid-encoded *rpoE*.

Since the anhydrotetracycline concentration in this experiment (25ng/mL) resulted in a stronger growth defect of the $P_{tet}::rpoE$ transformant (as compared to the $P_{ara}::rpoE$ transformant), lower concentrations were subsequently tested to exclude growth-stage dependent differences. Various concentrations of anhydrotetracycline, ranging from 12,5 to 0,25 ng/mL, were used to induce the expression of *rpoE* from the tetracycline-inducible promoter on plasmid pFS37 in the strain carrying the chromosomal *lptA(-152)→rapZ(+78)'-lacZ* fusion. For the purpose of this experiment, the strain carrying the *lptA→rapZ'-lacZ* reporter fusion was employed to determine to which extent the activation of the *lptAp1* promoter, by ectopic *rpoE* expression, results in read-through transcription into the *rapZ* gene. The latter strain was initially transformed with the *rpoE* expression plasmid (pFS37) or the corresponding VC (pFS36), respectively. Cells were grown as previously described (in 6.2.2.3.) with the exception that the pre-culture was grown in 120 mL LB and different concentrations of anhydrotetracycline were tested following splitting of the pre-culture. The expression of *rpoE* was induced with various concentrations (12,5 ng/mL, 10 ng/mL, 7,5

ng/mL, 5 ng/mL, 2,5 ng/mL, 2, ng/mL 1,5 ng/mL, 1 ng/mL in the first round and 1 ng/mL, 0,9 ng/mL, 0,8 ng/mL, 0,75 ng/mL, 0,7 ng/mL, 0,6 ng/mL, 0,5 ng/mL, 0,4 ng/mL, 0,3 ng/mL, 0,25 ng/mL in the second round) of anhydrotetracycline in two subsequent experiments. The growth of the cultures was monitored over 2 h by measuring OD₆₀₀ in 30 min intervals following the addition of arabinose. Additionally, samples for β -galactosidase activity measurements were taken at various times following the addition of anhydrotetracycline.

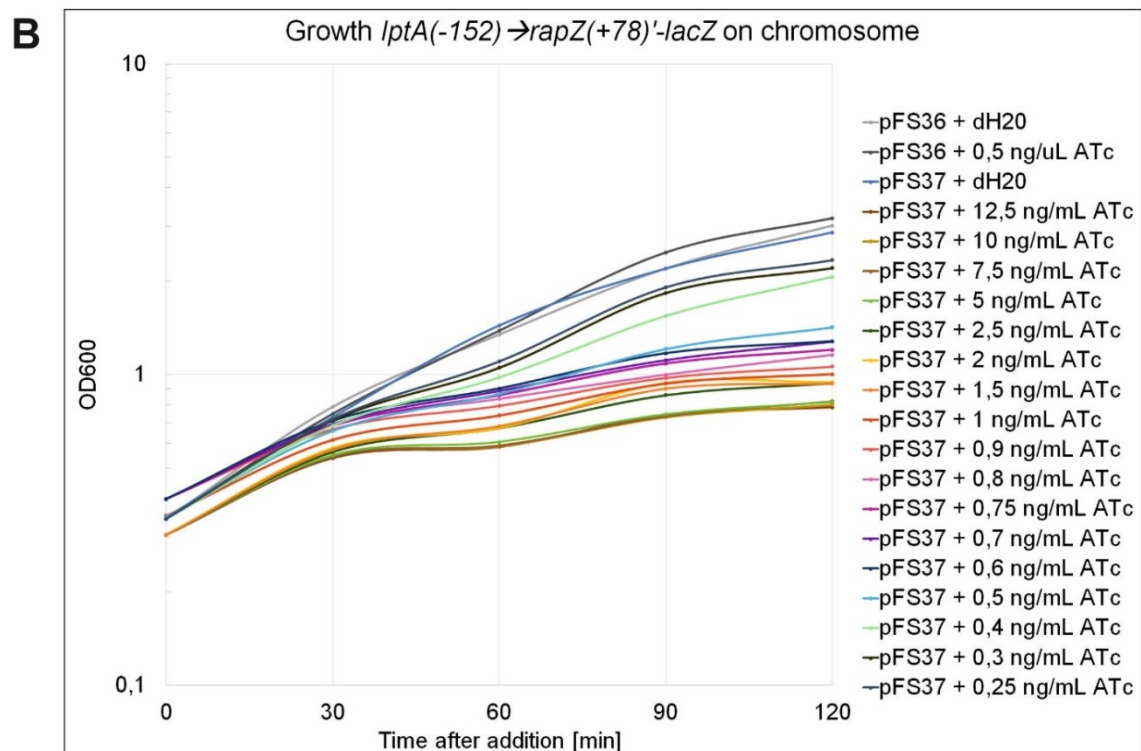
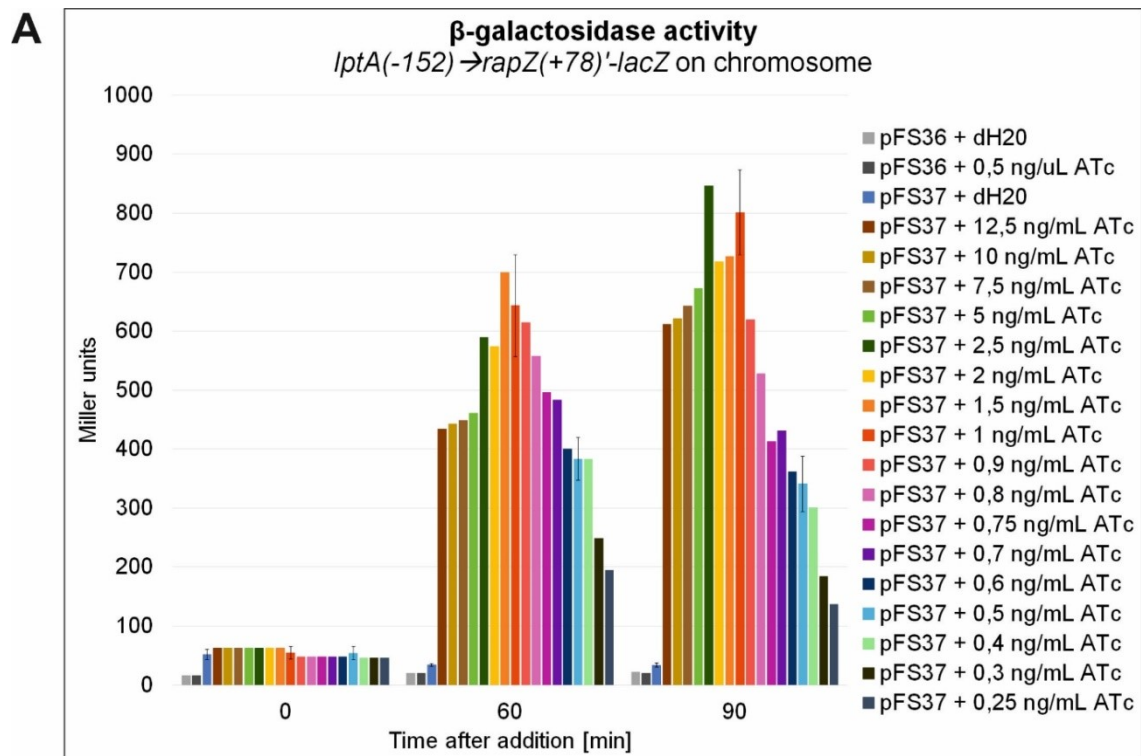


Figure 30. Pilot experiment to determine the optimal anhydrotetracycline concentration for ectopic *rpoE* expression. **A.** β -galactosidase activity assay addressing the expression of the chromosomal *lptA* $\rightarrow$ *rapZ'*-*lacZ* reporter fusion in *E. coli*. Strain Z1378 (*S4197* [*attB*:: *lptA*(-152) $\rightarrow$ *rapZ*(+78)'-*lacZ*]) was transformed with the *rpoE* expression plasmid (pFS37) or the corresponding VC (pFS36). Transformants were grown in LB supplemented with chloramphenicol +/- various concentrations of anhydrotetracycline (from 12,5 to 0,25 ng/mL). Miller unit values are presented as mean $\pm$ SD (when available) and derived from one or three independent measurements (n = 1-3). **B.** The growth of the cultures addressing the expression of the chromosomal *lptA*'-*lacZ* reporter fusion in *E. coli*.

Induction of the plasmid-encoded *rpoE* under the control of an anhydrotetracycline-inducible promoter in the strain carrying the *lptA* $\rightarrow$ *rapZ'*-*lacZ* reporter fusion led to a dramatic increase of the reporter gene fusion activity, correlating with the varying inducer concentrations (Figure 30A). A maximum induction of the fusion 60 and 90 min after the addition of anhydrotetracycline was achieved with 1 ng/mL anhydrotetracycline, resulting in a 20- and 25-fold induction of the β -galactosidase activity, respectively (Figure 30A, red columns). A similar fold-induction of the fusion was achieved previously (in 6.2.2.2.) using the arabinose-controlled *rpoE* expression plasmid 60 and 90 min after the addition of 0,2% arabinose, resulting in a 20- and 30-fold induction of the β -galactosidase activity, respectively (Figure 26A, dark green columns). The activities of the *P_{tet}::rpoE* transformants increased with decreasing anhydrotetracycline concentrations until the highest activities were achieved with 1 ng/mL anhydrotetracycline, resulting in $\sim$ 800 Miller units 90 min after *rpoE* induction. However, when the concentration of anhydrotetracycline was further decreased, the β -galactosidase activities of the *P_{tet}::rpoE* transformants decreased as well (Figure 30A). Furthermore, the growth rate of *P_{tet}::rpoE* transformants was improving with decreasing anhydrotetracycline concentrations (Figure 30B). After 2 h, the *P_{tet}::rpoE* transformant induced with 1 ng/mL anhydrotetracycline reached an OD₆₀₀ of $\sim$ 1.1. When using the concentrations of 0,4 ng/mL or lower, the growth-defect due to σ^E expression from a plasmid was abolished to a large extent, and the growth rate of those cultures is almost more comparable to the growth rate of the uninduced *P_{tet}::rpoE* transformant or the *P_{tet}* vector control (Figure 30B). Based on these results, the most suitable concentration of anhydrotetracycline used for the future experiments to induce expression of *rpoE* from the anhydrotetracycline-inducible promoter is 1 ng/mL. It is important to mention that *P_{tet}::rpoE* transformants still did not reach the same OD₆₀₀ as compared to the *P_{ara}::rpoE* transformants, yet the maximum induction of the *lptA* $\rightarrow$ *rapZ'*-*lacZ* reporter fusion is comparable

6.2.2.4. Impact of the σ^E -dependent accumulation of RapZ on the GlmY/GlmZ/*glmS* circuit using the arabinose-controlled *rpoE* expression plasmid

The post-transcriptional circuit controlling GlcN6P synthesis through the joint action of RapZ, GlmY and GlmZ has so far been studied under conditions not affecting RapZ levels.

Although the activity of RapZ is primarily regulated through interaction with the metabolite GlcN6P (Khan *et al.*, 2020), GlcN6P has no impact on RapZ amounts (Göpel *et al.*, 2013). Under normal growth conditions when GlcN6P levels in the cell are sufficient, high levels of GlmS are not required. Consequently, RapZ is mainly dedicated to recruit GlmZ to processing by RNase E, adjusting a basal GlmS level. A fraction of RapZ remains GlcN6P-free and activates QseE/QseF and thereby *glmY* expression to an intermediate level. Under GlcN6P starvation, RapZ hyper-activates QseE/QseF resulting in a burst of *glmY* expression, which leads to sequestration of RapZ and activation of *glmS* expression by full-length GlmZ (Khan *et al.*, 2020).

So far, the data confirm that ectopic expression of *rpoE* from a plasmid, resulting in over-activation of the σ^E ESR, triggers accumulation of RapZ as observed by the Western blot analysis (Figure 24) and reporter gene assays (Figure 30). The upregulation of *rapZ* expression by σ^E should increase both RapZ fractions, the GlcN6P-bound as well as the GlcN6P-free RapZ. In case of RapZ amounts exceeding GlmY amounts, part of the RapZ population is left un-sequestered and thereby free to recruit RNase E to process GlmZ and impede *glmS* translation. Additionally, higher amounts of GlcN6P-free RapZ should activate QseE/QseF TCS to upregulate *glmY* expression from the σ^{54} -dependent promoter which leads to sequestration of RapZ by GlmY into long-lasting complexes and increased *glmS* translation by full-length GlmZ. For the purpose of the following experiments, two strains carrying the desired reporter gene fusions, with the 5' ends of the respective genes fused to *lacZ*, on the chromosome, *glmY-5'-lacZ* (Z741) and *glmS-5'-lacZ* (Z854), were transformed with the *rpoE* expression plasmid (pFS28) or the corresponding VC (pBAD33).

The resulting transformants were used to determine *glmY* and *glmS* expression levels and consequently check whether the σ^E -dependent accumulation of RapZ prevents or activates *glmS* expression. Cells were grown as previously described (in 6.2.2.) and samples for β -galactosidase activity measurements were taken from the cultures over 2 h in 30 min intervals following the addition of arabinose.

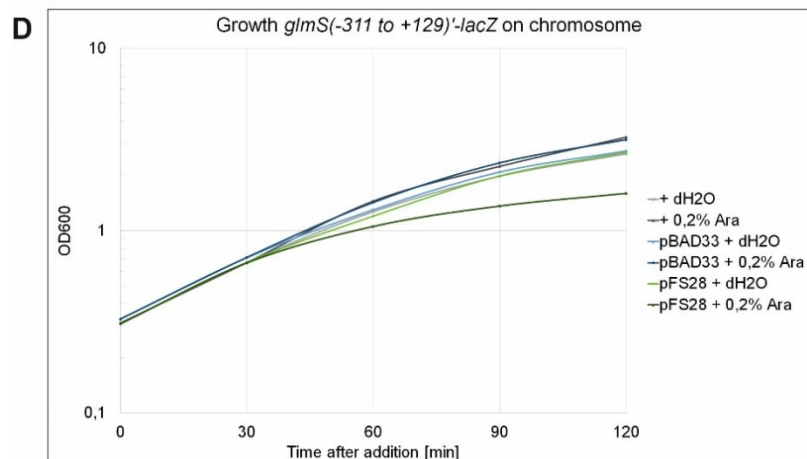
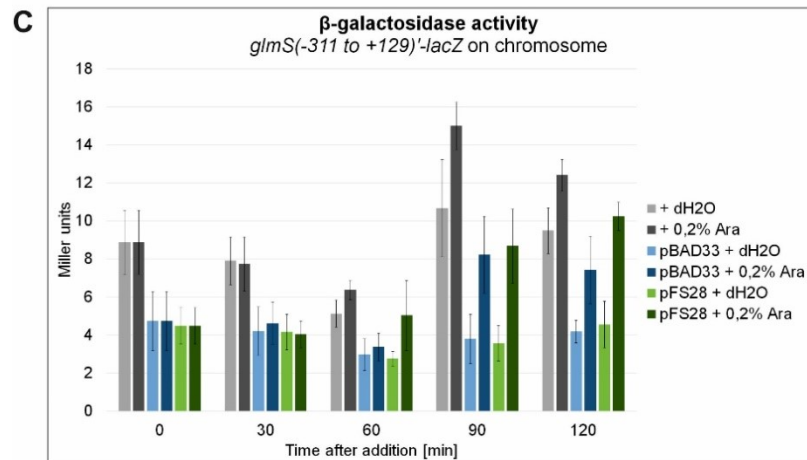
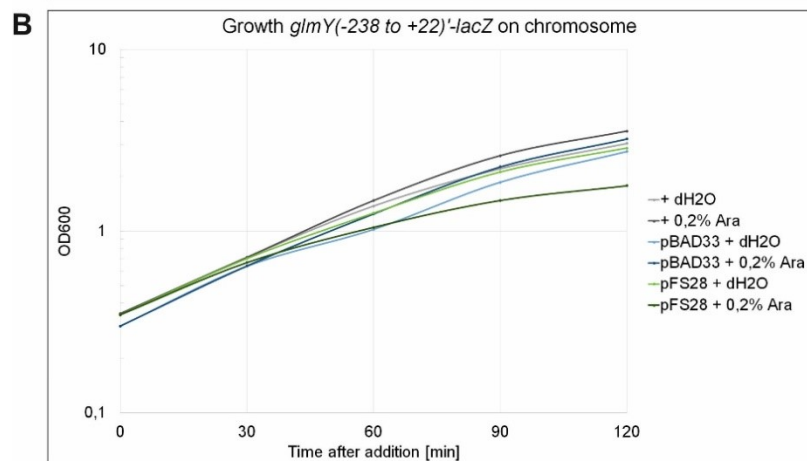
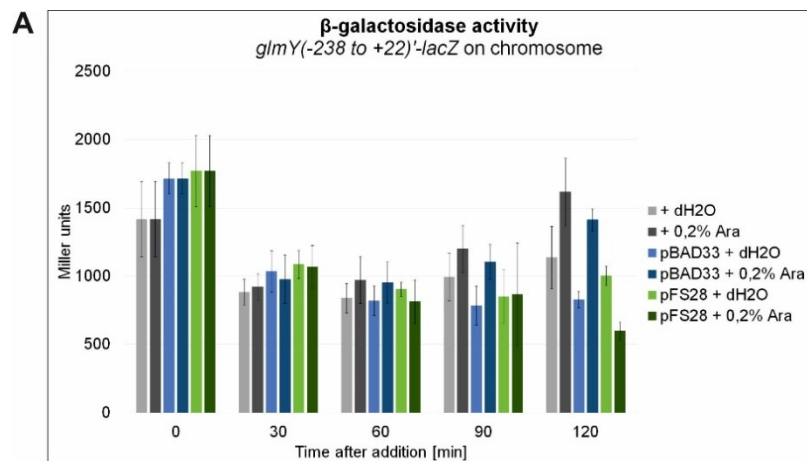


Figure 31. Impact of ectopic *rpoE* expression on the chromosomal *glmY'-lacZ* and *glmS'-lacZ* reporter fusion in *E. coli*. **A and C.** β -galactosidase activity assay addressing the expression of the chromosomal *glmY'-lacZ* (**A**) and *glmS'-lacZ* (**C**) reporter fusion in *E. coli*. Strains Z741 (*S4197 attB::[glmY'-lacZ]*) and Z854 (*S4197 attB::[glmS'-lacZ]*) were transformed with the *rpoE* expression plasmid (pFS28) or the corresponding VC (pBAD33). Transformants were grown in LB supplemented with chloramphenicol +/- 0,2% arabinose. Empty plasmid pBAD33 provided the VC and the untransformed *wild-type* strain was analyzed in parallel for comparison. Miller unit values are presented as mean $\pm$ SD that derived from three independent measurements (n = 3). **B and D.** The growth of the cultures addressing the expression of the chromosomal *glmY'-lacZ* (**B**) and *glmS'-lacZ* (**D**) reporter fusion in *E. coli*. OD₆₀₀ values are presented as mean that derived from three independent measurements (n = 3).

β -Galactosidase activities indicate that neither *glmY* nor *glmS* expression is upregulated when *rpoE* is ectopically expressed from the arabinose-inducible promoter on the plasmid (Figure 31A, B). Induction of the *rpoE* expression in the strain carrying the chromosomal *glmY'-lacZ* reporter fusion did not result in a significant difference in activities of the reporter gene fusion compared to the activities measured in the other strains (Figure 31A). The same is true for the strain carrying the chromosomal *glmS'-lacZ* reporter fusion (Figure 31B). Interestingly, the presence of arabinose resulted in 1,5 to 2-fold induction of the *glmS'-lacZ* reporter fusion that is most apparent 90 and 120 min after the addition of arabinose (Figure 31B, dark grey, blue and green columns). It is important to mention that RapZ stabilizes GlmY by binding (Khan *et al.*, 2020). Under GlcN6P starvation conditions, RapZ forms stable complexes with GlmY and subsequently protects GlmY from degradation. Therefore, the activities of the chromosomal *glmY'-lacZ* reporter fusion might not correctly represent the GlmY levels upon ectopic *rpoE* expression since the influence that RapZ has on sRNA stabilization is not reflected by the reporter gene assay. In order to consider this possibility, GlmY levels were subsequently analyzed by Northern blotting, allowing for direct comparison of the GlmY levels under the tested conditions.

To further investigate the downstream effects of ectopic *rpoE* expression, RapZ and GlmS levels were addressed by Western blot analysis and GlmZ and GlmY amounts by Northern blot analysis. For the purpose of this experiment, the *wild-type* (S4197) strain was initially transformed with the *rpoE* expression plasmid (pFS28) or the corresponding VC (pBAD33), respectively. Cells were grown as previously described (in 6.2.2.) and samples for preparation of total protein extracts and for total RNA extraction were harvested from the cultures at various times following the addition of arabinose. The samples were further analyzed by Western and Northern blotting.

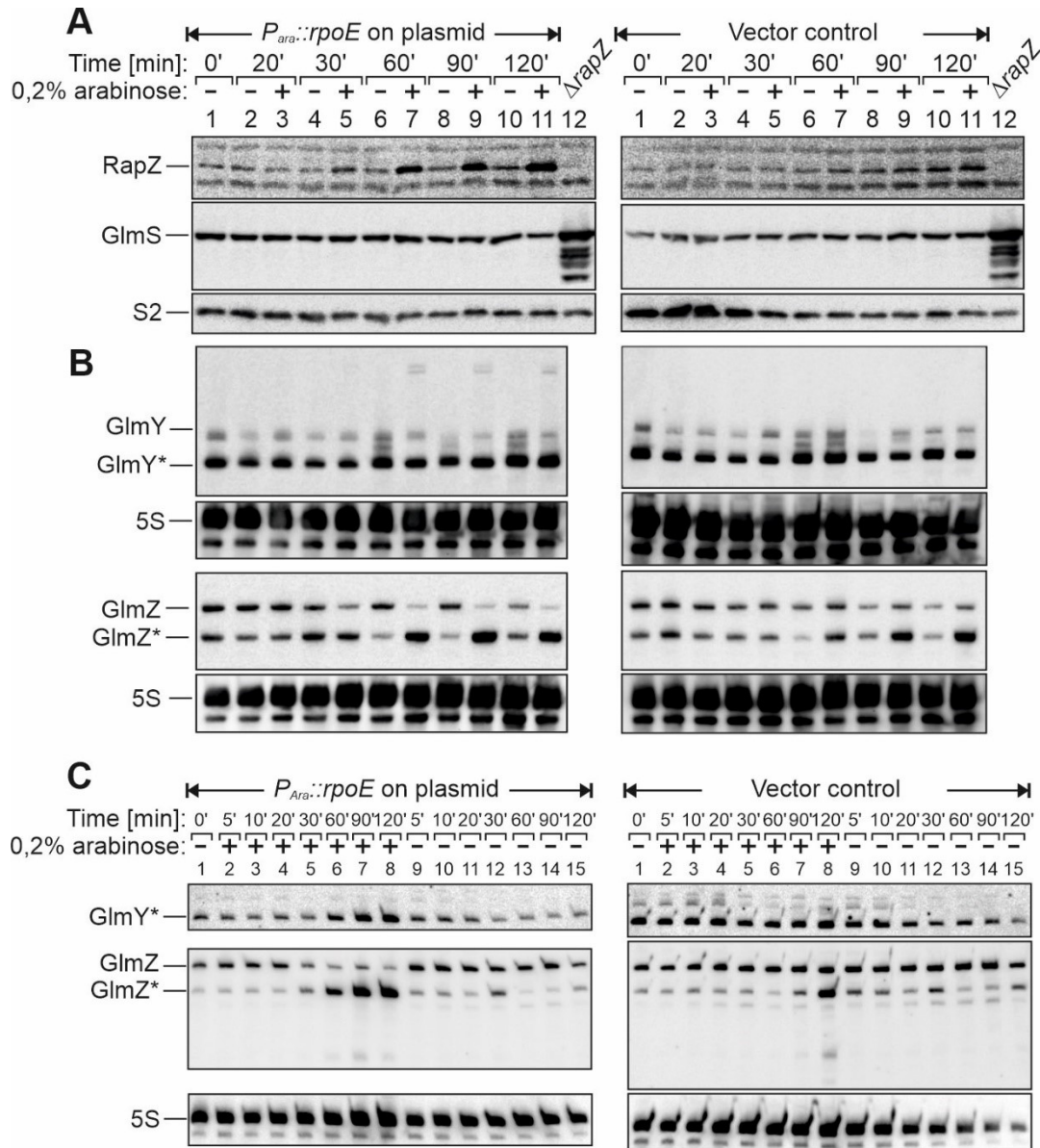


Figure 32. Western and Northern blots analyzing the impact of σ^E -mediated upregulation of RapZ for the GlmY/GlmZ/glmS circuit upon ectopic *rpoE* expression from the P_{ara} promoter. Strain S4197 (wild-type) was transformed with the *rpoE* expression plasmid (pFS28) or the corresponding VC (pBAD33). Transformants were grown in LB supplemented with chloramphenicol +/- 0,2% arabinose. Empty plasmid pBAD33 provided the VC and strain Z857 ($\Delta rapZ$) was included as additional control. For induction of P_{ara} promoter, 0,2% arabinose was added. Samples for preparation of total protein extracts and for total RNA extraction were harvested at the indicated times following the addition of arabinose. **A.** 7 μ L of total protein extracts (cell number ~ 0.035 OD₆₀₀) were separated on a 12,5% SDS-PAA gel and analyzed by Western blotting using polyclonal antisera directed against RapZ (top panel), GlmS (middle panel) and ribosomal protein S2 (bottom panel). Polyclonal antiserum directed against RapZ results in non-specific signals, therefore strain Z857 ($\Delta rapZ$) was included as additional control. Loading controls are provided by S2 signals. **B and C.** 3 μ g of total RNA was separated on an 8% urea-PAA gel and analyzed by Northern blotting using probes specific for GlmY (top panel), GlmZ (middle panel) and 5S rRNA (bottom panel; loading control).

Upon ectopic expression of *rpoE* from the arabinose-inducible promoter on plasmid pFS28, mimicking σ^E activation, a strong accumulation of protein RapZ was observed as previously (Figure 32A, left top panel). RapZ starts accumulating 30 min after induction of *rpoE* expression and its accumulation is even more pronounced at later points in time. In all other

samples, only basal levels of RapZ protein were detectable that appear comparable at all points in time (Figure 32A, left and right top panels). GlmY primary sRNA undergoes processing in its 3' end and the 5' cleavage product is the active form (labelled as GlmY*) that sequesters RapZ (Reichenbach *et al.*, 2009; Göpel *et al.*, 2013). The levels of GlmY did not significantly vary regardless of the *rpoE* ectopic expression and this result is in correspondence with the β -galactosidase activities measured by the reporter gene assay. However, it appears that at later points in time, 90 and 120 min following the σ^E overexpression, GlmY is present in slightly higher amounts (Figure 32B, left and right top panels). There is also an additional GlmY signal present on top of the membrane that was observed only upon ectopic *rpoE* expression (Figure 32B, left top panel). The latter RNA starts accumulating 30 min after induction of *rpoE* expression and is estimated to be ~ 300 bp longer than the processed form of GlmY RNA. Two perfectly overlapping promoters, a constitutively active σ^{70} promoter and the QseF-dependent σ^{54} promoter, control expression of the *glmY* gene (Reichenbach *et al.*, 2009). The additional GlmY RNA product could potentially result from a read-through transcription of the *glmY* gene into the *qseE* gene. In that case, the RNA product should be as well detectable using the RNA probe specific for *qseE*. The processed form of GlmZ is present in much higher amounts when *rpoE* is ectopically expressed from the plasmid as a consequence of the σ^E -mediated upregulation of RapZ. In contrast, the level of full-length GlmZ is decreased (Figure 32B, left bottom panel and Figure 32C, left bottom panel) compared to the non-induced conditions. RapZ binds GlmZ and recruits endoribonuclease RNase E to process and inactivate GlmZ. The increase of processed form of GlmZ and the corresponding decrease of full-length form of GlmZ is apparent 30 min after induction of *rpoE* expression when both forms are present in a ratio of 1:1 and is even more pronounced at later points in time. The addition of arabinose also resulted in enhanced GlmZ processing in the VC control (Figure 32B, right bottom panel and Figure 32C, right bottom panel). The increase of processed form of GlmZ is apparent 60 min after the addition of arabinose and even more evident at later points in time. Due to a decrease of the full-length active form of GlmZ upon ectopic *rpoE* expression, GlmS cannot be upregulated. Consequently, the levels of GlmS protein do not differ regardless of the *rpoE* ectopic expression (Figure 32A, left and right middle panel) and this result is in correspondence with the β -galactosidase activities measured by the reporter gene assay. The effect of this regulation on the target GlmS is not observed because in a rich growth medium GlmS is already only present at the basal level. The processed form of GlmZ still retains the ability to bind RapZ and therefore adjusts the basal level of GlmS through an inhibitory feed-back loop by partially sequestering RapZ and reducing further GlmZ processing (Durica-Mitic *et al.*, 2020). Hence, downregulation of GlmS is not possible even though there is more of GlmZ processing due to higher RapZ amounts. On account of the

additional effect of the inducer arabinose on the system, resulting in an enhanced GlnZ processing, the change of inducer and expression plasmid was necessary. Hence, a plasmid system allowing the expression of genes of interest under control of an anhydrotetracycline-inducible promoter (P_{tet}) was constructed (described in 6.2.2.3.) and used for ectopic *rpoE* expression in further experiments. Based on these former results (discussed in 6.2.2.3.), the optimal concentration of anhydrotetracycline, i.e. a concentration that led to similar phenotypes and growth rates as observed in the previous experiments with P_{ara} , was 1 ng/mL.

6.2.2.5. Impact of the σ^E -dependent accumulation of protein RapZ on the GlnY/GlnZ/*glnS* circuit using the anhydrotetracycline-controlled *rpoE* expression plasmid

To further investigate the downstream effects of *rpoE* ectopic expression from the newly constructed plasmid, RapZ and GlnS levels were addressed by Western blot analysis and GlnZ and GlnY amounts by Northern blot analysis. The *wild-type* (S4197) strain was initially transformed with the newly constructed *rpoE* expression plasmid (pFS37) or the corresponding VC (pFS36). Cells were grown as described above (in 6.2.2.) with the exception that *rpoE* expression was induced with 1 ng/mL anhydrotetracycline. Subsequently, samples for preparation of total protein extracts and for total RNA extraction were harvested from the cultures at various times following the addition of anhydrotetracycline. The samples were further analyzed by Western and Northern blotting.

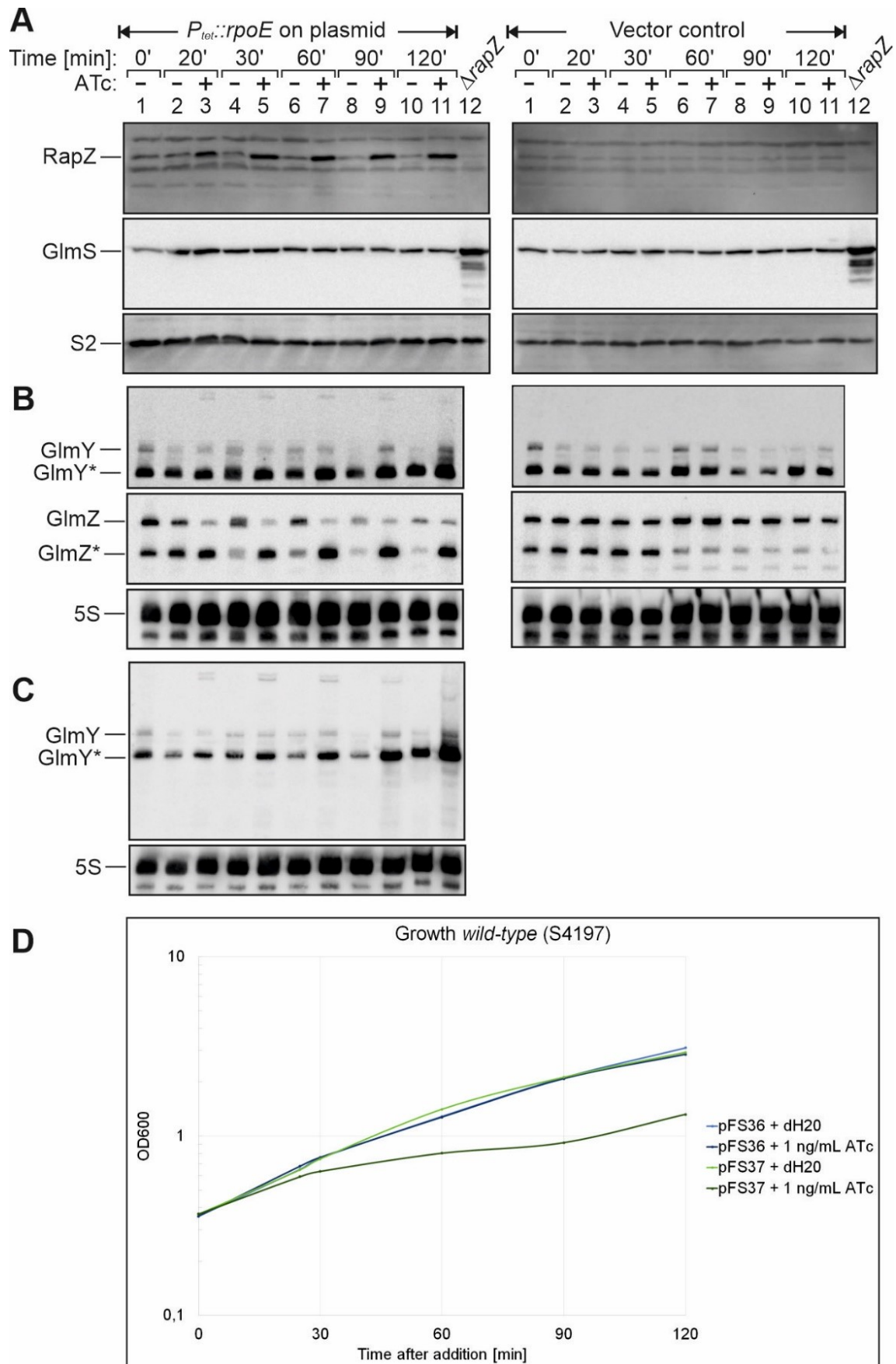


Figure 33. Western and Northern blots analyzing the impact of σ^E -mediated upregulation of RapZ for the GlmY/GlmZ/*glmS* circuit upon ectopic *rpoE* expression from the P_{tet} promoter. Strain S4197 (*wild-*

type) was transformed with plasmid pFS37 harboring *rpoE* under control of the inducible P_{tet} promoter or the corresponding VC (pFS36). Transformants were grown in LB supplemented with chloramphenicol +/- 1 ng/mL anhydrotetracycline. Empty plasmid pFS36 provided the VC and strain Z857 ($\Delta rapZ$) was included as additional control. For induction of the P_{tet} promoter, 1 ng/mL anhydrotetracycline was added. Samples for preparation of total protein extracts and for total RNA extraction were harvested at the indicated times following the addition of anhydrotetracycline. **A.** 7 μ L of total protein extracts (cell number ~ 0.035 OD₆₀₀) were separated on a 12.5% SDS-PAA gel and analyzed by Western blotting using polyclonal antisera directed against RapZ (top panel), GlmS (middle panel) and ribosomal protein S2 (middle panel). Polyclonal antiserum directed against RapZ results in non-specific signals, therefore strain Z857 ($\Delta rapZ$) was included as additional control. Loading controls are provided by S2 signals. **B and C.** 3.5 μ g of total RNA was separated on an 8% urea-PAA gel and analyzed by Northern blotting using probes specific for GlmY (top panel), GlmZ (middle panel) and 5S rRNA (bottom panel; loading control). **D.** The growth of transformants upon ectopic *rpoE* expression from the P_{tet} promoter.

Upon ectopic expression of *rpoE* from the anhydrotetracycline-inducible promoter on plasmid pFS37, mimicking σ^E activation, a strong accumulation of RapZ was observed as before (Figure 33A, top left panel). In contrast to the plasmid system with P_{ara} that was used before, RapZ starts accumulating already 20 min after induction of *rpoE* expression. In the other samples lacking the inducer or harboring the empty VC, only basal levels of RapZ protein were detectable that appear comparable at all points in time. The levels of GlmY are elevated when *rpoE* is overexpressed from the plasmid (Figure 33C, top left panel) as opposed to the plasmid system with P_{ara} where GlmY is present in slightly higher amounts only at the three latest points in time. There is as well an additional GlmY signal present at the top of the membrane that is observed only when *rpoE* is expressed ectopically (Figure 33C, left top panel) and starts accumulating already 20 min after induction of *rpoE* expression. Furthermore, the processed form of GlmZ is present in much higher amounts when *rpoE* is overexpressed from the plasmid and the level of full-length GlmZ is decreased as a consequence of the σ^E -mediated upregulation of RapZ (Figure 33B, left middle panel). RapZ binds GlmZ and recruits endoribonuclease RNase E to process GlmZ. Before or without induction of *rpoE*, the full-length and processed forms of GlmZ are present in approximately equal amounts with a slight increase of the full-length form towards the later timepoints. However, this ratio shifts already 20 min after the ectopic *rpoE* expression when the processed form of GlmZ is already dominant and full-length GlmZ is depleted. When using the plasmid system with the anhydrotetracycline-inducible P_{tet} promoter, the enhanced GlmZ processing in the VC control that was previously observed upon the addition of arabinose is abolished, confirming that arabinose alone can already trigger GlmZ processing to some extent (Figure 33B, right middle panel). The levels of GlmS protein do not differ regardless of the *rpoE* ectopic expression (Figure 33A, left and right middle panel) although the GlmZ processing is enhanced upon ectopic *rpoE* expression. The effect of this regulation on the target GlmS is not observed because in a rich growth medium, GlmS is already only present at the basal level. Hence, further downregulation of GlmS is not possible even though there is more of GlmZ processing due to higher RapZ amounts.

6.2.2.6. Impact of the σ^E -dependent accumulation of protein RapZ under GlcN6P depletion conditions

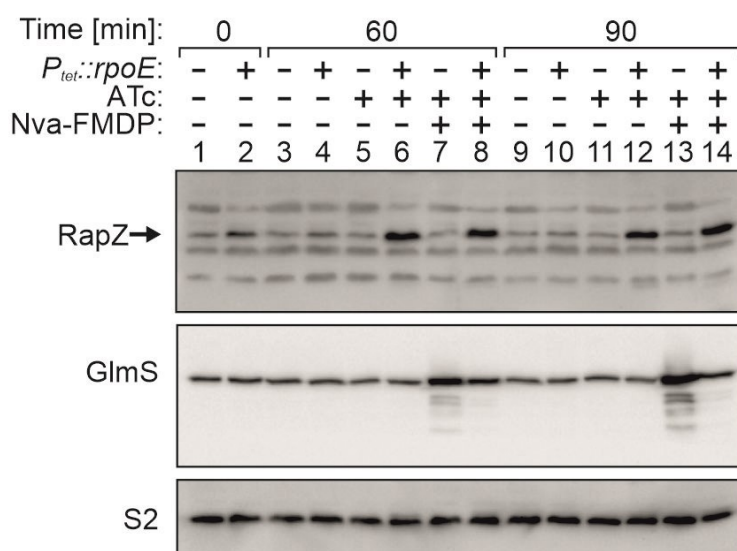
The RNase adaptor protein RapZ post-transcriptionally controls synthesis of GlmS (Göpel *et al.*, 2014) in response to the level of GlcN6P. The enzyme GlmS synthesizes GlcN6P from fructose-6-phosphate and glutamine, catalyzing the first committed step in the biosynthesis of cell envelope components and is consequently a good target for antibiotic treatment. Nva-FMDP is a synthetic derivative of modified dipeptide antibiotics that selectively inhibit GlmS enzymatic activity (Milewski *et al.*, 1986). It is hydrolyzed in the cytoplasm to release its active moiety FMDP that acts as glutamine analogue and inhibits GlmS by covalently binding to a residue in the glutamine binding pocket, resulting in an irreversible inhibition of GlmS that in turn leads to GlcN6P depletion (Milewski *et al.*, 1986; Kucharczyk *et al.*, 1990). GlcN6P depletion triggers accumulation of sRNA GlmY which in turn sequesters RapZ from processing GlmZ through molecular mimicry. As a result, full-length GlmZ activates translation of *glmS* mRNA in an Hfq-dependent manner. Thus, GlcN6P deprivation, induced e.g. by Nva-FMDP, leads to activation of *glmS* expression in a dose-dependent manner (Khan *et al.*, 2016), allowing the bacteria to counteract these antibiotics and to replenish their GlcN6P pools.

So far, the results obtained in this study confirm that the σ^E -mediated upregulation of RapZ, achieved by ectopic expression of *rpoE* from a plasmid, leads to enhanced GlmZ processing but consequently does not affect the already basal level of GlmS. To investigate whether the σ^E -mediated upregulation of RapZ impacts GlmS levels and in turn synthesis of LPS and peptidoglycan precursors under conditions of GlcN6P depletion, Nva-FMDP was used to inhibit GlmS enzymatic activity. Under these conditions, GlmY accumulates and sequesters RapZ from GlmZ processing, resulting in higher GlmS levels that overcome GlmS enzymatic inhibition caused by Nva-FMDP (Khan *et al.*, 2016). Nva-FMDP was used in combination with ectopic *rpoE* expression to determine which of these conditions is more dominant, the upregulation of GlmS due to GlcN6P depletion or the enhanced GlmZ processing due to σ^E -mediated upregulation of RapZ.

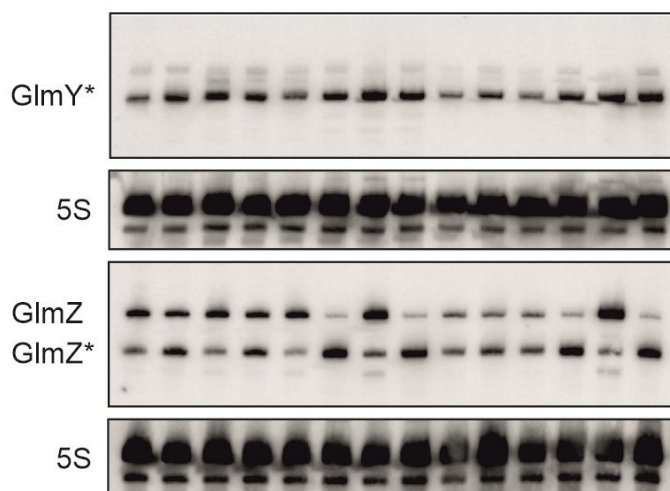
For the purpose of this experiment, *wild-type* (S4197) strain was initially transformed with the *rpoE* expression plasmid (pFS37) or the corresponding VC (pFS36), respectively. Pre-cultures of transformants were inoculated from the overnight cultures to an OD₆₀₀ of 0.1 and grown at 37°C in 25 mL LB supplemented with chloramphenicol until reaching an OD₆₀₀ of ~ 0.3. Next, each pre-culture was split into three 6 mL sub-cultures, one of which was supplemented with 1 ng/mL anhydrotetracycline with or without 60 µg/mL Nva-FMDP whereas the other sub-culture remained untreated (addition of dH₂O). 1 ng/mL anhydrotetracycline was added to induce expression of *rpoE* from the plasmid and 60 µg/mL

Nva-FMDP was added to inhibit GlmS enzymatic activity. 60 $\mu\text{g/mL}$ is the sub-inhibitory concentration of Nva-FMDP at which the growth of the *wild-type* strain is not impaired and the *glmS* expression is activated to overcome inhibition by the antibiotic (Khan *et al.*, 2016). Subsequently, samples for preparation of total protein extracts and for total RNA extraction were harvested from the cultures at various times following the addition of anhydrotetracycline with or without Nva-FMDP. The samples were further analyzed by Western and Northern blotting.

A



B



C

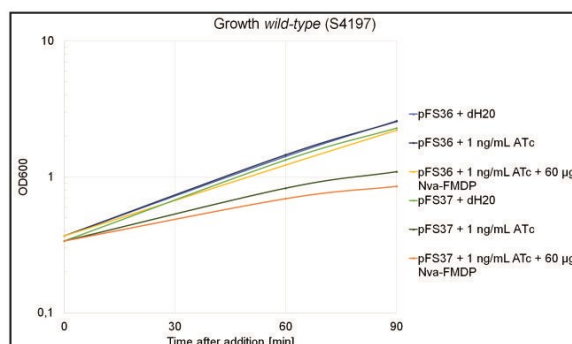


Figure 34. Western and Northern blots analyzing the impact of σ^E -mediated upregulation of RapZ for the GlmY/GlmZ/*glmS* circuit upon ectopic *rpoE* expression from the P_{tet} promoter and addition of GlmS inhibitor Nva-FMDP. Strain S4197 (*wild-type*) was transformed with the *rpoE* expression plasmid (pFS37) or the corresponding VC (pFS36). Transformants were grown in LB supplemented with chloramphenicol +/- 1 ng/mL anhydrotetracycline with or without 60 μ g/mL Nva-FMDP. Empty plasmid pFS36 provided the VC. For induction of the P_{tet} promoter, 1 ng/mL anhydrotetracycline was added. For inhibition of GlmS enzymatic activity, 60 μ g/mL Nva-FMDP was added. Samples for preparation of total protein extracts and for total RNA extraction were harvested at the indicated times following the addition of anhydrotetracycline and/or Nva-FMDP. **A.** 7 μ L of total protein extracts (cell number $\sim$ 0.035 OD₆₀₀) were separated on a 12,5% SDS-PAA gel and analyzed by Western blotting using polyclonal antisera directed against RapZ (top panel), GlmS (middle panel) and ribosomal protein S2 (middle panel). Polyclonal antiserum directed against RapZ results in non-specific signals, therefore strain Z857 ($\Delta rapZ$) was included as additional control. Loading controls are provided by S2 signals. **B and C.** 3,5 μ g of total RNA was separated on an 8% urea-PAA gel and analyzed by Northern blotting using probes specific for GlmY (top panel), GlmZ (middle panel) and 5S rRNA (bottom panel; loading control). **C.** The growth of transformants upon ectopic *rpoE* expression from the P_{tet} promoter and addition of GlmS inhibitor Nva-FMDP.

As already observed before, ectopic expression of *rpoE* from an anhydrotetracycline-inducible promoter on a plasmid, leads to strong accumulation of protein RapZ (Figure 34A, top panel, lane 6 and 8). Additionally, the levels of GlmY are slightly elevated when *rpoE* is overexpressed from the plasmid (Figure 34B, top panel, lane 6 and 12) as opposed to the non-induced conditions (Figure 34B, top panel, lanes 3 to 5 and 9 to 11) although the difference is more evident 90 min after induction of *rpoE* expression. As a consequence of the σ^E -mediated upregulation of RapZ, the processed form of GlmZ is present in much higher amounts when *rpoE* is overexpressed and the level of full-length GlmZ is decreased (Figure 34B, middle panel, lane 6 and 12) in comparison to the non-induced conditions (Figure 34B, middle panel, lane 3 to 5 and 9 to 11). The levels of GlmS protein do not differ regardless of the *rpoE* ectopic expression (Figure 34A, middle panel, lane 3 to 6 and 9 to 12) since GlmS is already only present at the basal level in the rich growth medium. Thus, further downregulation of GlmS cannot be observed even though GlmZ processing is enhanced due to higher RapZ amounts. The addition of 60 μ g/mL Nva-FMDP alone does not affect the levels of RapZ (Figure 34A, top panel, lane 7 and 13) as the amounts of protein appear comparable to the amounts that are detected in the uninduced samples (Figure 34A, middle panel, lane 3 to 5 and 9 to 11). On the contrary, GlcN6P depletion, achieved by the addition of GlmS inhibitor Nva-FMDP, elevates the levels of GlmY and full-length GlmZ (Figure 34B, top and middle panel, lane 7 and 13). GlmY in turn sequesters RapZ from GlmZ processing, and, as a consequence, the level of full-length GlmZ is increased to activate translation of the *glmS* mRNA. As a result, GlmS levels are strongly elevated (Figure 34A, middle panel, lane 7 and 13) to overcome GlmS enzymatic inhibition, caused by Nva-FMDP, and GlcN6P pools can be replenished. The addition of 60 μ g/mL Nva-FMDP to the strain which ectopically expressed *rpoE* from the P_{tet} promoter on a plasmid leads to strong accumulation of RapZ (Figure 34A, top panel, lane 8 and 14) and elevates the levels of GlmY (Figure 34B, top panel, lane 8 and 14). The higher levels of RapZ that are achieved by ectopic *rpoE* expression stimulate the cleavage of full-length GlmZ. Consequently, the level of the

processed form of GlmZ is increased and the level of full-length GlmZ is decreased (Figure 34B, middle panel, lane 8 and 14) as opposed to the condition when Nva-FMDP is added alone (Figure 34B, middle panel, lane 7 and 13). Despite the GlcN6P depletion that leads to the lack of the cell envelope precursors, full-length GlmZ is still cleaved and consequently there is no accumulation of GlmS (Figure 34A, middle panel, lane 8 and 14). Furthermore, it is important to mention that the growth rate of $P_{tet}::rpoE$ transformants which grow under combined conditions of induced cell envelope stress and GlcN6P depletion is even further decreased as compared to the induced cell envelope stress conditions alone (Figure 34C). Increased GlmS levels are only observed upon the addition of Nva-FMDP alone (Figure 34A, middle panel, lane 7 and 13).

In sum, these data indicate that the σ^E -mediated upregulation of RapZ is a more dominant cue under combined conditions of cell envelope stress and GlcN6P depletion. As a result of the σ^E -dependent accumulation of RapZ, the cleavage of the active full-length GlmZ is enhanced and the upregulation of GlmS due to GlcN6P deprivation is abolished. Upon ectopic expression of *rpoE*, mimicking σ^E activation, RapZ prevents upregulation of GlmS under conditions of GlcN6P starvation. Hence, *de novo* synthesis of the cell envelope precursors is prevented during the σ^E -response. This mechanism could allow the bacteria to first repair the damage in the envelope without activating its biosynthesis pathway from the very initial steps.

6.3. Compounds eliciting expression from the *lptAp1* promoter

The σ^E -dependent *lptAp1* promoter could potentially explain the observed accumulation of RapZ in response to *rpoE* expression. The latter promoter was shown to be selectively activated upon depletion of the LPS transport machinery, suggesting that it responds to LPS biogenesis defects and trafficking problems (Martorana *et al.*, 2011; Martorana *et al.*, 2021). Lipopolysaccharides (LPS) are a major component of the Gram-negative bacteria outer membrane that to a large extent contribute to the permeability barrier properties of the OM (Raetz and Whitfield, 2002). LPS is synthesized in the cytoplasm and further transported from the IM, across the periplasm, to the cell surface where it is inserted into the OM. The translocation of LPS to the outer leaflet of the OM is achieved by a dedicated LPS transport (Lpt) machinery that spans from the inner to the outer membrane as a so-called Lpt bridge (Wu *et al.*, 2006). Due to its importance in Gram-negative bacteria, LPS and proteins involved in its transport and biosynthesis represent crucial antibiotic targets. Thanatin is a naturally occurring 21-residue long cationic antimicrobial peptide (CAMP), isolated from the insect *Podisus maculiventris* (spined soldier bug), that contains one disulfide bond and exhibits antimicrobial activity against a broad spectrum of organisms, including *E. coli* (Fehlbaum *et al.*, 1996). The minimal inhibitory concentration (MIC) of thanatin against *E. coli* was reported to be below 1,5 μ M (Vetterli *et al.*, 2018). Thanatin is one of the few known inhibitors of the LPS transport machinery. It binds LptA and LptD, disrupting the interactions required for the correct Lpt complex assembly (Vetterli *et al.*, 2018), which results in LptA degradation as well as the accumulation of colanic-acid modified LPS (Moura *et al.*, 2020). The disassembly of the Lpt machinery leads to the inhibition of LPS transport to the OM and defects in membrane assembly (Moura *et al.*, 2020). Since the σ^E -dependent *lptAp1* promoter responds to LPS biogenesis defects and trafficking problems, thanatin could elicit transcription from the latter promoter. To test this hypothesis, the effect of thanatin on the *lptAp1* promoter and the possible impact on the RapZ/GlmY/GlmZ/*glmS* circuit were addressed.

6.3.1. Thanatin activates the *lptAp1* promoter

The activation of the *lptAp1* promoter upon the addition of thanatin was tested through the use of *E. coli* strains carrying suitable *lacZ* reporter gene fusions. At first, the strain carrying the chromosomal *lptA*(-152 to +78)-*lacZ* fusion was used to determine whether the *lptAp1* promoter gets activated upon treatment with thanatin. Subsequently, the strain carrying the long chromosomal *lptA*(-152) $\rightarrow$ *rapZ*(+78)-*lacZ* fusion was used to check if the latter activation would lead to read-through transcription into the *rapZ* gene encoded in the *rpoN* operon.

Strains were first grown overnight in 5 mL LB at 37°C. The next morning, pre-cultures were inoculated from the overnight cultures to an OD₆₀₀ of 0.05 and grown at 37°C in 25 mL LB until reaching an OD₆₀₀ of ~ 0.1 (according to Moura *et al.*, 2020). Next, each pre-culture was split into two 5 mL cultures, one of which was supplemented with 5,25 µg/mL thanatin (1,5 x MIC as determined by Moura *et al.*, 2020) and the other with dH₂O as mock control. Subsequently, samples for β-galactosidase activity measurements were taken from the cultures at various times following the addition of thanatin.

The addition of thanatin (5,25 µg/mL final concentration) to the strain carrying the chromosomal *lptA*'-lacZ reporter fusion led to an increase in the activity of the reporter fusion (Figure 35A, orange columns), likely via the *lptAp1* promoter, upon treatment with thanatin. A maximum induction of the fusion was reached 60 and 90 min after the addition of thanatin, resulting in a 4,5-fold increase of the β-galactosidase activity. Those data confirm that thanatin activates the *lptAp1* promoter. Subsequently, the strain carrying the long chromosomal *lptA*(-152)→*rapZ*(+78)'-lacZ fusion was used to check if the latter activation is as well a signal that can regulate the whole *rpoN* locus all the way downstream to *rapZ*. Indeed, the addition of thanatin to the strain carrying the long chromosomal *lptA*(-152)→*rapZ*(+78)'-lacZ reporter fusion led to an increase in the activity of the reporter fusion (Figure 35C, orange columns), resulting in a maximum 2-fold induction of the β-galactosidase activity 90 and 120 min after the addition of thanatin. Together, those data confirm that thanatin likely elicits the activation of the *lptAp1* promoter. Additionally, *rapZ* expression increases upon treatment with thanatin and that is perhaps mediated by read-through transcription from the upstream located *lptAp1* promoter. Furthermore, thanatin is causing substantial growth inhibition of *E. coli* cells. The growth-defect becomes evident 45 min after the addition of thanatin to the culture and after 1 h, the cells treated with thanatin reached a maximum OD₆₀₀ of ~ 0.3, while the untreated cells were in an OD₆₀₀ range of 0.55 to 0.65 (Figure 35B, D). At later points in time, 90 and 120 min after treatment with thanatin, the cells started lysing and the OD₆₀₀ decreased. Thanatin is highly efficient in inhibiting the growth of bacteria, including *E. coli*, at low concentrations (Vetterli *et al.*, 2018). Consequently, the addition of thanatin impeded the cell growth, as expected.

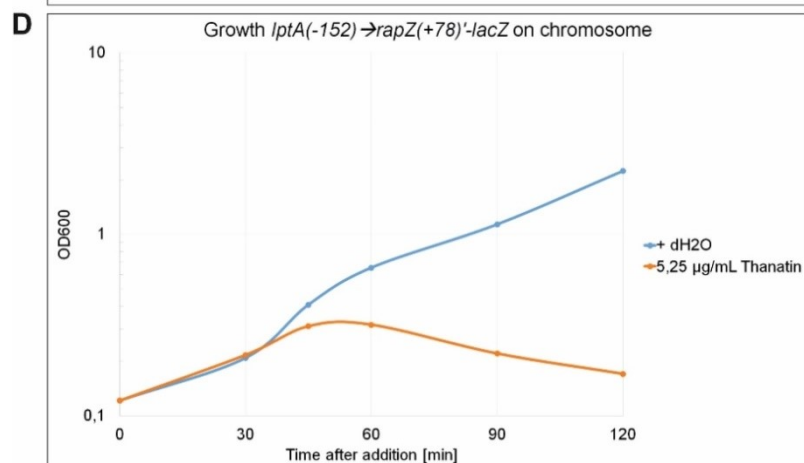
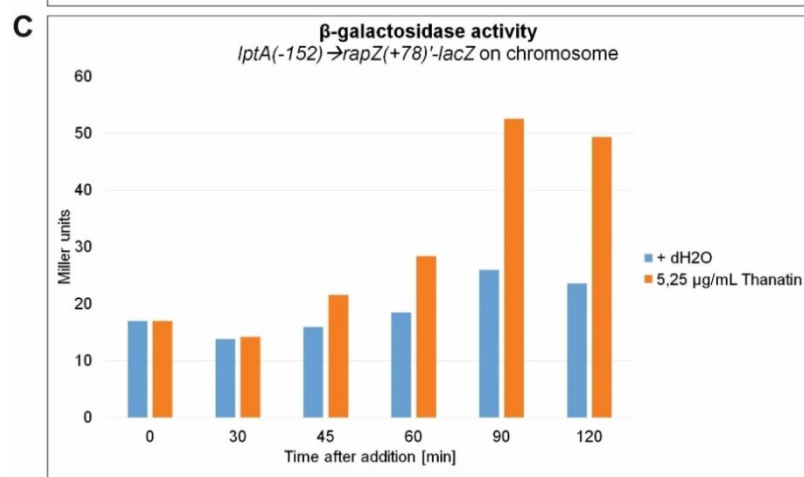
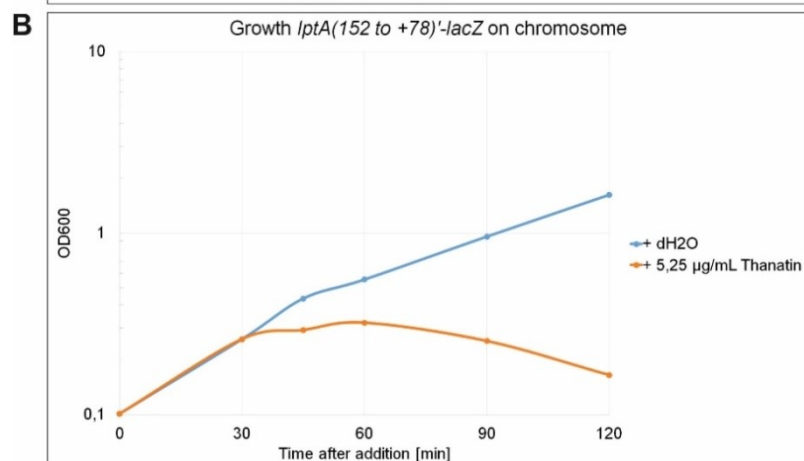
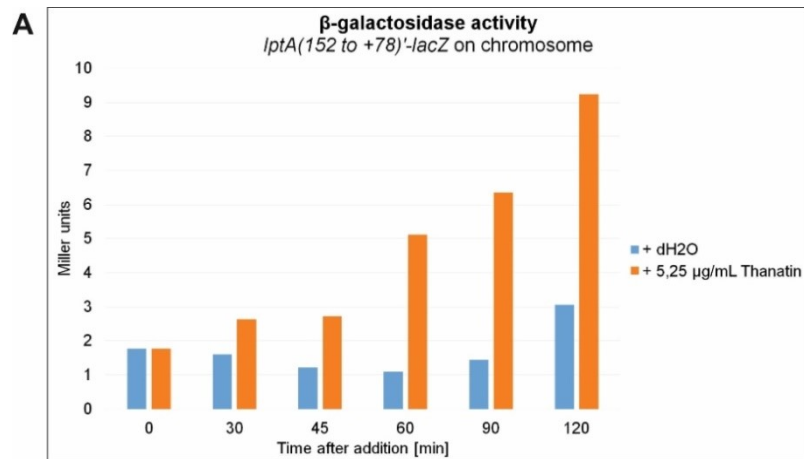


Figure 35. Thanatin activates *lptAp1* promoter and leads to upregulation of *rapZ'-lacZ* . A and C. β -galactosidase activity assay addressing the expression of the chromosomal *lptA'-lacZ* (A) and *lptA $\rightarrow$ rapZ'-lacZ* (C) reporter fusion in *E. coli*. Cultures of strains Z1303 (*S4197 [attB::lptA(-152 to +78)']-lacZ*) and Z1378 (*S4197 [attB::lptA(-152) $\rightarrow$ rapZ(+78)']-lacZ*) were grown in LB +/- 5,25 μ g/mL thanatin. Miller unit values derived from one measurement (n = 1). **B and D.** The growth of the cultures addressing the expression of the chromosomal *lptA'-lacZ* (B) and *lptA $\rightarrow$ rapZ'-lacZ* (D) reporter fusion in *E. coli* in the presence and absence of thanatin at 5,25 μ g/mL concentration.

6.3.2. Impact of thanatin on the RapZ/GlmY/GlmZ/*glmS* circuit

Thanatin is an inhibitor of the Lpt machinery, consequently leading to the inhibition of LPS transport and defects in membrane assembly (Moura *et al.*, 2020). So far, the obtained results confirm that thanatin likely elicits the *lptAp1* promoter which is believed to respond solely to LPS biogenesis defects. The activation of the latter promoter upon the addition of thanatin likely leads to read-through transcription into the *rapZ* gene, as a 2-fold induction of the β -galactosidase activity in the strain carrying the chromosomal *lptA $\rightarrow$ rapZ'-lacZ* reporter gene fusion could be observed. The activity of protein RapZ is primarily regulated through interaction with the metabolite GlcN6P (Khan and Görke, 2020). The increased *rapZ* transcription as a consequence of activation of the *lptAp1*, elicited by thanatin, could result in elevated RapZ protein levels and account for activation of the *glmY* expression in a QseF-dependent manner. In case of RapZ amounts exceeding GlmY amounts, part of the RapZ population is left un-sequestered and thereby free to recruit RNase E to GlmZ and impede *glmS* translation. To study the consequences of thanatin for accumulation of RapZ protein and to further analyze the impact it might have on the GlmY/GlmZ/*glmS* circuit, RapZ and GlmS levels were addressed by Western blot analysis and GlmZ and GlmY amounts by Northern blot analysis. In addition, *glmY* expression levels were quantitatively monitored by the reporter gene assay in the strain carrying the chromosomal *glmY'-lacZ* reporter gene fusion.

Initially, a *wild-type* strain (Z367) was grown as previously described (in 6.3.1.) and samples were harvested at various times following the treatment with thanatin. Next, total protein extracts were prepared and 10 μ L of the latter (cell number $\sim$ 0.05 OD₆₀₀) were separated on a 12,5% or 15% SDS-PAA gel and subsequently analyzed by Western blotting using polyclonal antisera directed against RapZ, GlmS and ribosomal protein S2 (loading control). After blotting, the SDS-PAA gel was stained with Coomassie blue to serve as an additional loading control.

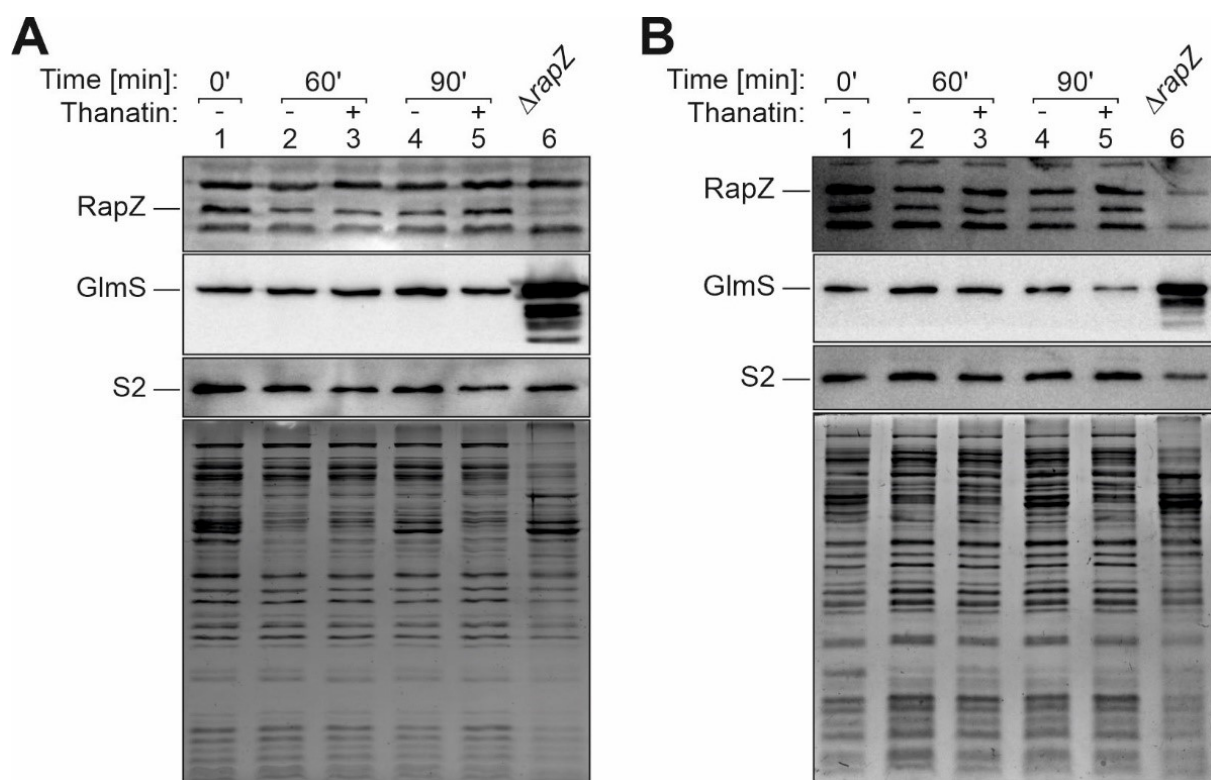


Figure 36. Western blot analysis of RapZ and GlmS levels upon treatment with thanatin. Cultures of *wild-type* strain Z367 were grown in LB +/- 5,25 µg/mL thanatin. Samples were harvested at the indicated times and total protein extracts were prepared. Of the latter, 10 µL (cell number ~ 0.05 OD₆₀₀) were separated on a 12,5% **(A)** or 15% **(B)** SDS-PAA gel and analyzed by Western blotting using polyclonal antisera directed against RapZ (top panel), GlmS (middle panel) and ribosomal protein S2 (middle panel). Polyclonal antiserum directed against RapZ results in non-specific signals, therefore strain Z857 ($\Delta rapZ$) was included as additional control. Loading controls are provided by S2 signals and Coomassie blue stained SDS-PAA gel (bottom panel).

Table 29. Western blot quantifications according to the program GelQuant.NET. The signal intensities of the RapZ and GlmS protein bands were normalized to the signal intensities of the loading controls provided by S2 protein signals. Lane 1 (prior to thanatin treatment) was selected as the reference lane (100% signal intensity) and determined the normalization factor for each lane. Signal intensity values are presented as average and derived from three independent quantifications of each blot.

Lane	RapZ (A)	GlmS (A)	RapZ (B)	GlmS (B)
1 (0')	100	100	100	100
2 (H₂O 60')	47	96	68	102
3 (Thanatin 60')	66	129	81	106
4 (H₂O 90')	66	145	55	86
5 (Thanatin 90')	106	125	93	53
6 ($\Delta rapZ$)	0	212	0	208

The addition of thanatin resulted in slightly elevated levels of RapZ as compared to the untreated cells (Figure 36A, B, top panel, lanes 4 and 5; Table 29). The effect is observed solely 90 min after treatment with thanatin and this result is in correspondence with the β -galactosidase activities measured by the reporter gene assay that resulted in a 2-fold induction of the *lptA*→*rapZ'*-*lacZ* reporter gene fusion 90 min after the addition of thanatin. The levels of GlmS protein do not differ much regardless of the thanatin treatment (Figure

36A, B, middle panel; Table 29) reflecting that GlmS is already only present at the basal level. The results obtained by Western blot analysis and reporter gene assays confirm that thanatin elicits an envelope stress response which in turn triggers slight accumulation of protein RapZ.

Next, the *wild-type* strain and the isogenic $\Delta rapZ$ strain, carrying the chromosomal *glmY*-5'-*lacZ* reporter gene fusion, were used to determine *glmY* expression levels and consequently check whether the accumulation of RapZ, achieved by the addition of thanatin, activates *glmY* expression from the σ^{54} -dependent promoter in a QseF-dependent manner. The isogenic $\Delta rapZ$ strain served as negative control in the experiment since RapZ is required for full activity of the *glmY* σ^{54} promoter. The latter strains were grown as previously described (in 6.3.1.). Subsequently, samples for determination of β -galactosidase activities, for preparation of total protein extracts and for total RNA extraction were harvested from the cultures 0 and 90 min following the addition of thanatin; i.e. when the effect on RapZ levels was most notable. The samples were further analyzed by quantitative β -galactosidase activity assay as well as Western and Northern blotting.

Two perfectly overlapping promoters, one of which is σ^{70} - and one is σ^{54} -dependent control expression of the *glmY* gene in *E. coli* (Reichenbach *et al.*, 2009). The constitutively active σ^{70} promoter allows for a basal level of *glmY* expression during the exponential phase. The σ^{54} promoter is controlled by the TCS QseE/QseF and requires activation in a QseF-dependent manner that can be initiated by GlcN6P-free RapZ. In agreement with previous results (Khan *et al.*, 2020), the β -galactosidase activities indicate that the $\Delta rapZ$ mutation reduces *glmY* expression levels before the treatment with thanatin or dH₂O as compared to the *wild-type* (at $t = 0$; Figure 37A, light and dark blue columns). In the *wild-type*, the addition of thanatin at 5,25 μ g/mL concentration resulted in a ~ 2 -fold higher activity of the *glmY*'-*lacZ* reporter fusion as compared to the untreated cells 90 min after the addition (Figure 37A, light blue and orange columns). However, a similar increase in the activity of the reporter fusion ($\sim 1,7$ -fold) was observed in the $\Delta rapZ$ mutant upon thanatin treatment (Figure 37A, dark blue and yellow columns). Since RapZ is required for full activity of the *glmY* σ^{54} promoter and the activity of the *glmY*'-*lacZ* reporter fusion is induced to a similar extent in the $\Delta rapZ$ mutant as well, the increased *glmY* expression upon thanatin addition is not initiated by the elevated RapZ levels. Considering that the influence of RapZ on GlmY stabilization is not reflected by the reporter gene assay, samples for total RNA extraction, that were harvested alongside of the samples used for determination of β -galactosidase activities, were afterwards analyzed by Northern blotting in order to compare the GlmY levels directly.

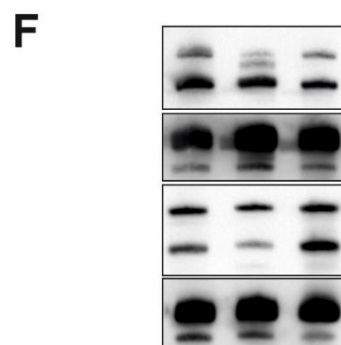
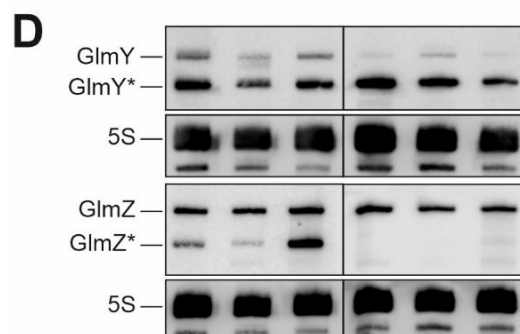
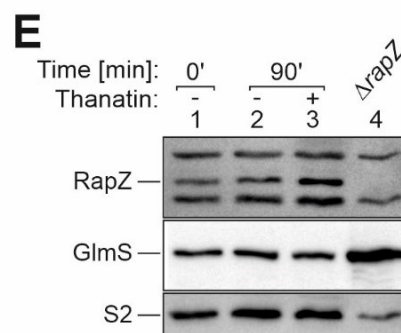
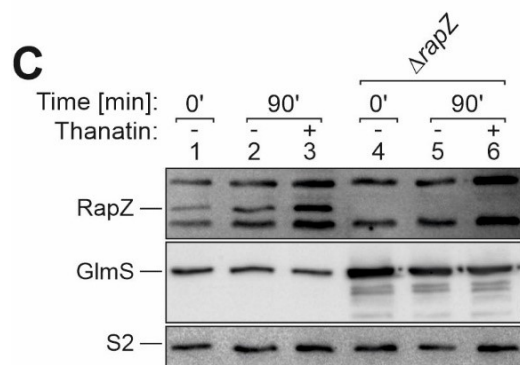
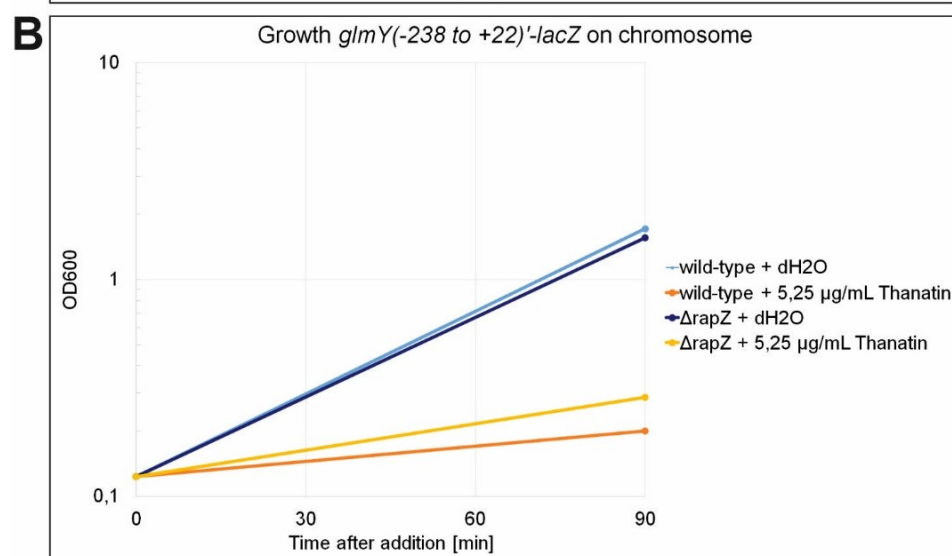
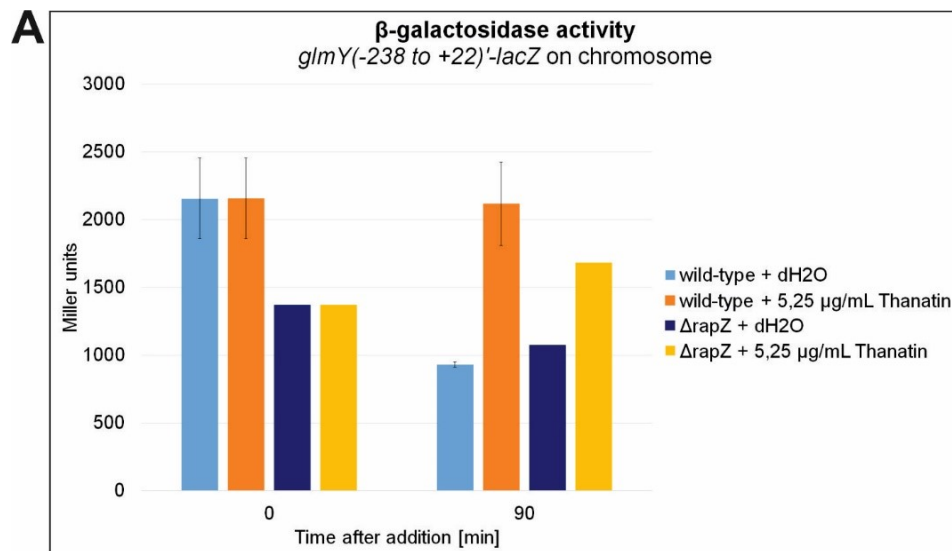


Figure 37. Analysis of the impact of thanatin on the RapZ/GlmY/GlmZ/*glmS* circuit. Cultures of strains Z741 (*S4197 attB::[glmY-5'-lacZ]*) and Z833 (*S4197 attB::[glmY-5'-lacZ], ΔrapZ*) were grown in LB +/- 5,25 µg/mL thanatin. Samples for determination of β-galactosidase activities, for preparation of total protein extracts and for total RNA extraction were taken from the cultures 0 and 90 min following the addition of thanatin. **A.** β-galactosidase activity assay addressing the expression of the chromosomal *glmY'-lacZ* reporter fusion in *E. coli*. Miller unit values are presented as mean ± SD (when available) and derived from one or two independent measurements (n = 1-2). **B.** The growth of the cultures addressing the expression of the chromosomal *glmY'-lacZ* reporter fusion in *E. coli* in the presence and absence of thanatin at 5,25 µg/mL concentration. **C and E.** 7 µL of total protein extracts (cell number ~ 0.035 OD₆₀₀) were separated on a 12,5% SDS-PAA gel and analyzed by Western blotting using polyclonal antisera directed against RapZ (top panel), GlmS (middle panel) and ribosomal protein S2 (middle panel). Polyclonal antiserum directed against RapZ results in non-specific signals, therefore strain Z857 (*ΔrapZ*) was included as additional control. Loading controls are provided by S2 signals. **D and F.** 3,5 µg of total RNA was separated on an 8% urea-PAA gel and analyzed by Northern blotting using probes specific for GlmY (top panel), GlmZ (middle panel) and 5S rRNA (bottom panel; loading control). Figures C, D and E, F each represent an independent biological replicate.

Table 30. Western blot quantifications according to the program GelQuant.NET. The signal intensities of the RapZ and GlmS protein bands were normalized to the signal intensities of the loading controls provided by S2 protein signals. Lane 1 (prior to thanatin treatment) was selected as the reference lane (100% signal intensity) and determined the normalization factor for each lane. Signal intensity values are presented as average and derived from three independent quantifications of each blot.

Lane	RapZ (C)	GlmS (C)	RapZ (E)	GlmS (E)
1 (0' wt)	100	100	100	100
2 (H ₂ O 90' wt)	137	93	125	94
3 (Thanatin 90' wt)	248	89	199	93
4 (0' ΔrapZ)	0	190	0	513
5 (H ₂ O 90' ΔrapZ)	0	192	/	/
6 (Thanatin 90' ΔrapZ)	0	121	/	/

Table 31. Northern blot quantifications according to the program GelQuant.NET. The signal intensities of the GlmY and full-length GlmZ RNA bands were normalized to the signal intensities of the loading controls provided by 5S rRNA signals. Lane 1 and lane 3 (prior to thanatin treatment) were selected as the reference lanes (100% signal intensity) for each strain (*wild-type* and *ΔrapZ*) and determined the normalization factor for each lane. Signal intensity values are presented as average and derived from three independent quantifications of each blot.

Lane	GlmY (D)	Full-length GlmZ (D)	GlmY (F)	Full-length GlmZ (F)
1 (0' wt)	100	100	100	100
2 (H ₂ O 90' wt)	82	98	86	83
3 (Thanatin 90' wt)	90	119	85	107
4 (0' ΔrapZ)	100	100	/	/
5 (H ₂ O 90' ΔrapZ)	97	85	/	/
6 (Thanatin 90' ΔrapZ)	87	80	/	/

As observed before, the addition of thanatin induces an ESR that results in elevated levels of RapZ as compared to the untreated cells (Figure 37C, E, top panel, lanes 2 and 3; Table 30). GlmY is present in slightly higher amounts before thanatin or dH₂O was added (at *t* = 0; Figure 37D, F, top panel, lanes 1 and 4; Table 31) and this result is in agreement with the measured β-galactosidase activities. The levels of GlmY in the *wild-type* strain do not

significantly vary regardless of the thanatin treatment (Figure 37D, F, top panel, lanes 2 and 3; Table 31). However, it seems that in the $\Delta rapZ$ mutant, GlmY is present in slightly lower amounts upon the addition of thanatin as compared to the untreated cells (Figure 37D, top panel, lanes 5 and 6; Table 31). The observed differences could be due to a blotting artefact or loading unequal amounts of RNA as the 5S rRNA signal in lane 6 seems slightly weaker (indicated by quantifications in Table 31). RapZ is required for stabilization of GlmY (Khan *et al.*, 2020) and consequently, the $\Delta rapZ$ mutation results in lower levels of GlmY since it is not protected from degradation. Contrary to previous results (Reichenbach *et al.*, 2009), GlmY levels are not collectively decreased in the $\Delta rapZ$ mutant as compared to the *wild-type* (Figure 37D, top panel, lanes 5 and 6; Table 31). This could be due to the growing conditions that were used since the pre-cultures were inoculated from the overnight cultures to an OD₆₀₀ of 0.05 and grown only until reaching an OD₆₀₀ of ~ 0.1 (and not 0.3) which results in just one cell division. Upon the addition of thanatin, the cells are barely dividing further (Figure 37B, light blue and orange columns). Since GlmY and LacZ-fusion proteins tend to accumulate in the overnight cultures, GlmY levels observed by Northern blots and β -galactosidase activities of the *glmY'-lacZ* reporter fusion might be carryover from the overnight accumulation, which is simply out-diluted and reduced in the untreated samples where the cells are growing and dividing further. Additionally, the level of GlmZ processing depends on the availability of RapZ for interaction, which is also regulated by GlmY. As a consequence of higher RapZ amounts, the processed form of GlmZ is accumulating upon the addition of thanatin (Figure 37D, F, middle panel, lanes 2 and 3). RapZ binds full-length GlmZ and recruits endoribonuclease RNase E to process and inactivate GlmZ. Since RapZ is required for the cleavage of GlmZ by RNase E (Göpel *et al.*, 2013; Göpel *et al.*, 2016), the processing of GlmZ is abolished in the $\Delta rapZ$ mutant (Figure 37D, middle panel, lanes 4, 5 and 6). As a consequence of the accumulation of full-length GlmZ that triggers *glmS* expression, GlmS protein levels are highly elevated in the $\Delta rapZ$ mutant (Figure 37C, middle panel, lanes 4, 5 and 6; Table 30). In the *wild-type*, the levels of GlmS do not differ much regardless of the thanatin treatment (Figure 37C, E, middle panel, lanes 2 and 3; Table 30) as selective accumulation of the processed form of GlmZ, triggered by higher RapZ amounts, prevents upregulation of GlmS. Furthermore, GlmS is already only present at the basal level in a rich growth medium that was used and as a consequence, further downregulation of GlmS is not possible. The results obtained by Western and Northern blot analysis further confirm that thanatin likely elicits a response from *lptAp1*, leading to higher RapZ amounts, which in turn trigger processing of full-length GlmZ by recruitment of RNase E.

6.3.3. Impact of thanatin on the RapZ/GlmY/GlmZ/*glmS* circuit in combination with the GlmS inhibitor Nva-FMDP

To further investigate whether the upregulation of RapZ, caused by thanatin, impacts GlmS levels and in turn synthesis of precursors required for cell envelope components under conditions of GlcN6P starvation, Nva-FMDP was used. It is a synthetic derivative of an antibiotic that selectively inhibits GlmS enzymatic activity which in turn results in depletion of intracellular GlcN6P (Chmara *et al.*, 1998). A recent study has demonstrated that Nva-FMDP upregulates GlmY and GlmZ to achieve *glmS* expression in a dose-dependent manner, thereby overcoming GlmS enzymatic inhibition and rendering Nva-FMDP ineffective (Khan *et al.*, 2016). Nva-FMDP was used in combination with thanatin to determine which of these conditions is more dominant, the upregulation of GlmS due to GlcN6P depletion, caused by Nva-FMDP, or the enhanced GlmZ processing due to upregulation of RapZ, caused by thanatin. For the purpose of this experiment, the *wild-type* strain carrying the chromosomal *glmY-5'-lacZ* reporter gene fusion was used to determine *glmY* expression levels and consequently check to which extent *glmY* expression is activated upon the addition of Nva-FMDP with or without thanatin. The latter strain was first grown overnight in 5 mL LB at 37°C. The next morning, the pre-culture was inoculated from the overnight culture to an OD₆₀₀ of 0.05 and grown at 37°C in 25 mL LB until reaching an OD₆₀₀ of ~ 0.1. Next, the pre-culture was split into three 5 mL cultures, one of which received Nva-FMDP at 60 µg/mL sub-inhibitory concentration with or without thanatin at a concentration of 5,25 µg/mL (1,5 x MIC as determined by Moura *et al.*, 2020) whereas the other sub-culture remained untreated (addition of dH₂O). 60 µg/mL is the sub-inhibitory concentration of Nva-FMDP at which the growth of the *wild-type* strain is not impaired and *glmS* expression is activated to some degree to overcome inhibition by the antibiotic (Khan *et al.*, 2016). Subsequently, samples for determination of β-galactosidase activities, for preparation of total protein extracts and for total RNA extraction were taken from the cultures 0 and 90 min following the addition of Nva-FMDP with or without thanatin. The samples were further analyzed by the quantitative β-galactosidase activity assay and Western and Northern blotting.

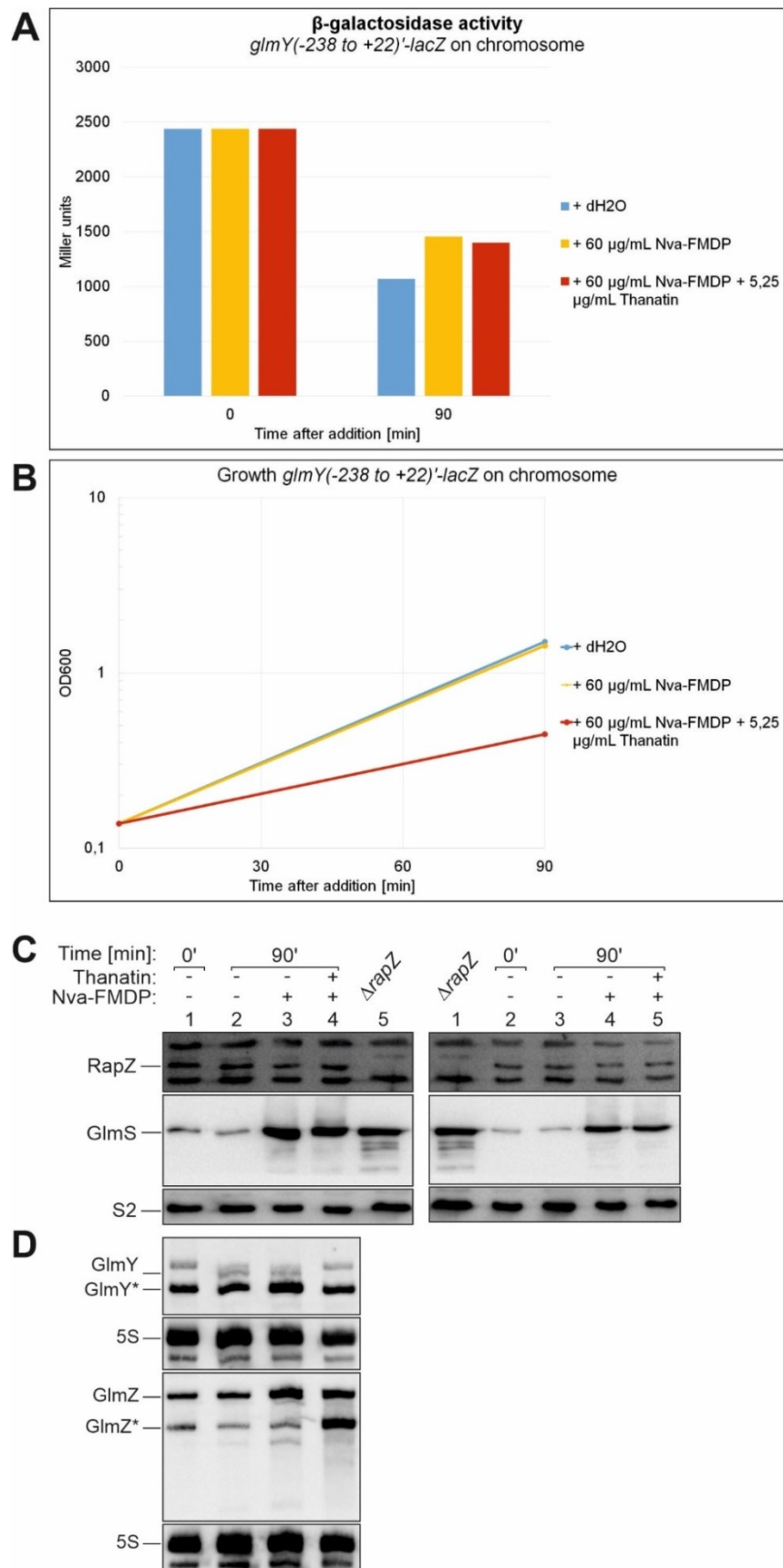


Figure 38. Analysis of the impact of thanatin on the RapZ/GlmY/GlmZ/*glmS* circuit upon addition of GlmS inhibitor Nva-FMDP. Cultures of strain Z741 (*S4197 attB::[glmY-5'-lacZ]*) were grown in LB +/- 5,25 µg/mL thanatin with or without 60 µg/mL Nva-FMDP. Samples for determination of β -galactosidase

activities, for preparation of total protein extracts and for total RNA extraction were taken from the cultures 0 and 90 min following the addition of Nva-FMDP with or without thanatin. **A.** β -galactosidase activity assay addressing the expression of the chromosomal *glmY'-lacZ* reporter fusion in *E. coli*. Miller unit values derived from one measurement ($n = 1$). **B.** The growth of the cultures addressing the expression of the chromosomal *glmY'-lacZ* reporter fusion in *E. coli* in the presence and absence of Nva-FMDP at 60 $\mu\text{g/mL}$ concentration and thanatin at 5,25 $\mu\text{g/mL}$ concentration. **C.** 7 μL of total protein extracts (cell number $\sim 0.035 \text{ OD}_{600}$) were separated on a 12,5% SDS-PAA gel and analyzed by Western blotting using polyclonal antisera directed against RapZ (top panel), GlmS (middle panel) and ribosomal protein S2 (middle panel). Polyclonal antiserum directed against RapZ results in non-specific signals, therefore strain Z857 (ΔrapZ) was included as additional control. Loading controls are provided by S2 signals. Figure C represents two independent technical replicates. **D.** 3,5 μg of total RNA was separated on an 8% urea-PAA gel and analyzed by Northern blotting using probes specific for GlmY (top panel), GlmZ (middle panel) and 5S rRNA (bottom panel; loading control).

Table 32. Western blot quantifications according to the program GelQuant.NET. The signal intensities of the RapZ and GlmS protein bands were normalized to the signal intensities of the loading controls provided by S2 protein signals. Lane 1 (for C1) and lane 2 (for C2; prior to thanatin treatment) were selected as the reference lane (100% signal intensity) and determined the normalization factor for each lane. Signal intensity values are presented as average and derived from three independent quantifications of each blot.

Lane	RapZ (C1)	GlmS (C1)	RapZ (C2)	GlmS (C2)
1 (0' wt/ ΔrapZ)	100	100	0	359
2 (H_2O 90' / 0' wt)	102	78	100	100
3 (Thanatin 90' / H_2O 90')	100	266	88	91
4 (Thanatin + Nva-FMDP 90' / Thanatin 90')	113	255	82	451
5 (ΔrapZ / Thanatin + Nva-FMDP 90')	0	157	95	445

Table 33. Northern blot quantifications according to the program GelQuant.NET. The signal intensities of the GlmY and full-length GlmZ RNA bands were normalized to the signal intensities of the loading controls provided by 5S rRNA signals. Lane 1 (prior to thanatin treatment) was selected as the reference lane (100% signal intensity) and determined the normalization factor for each lane. Signal intensity values are presented as average and derived from three independent quantifications of each blot ($n = 3$).

Lane	GlmY (D)	Full-length GlmZ (D)
1 (0' wt)	100	100
2 (H_2O 90')	99	116
3 (Thanatin 90')	137	179
4 (Thanatin + Nva-FMDP 90')	112	158

In agreement with previous results (Khan *et al.*, 2020), the addition of Nva-FMDP at 60 $\mu\text{g/mL}$ concentration led to an almost 1,5-fold increase in the activity of the *glmY'-lacZ* reporter fusion as compared to the untreated cells (Figure 38A, blue and yellow columns). GlcN6P depletion, caused by Nva-FMDP, triggers accumulation of a decoy sRNA GlmY to sequester RapZ from processing GlmZ and in turn activate the translation of *glmS* mRNA in a concentration-dependent manner to replenish GlcN6P. A similar increase in the activity of the reporter fusion was observed upon Nva-FMDP treatment in combination with thanatin (Figure 38A, blue and red columns). Furthermore, the addition of Nva-FMDP at 60 $\mu\text{g/mL}$ sub-inhibitory concentration did not impair the growth of the *wild-type* strain, while the

addition of thanatin on top of Nva-FMDP caused substantial growth inhibition as observed before (Figure 38B). RapZ protein levels are not affected by the addition of 60 $\mu\text{g/mL}$ Nva-FMDP alone as the amounts of protein appear comparable to the amounts that are achieved in untreated cells (Figure 38C, top panel, lanes 2, 3 on the left and lanes 3, 4 on the right; Table 32). GlcN6P depletion, caused by GlmS inhibitor Nva-FMDP, increases the levels of GlmY and GlmZ (Figure 38D, top and middle panel, lane 3; Table 33). The processed GlmY that accumulates sequesters RapZ to inhibit the processing of GlmZ. Consequently, the level of full-length GlmZ is increased and in turn triggers *glmS* expression. As a result, GlmS levels are highly elevated (Figure 38C, middle panel, lanes 2, 3 on the left and 3, 4 on the right; Table 32) to overcome GlmS enzymatic inhibition, caused by Nva-FMDP. The addition of thanatin on top of Nva-FMDP had a weaker impact on the levels of RapZ compared to the previous experiments (Figure 38C, top panel, lane 4 on the left and lane 5 on the right; Table 32). GlmY amounts do not differ much from the amounts that are present in the untreated cells and seem lower than the amounts achieved by the addition of Nva-FMDP alone (Figure 38D, top panel, lane 4; Table 33). As a consequence of the slightly higher RapZ amounts, the processing of GlmZ is enhanced and the processed form of GlmZ is accumulating (Figure 38D, middle panel, lane 4). Simultaneously, the level of full-length GlmZ is increased as well to activate the translation of *glmS* mRNA and counteract GlcN6P depletion that is caused by Nva-FMDP (Figure 38D, middle panel, lane 4; Table 33). As a result, GlmS levels are highly elevated to overcome GlmS enzymatic inhibition and do not differ much from the amounts that are achieved when Nva-FMDP is added alone (Figure 38C, middle panel, lane 4 on the left and lane 5 on the right; Table 32). There is a slight decrease in GlmS amounts when thanatin is added in combination with Nva-FMDP compared to the amounts achieved by the addition of Nva-FMDP alone (indicated by quantifications in Table 32) but the difference seems insignificant. Therefore, the effect of thanatin on the system in combination with Nva-FMDP is not strong enough to prevent GlmS upregulation under conditions of GlcN6P starvation even though the processing of GlmZ is enhanced due to slightly higher RapZ amounts. The contrary was observed when the σ^E ESR was triggered by ectopic expression of *rpoE* (6.2.2.6.), yet it is important to mention that ectopic expression of *rpoE* from a plasmid results in an artificial over-activation of the *lptAp1* promoter that leads to saturated amounts of RapZ, which are exceeding the physiological requirements. Induction of *rpoE* expression from a plasmid led to an almost 30-fold increase in the activity of the *lptA*→*rapZ*'-lacZ reporter fusion (6.2.2.2.) as compared to the addition of thanatin that resulted in a maximum 2-fold induction of the reporter fusion (6.3.1.). The results obtained from this experiment demonstrate that the upregulation of GlmS due to GlcN6P depletion is a more dominant cue under combined conditions of cell envelope stress, triggered by thanatin, and GlcN6P depletion.

6.4. Genetic alterations activating the *lptAp1* promoter

Activation of the *lptAp1* promoter explains the observed accumulation of RapZ in response to *rpoE* expression. A recent study has demonstrated through analysis of conditional *lptA* and *lptC* mutants that this promoter is selectively activated upon depletion of the LPS transport machinery, suggesting that it responds to problems in LPS traffic (Martorana *et al.*, 2011). However, beyond depletion of the *lpt* genes themselves, no signals are known that would lead to activation of the latter promoter. In order to search for mutations and genes that cause the activation of the σ^E -dependent *lptAp1* promoter, a random transposon mutagenesis screen was carried out in a strain containing a chromosomal *lptA'*-*lacZ* reporter gene fusion. For this purpose, the miniTn10 delivery system was used (as described in 5.2.3.). The temperature sensitive plasmid pKESK18 encodes the miniTn10 transposon with a chloramphenicol resistance cassette (miniTn10-Cam^r) and the Tn10 transposase (Madhusudan *et al.*, 2005). The temperature shift to 42°C induces transposition and mediates loss of the plasmid, resulting in disruption of genes by a random insertion of the mini-Tn10 transposon into the chromosome. To identify gene disruptions that activate the *lptAp1* promoter, the expression levels of *lptA*(-152 to +78)-*lacZ* reporter fusion, were screened phenotypically on LB-X-gal plates and later quantitatively monitored.

6.4.1. Construction of a suitable *lptA'*-*lacZ* reporter gene fusion for screening

To screen for mutations that activate the *lptAp1* promoter, a previously constructed, suitable *lptA*(-152 to +78)-*lacZ* fusion that is comprising only the *lptAp1* and the *lptB* promoters (but no downstream promoters; Figure 39), was inserted ectopically into the λ -*attB*-site in the chromosome of *E. coli*-K12 *wild-type* strain S4197 (as described in 5.2.2.3.1.). The latter reporter construct consists of the -152 to +78 region of the *lptA* gene that is fused to the reporter gene *lacZ*. The integration of the *lptA'*-*lacZ* reporter construct into the chromosomal lambda attachment site was successful only in the MG1655 strain carrying a FLAG-tagged *ptsN* allele (*ptsN*-3xFLAG), generating strain Z1297.

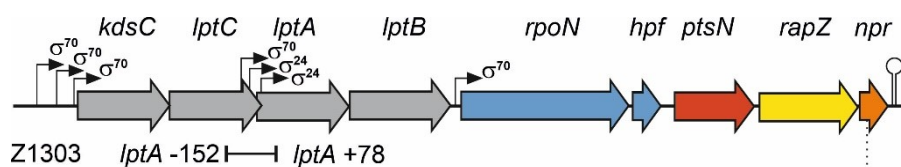


Figure 39. The *E. coli* *rpoN* operon and the *lptA'*-*lacZ* reporter fusion used for screening. The strain Z1303 carrying the chromosomal *lptA*(-152 to +78)-*lacZ* reporter gene fusion, comprising only the *lptAp1* and the *lptB* promoters, was used for screening to find mutations that activate the σ^E -dependent *lptAp1* promoter.

To check whether the *lptA*(-152 to +78)-*lacZ* reporter fusion is suitable for screening, a spotting assay was carried out to evaluate the difference at the level of phenotypes on X-Gal

indicator plates. For the purpose of this experiment, the constructed Z1297 strain was transformed with the *rpoE* expression plasmid (pFS28) or the corresponding VC (pBAD33), respectively. Bacteria were grown and spotted onto LB plates supplemented with chloramphenicol and X-Gal either containing or lacking arabinose as previously described (in 6.2.1.).

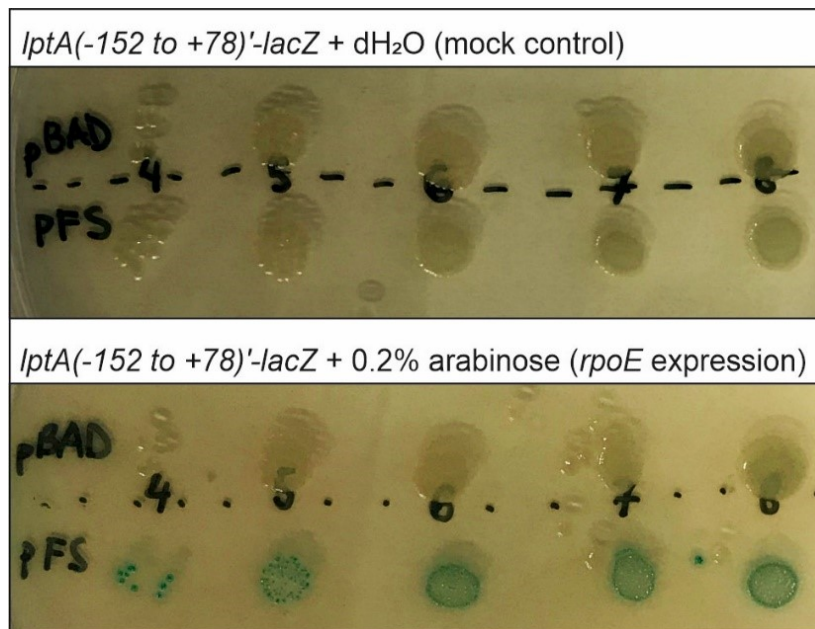


Figure 40. Impact of ectopic *rpoE* expression on the chromosomal *lptA'*-*lacZ* reporter fusion in *E. coli*. Strain Z1297 (*S4197 ptsN-3xFLAG [attB::lptA(-152 to +78)'-lacZ]*) was transformed with the *rpoE* expression plasmid (pFS28) or the corresponding VC (pBAD33). Overnight cultures of transformants were adjusted to 1 OD₆₀₀ = 10⁹ cells/mL and 10-fold serial dilutions were prepared in 1 mL LB medium, ranging from 10⁸ to 10⁴ cells/mL (spotted from right to left). 2 μ L of each serial dilution was spotted onto LB plates supplemented with chloramphenicol and X-Gal either containing or lacking 0,2% arabinose to induce expression of *rpoE*. The plates were incubated overnight at 37°C.

It is most important for the screening procedure to clearly recognize differences at the level of phenotypes, which allows specific selection for colonies that are exhibiting increased expression of the *lacZ* reporter fusion amongst hundreds of others on the same plate. As expected, ectopic expression of *rpoE* from an arabinose-inducible promoter on a plasmid, mimicking σ^E activation, led to activation of the σ^E -dependent *lptAp1* promoter which resulted in a strong blue colored phenotype of the transformants on an LB-X-Gal plate (Figure 40). An obvious difference in phenotype of the induced and the uninduced *rpoE* transformants was observed, rendering the *lptA(-152 to +78)'-lacZ* reporter fusion suitable for phenotypic screening. Hence, the general transduction by phage *T4-GT7* (Wilson *et al.*, 1979) was carried out to introduce the spectinomycin resistance tagged *lptA(-152 to +78)'-lacZ* fusion from the strain Z1297, carrying a FLAG-tagged *ptsN* allele, into the *wild-type* strain S4197 (as described in 5.2.2.1.), generating strain Z1303.

6.4.2. Finding signals affecting activity of the *lptAp1* promoter by transposon mutagenesis screen using a miniTn10 delivery system

Once constructed, the strain carrying the chromosomal *lptA*(-152 to +78)-*lacZ* reporter gene fusion was subjected to a random transposon mutagenesis screen (as described in 5.2.3.) using the mTn10 delivery plasmid pKESK18 (Madhusudan *et al.*, 2005). For the purpose of this experiment, the latter strain was initially transformed with the plasmid pKESK18. A single transformant was inoculated in 100 mL LB supplemented with kanamycin and grown overnight at 28°C. The next day, the overnight culture was adjusted to an appropriate dilution factor (10^{-3} and 10^{-4} -fold), and 100 μ L of the diluted culture were plated on LB-agar supplemented with chloramphenicol and X-Gal as well as on MacConkey-lactose agar plates supplemented with chloramphenicol. The plates were subsequently incubated overnight at 42°C to induce transposition and mediate loss of the plasmid. Additionally, 100 μ L of the diluted culture were plated on LB-agar supplemented with kanamycin and incubated overnight at 28°C to determine the total cell count of transformants in order to calculate the transposition rate. On the following day, colonies exhibiting a more intense, blue coloration on LB-X-Gal plates were isolated and re-streaked on fresh LB-X-Gal plates supplemented with chloramphenicol to re-evaluate the phenotypes. To allow for the direct comparison of the phenotypes, the *wild-type* strain, exhibiting an unaltered phenotype, was transformed with the VC carrying the chloramphenicol resistance cassette (pBAD33) and additionally re-streaked on the fresh plates next to the mutants. Eight screening rounds were performed in total with 100-150 plates each, which were inoculated with 100 μ L of the diluted overnight culture of the strain carrying the chromosomal *lptA*'-*lacZ* that was initially transformed with the plasmid pKESK18. Re-streaked mutants still exhibiting a strong blue colored phenotype were first tested for kanamycin resistance to verify loss of the plasmid and analyzed by colony PCR (as described in 5.2.4.3.2.), using primers S357 and BG621, to exclude the mutants carrying the transposon insertion in the *lacZ* reporter construct. In each round of screening, the selection procedure yielded ~ 2-4 appropriate mutants for further investigation. Subsequently, the *lptA* expression levels of those mutants were quantitatively monitored by the β -galactosidase activity assays (as described in 5.2.7.1.). For this purpose, the mutants were grown overnight at 37°C in 5 mL LB supplemented with chloramphenicol. The next morning, fresh cultures were inoculated from the overnight cultures to an OD₆₀₀ of 0.1 and grown at 37°C in 10 mL LB supplemented with chloramphenicol until reaching an OD₆₀₀ of ~ 1. Samples for β -galactosidase activity measurements were taken from the cultures at an OD₆₀₀ of ~ 0.3 and ~ 1 to evaluate expression levels in the early and late exponential growth phase, respectively. Additionally, samples from the overnight cultures were taken for the reporter gene assay to evaluate expression levels in the stationary growth

phase (OD_{600} of ~ 2 to 5). Subsequently, the genome positions of the miniTn10 transposon leading to increased expression of the *lacZ* reporter gene fusion were mapped by semi-random two-step PCR (as described in 5.2.4.3.3.). In order to determine genes, interrupted by the transposon insertion, the obtained transposon flanking sequences were BLAST® searched against the *E. coli*-K12 MG1655 genome (NCBI reference taxid:511145) in the NCBI database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Furthermore, the obtained transposon flanking sequences were subsequently aligned using vectorNTI software to determine the exact position and orientation of transposon insertions.

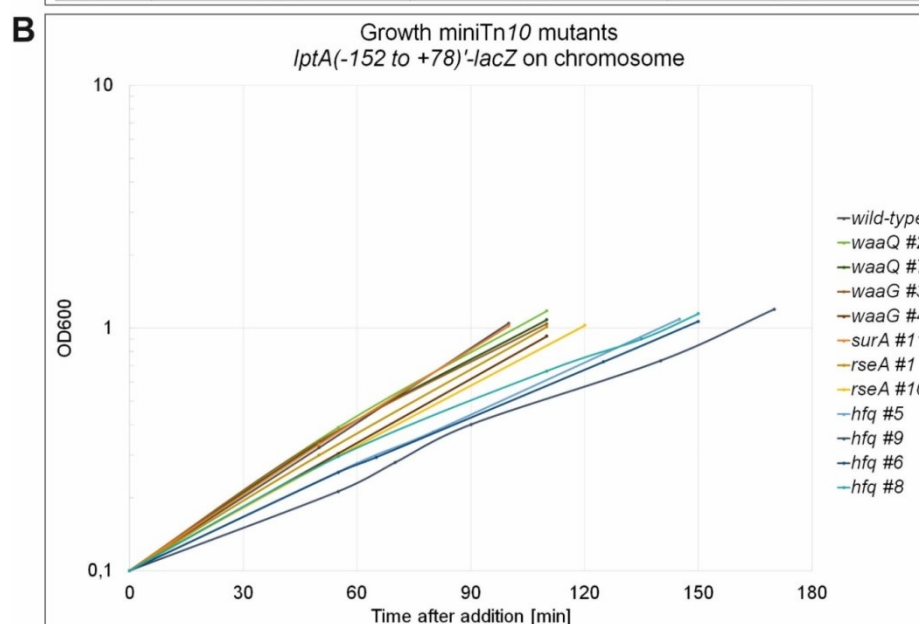
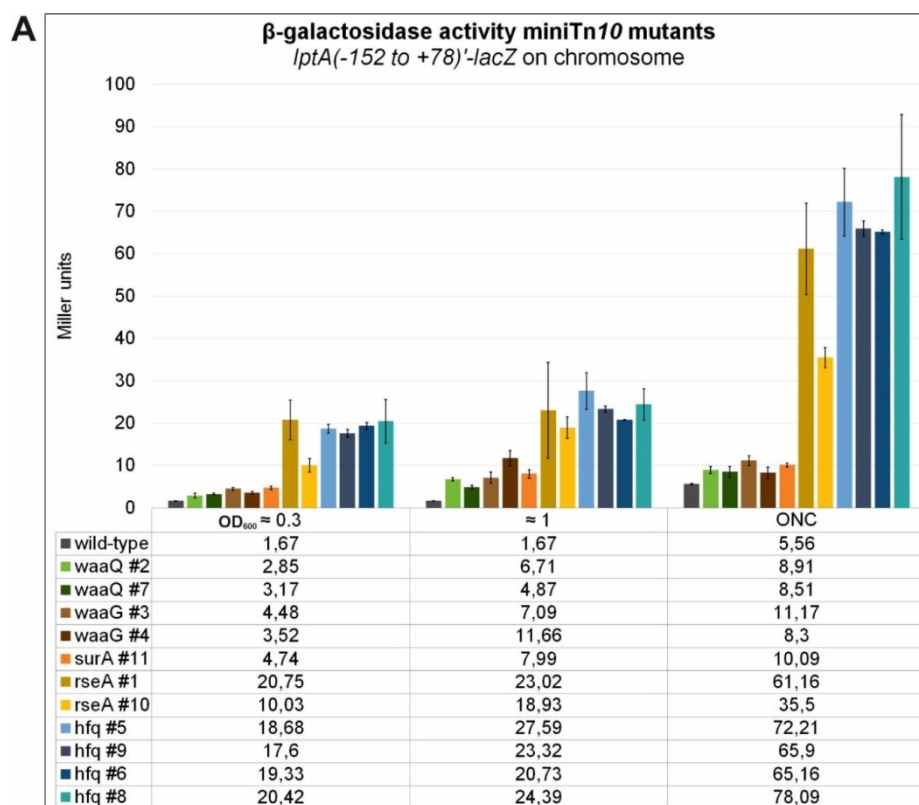


Figure 41. Genes hit in the transposon mutagenesis screen for activation of the *lptAp1* promoter. A. β -galactosidase activity assay addressing the expression of the chromosomal *lptA*'-lacZ reporter fusion in strain Z1303 (*S4197 [attB::lptA(-152 to +78)'*-lacZ]) screening candidates obtained by transposon mutagenesis using pKESK18. Transposon insertion mutants were selected on chloramphenicol plates and the screening candidates exhibiting a strong lactose⁺ phenotype on LB-X-Gal plates were further analyzed by the quantitative β -galactosidase activity assay. Cultures of the screening candidates were grown in LB supplemented with chloramphenicol. Samples for β -galactosidase activity assay were taken from the overnight cultures (OD₆₀₀ of 2-5) and at an OD₆₀₀ of ~ 0.3 and ~ 1 . Screening candidates are labeled with the name of the gene that was interrupted by the transposon insertion and with the number that was assigned to them during the transposon mutagenesis screening procedure. Miller unit values are presented as mean $\pm$ SD that derived from at least three independent measurements (n = 3 - 5). **B.** The growth of the cultures addressing the expression of the chromosomal *lptA*'-lacZ reporter fusion in *E. coli*. OD₆₀₀ values are presented as mean (n = 3-5).

Table 34. Screening candidates obtained by transposon mutagenesis of strain Z1303 (*S4197 [attB::lptA(-152 to +78)'*-lacZ]) using pKESK18. Table of screening candidates including the genes, interrupted by the transposon insertion, and their protein function, the orientation and the genome position of the miniTn10 transposon insertions with respect to the orientation of the interrupted gene in *E. coli*-K12 MG1655. To determine position and orientation of transposon insertions, the obtained transposon flanking sequences were BLAST[®] searched against the *E. coli*-K12 MG1655 genome (NCBI reference taxid:511145) in the NCBI database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and aligned in vector NTI software.

Screening candidate	Gene	Orientation	Position	Protein function
SC #2	<i>waaQ</i>	→←→	<i>waaQ</i> ::mTn10 (+96 - +64)	LPS core heptosyltransferase III
SC #7	<i>waaQ</i>	→←→	<i>waaQ</i> ::mTn10 (+914 - +908)	LPS core heptosyltransferase III
SC #3	<i>waaG</i>	→←→	<i>waaG</i> ::mTn10 (+173 - +164)	LPS glucosyltransferase I
SC #4	<i>waaG</i>	→→→	<i>waaG</i> ::mTn10 (+164 - +173)	LPS glucosyltransferase I
SC #11	<i>surA</i>	→→→	<i>surA</i> ::mTn10 (+51 - +60)	Periplasmic chaperone involved in the biogenesis of β -barrel OMPs
SC #1	<i>rseA</i>	→←→	<i>rseA</i> ::mTn10 (+1 - -8)	Anti-sigma factor antagonizing σ^E function
SC #10	<i>rseA</i>	→→→	<i>rseA</i> ::mTn10 (+435 - +444)	Anti-sigma factor antagonizing σ^E function
SC #5	<i>hfq</i>	→→→	<i>hfq</i> ::mTn10 (+51 - +60)	RNA-chaperone facilitating sRNA-mRNA interactions
SC #9	<i>hfq</i>	→→→	<i>hfq</i> ::mTn10 (+86 - +95)	RNA-chaperone facilitating sRNA-mRNA interactions
SC #6	<i>hfq</i>	→←→	<i>hfq</i> ::mTn10 (+15 - +14)	RNA-chaperone facilitating sRNA-mRNA interactions
SC #8	<i>hfq</i>	→←→	<i>hfq</i> ::mTn10 (+14 - +5)	RNA-chaperone facilitating sRNA-mRNA interactions

To identify gene disruptions that activate the *lptAp1* promoter, the expression levels of the *lptA*(-152 to +78)'-lacZ reporter fusion, comprising the *lptAp1* promoter, were quantitatively monitored in the exponential and the stationary growth phase of the transposon mutants. The genome positions of the miniTn10 transposon insertions were mapped by semi-random two-

step PCR for 11 screening candidates, which significantly increased expression of the *lptA'*-*lacZ* reporter fusion compared to the *wild-type* strain. The exact position and orientation of all identified transposon insertions are shown in Table 34. The affected genes can be classified into 4 classes; proteins involved in the assembly of the core region of the LPS (WaaQ, WaaG), periplasmic chaperons (SurA), proteins involved in the σ^E ESR (RseA) and RNA binding proteins (Hfq) (Table 34). The results revealed different mutants with the transposon insertions in the same gene. Two insertions were mapped in *waaQ*, *waaG* and *rseA*, while only one insertion site was identified in *surA*. The gene that was most often hit by transposon insertion is *hfq*, given that 4 out of 11 insertions were mapped in different genome positions in the coding region of the *hfq* coding sequence. The screening candidates with transposon insertions in *hfq* exhibited the highest induction of *lptA* expression (Figure 41), implying the possibility that *lptA* may be regulated on the post-transcriptional level. The RNA-binding chaperone Hfq is an important global regulator of gene expression in *E. coli*, stimulating RNA-RNA pairing and consequently affecting many cellular processes (Moller *et al.*, 2002). Furthermore, Hfq regulates the activity of σ^E through OMP mRNA levels (Guisbert *et al.*, 2007). Two insertions each were mapped in the coding region of genes *waaQ* and *waaG*, encoding for the proteins that are involved in the assembly of the oligosaccharide region of the LPS, and one in the coding region of gene *surA*, encoding for the periplasmic chaperone that is involved in the biogenesis of the β -barrel OMPs. Transposon insertions in the genes which are involved in LPS biogenesis and composition, *waa* and *surA* respectively, imply that *lptAp1* promoter is activated upon LPS or outer membrane stress. Two insertions were mapped in the coding region of gene *rseA*, encoding for the anti-sigma factor that sequesters σ^E at the inner membrane and consequently represses its transcriptional activity (Walsh *et al.*, 2003).

6.4.3. Confirmation of the transposon screening candidates by measuring *lptA'*-*lacZ* in strains with corresponding clean deletions

In order to confirm the role of the genes interrupted by the transposon insertions in the activation of the *lptAp1* promoter, clean deletion mutants were established in the *wild-type* strain carrying the chromosomal *lptA'*-*lacZ* reporter gene fusion. The general transduction by phage *T4-GT7* (Wilson *et al.*, 1979) was carried out to introduce the desired alleles, tagged with the kanamycin resistance cassette, from the Keio collection donor strains (Baba *et al.*, 2006) into the *wild-type* strain Z1303, carrying the chromosomal *lptA*(-152 to +78)-*lacZ* reporter fusion (as described in 5.2.2.1.). The Keio collection comprises in-frame, single-gene deletion mutants of all non-essential genes in *E. coli*-K12 *MG1655* (Baba *et al.*, 2006). The constructed strains were subsequently cured from the acquired kanamycin resistance cassettes using the FLP-FRT recombination system (Datsenko and Wanner, 2000) to

establish the corresponding clean gene deletions (as described in 5.2.2.2.). Clean deletion mutants of the *hfq*, *rseA*, *waaQ* and *waaG* genes were confirmed by colony PCR analysis. Subsequently, the *lptA* expression levels of those mutants were quantitatively monitored by the β -galactosidase activity assays (as described in 5.2.7.1.). For this purpose, the mutants were grown as previously described (in 6.4.2.).

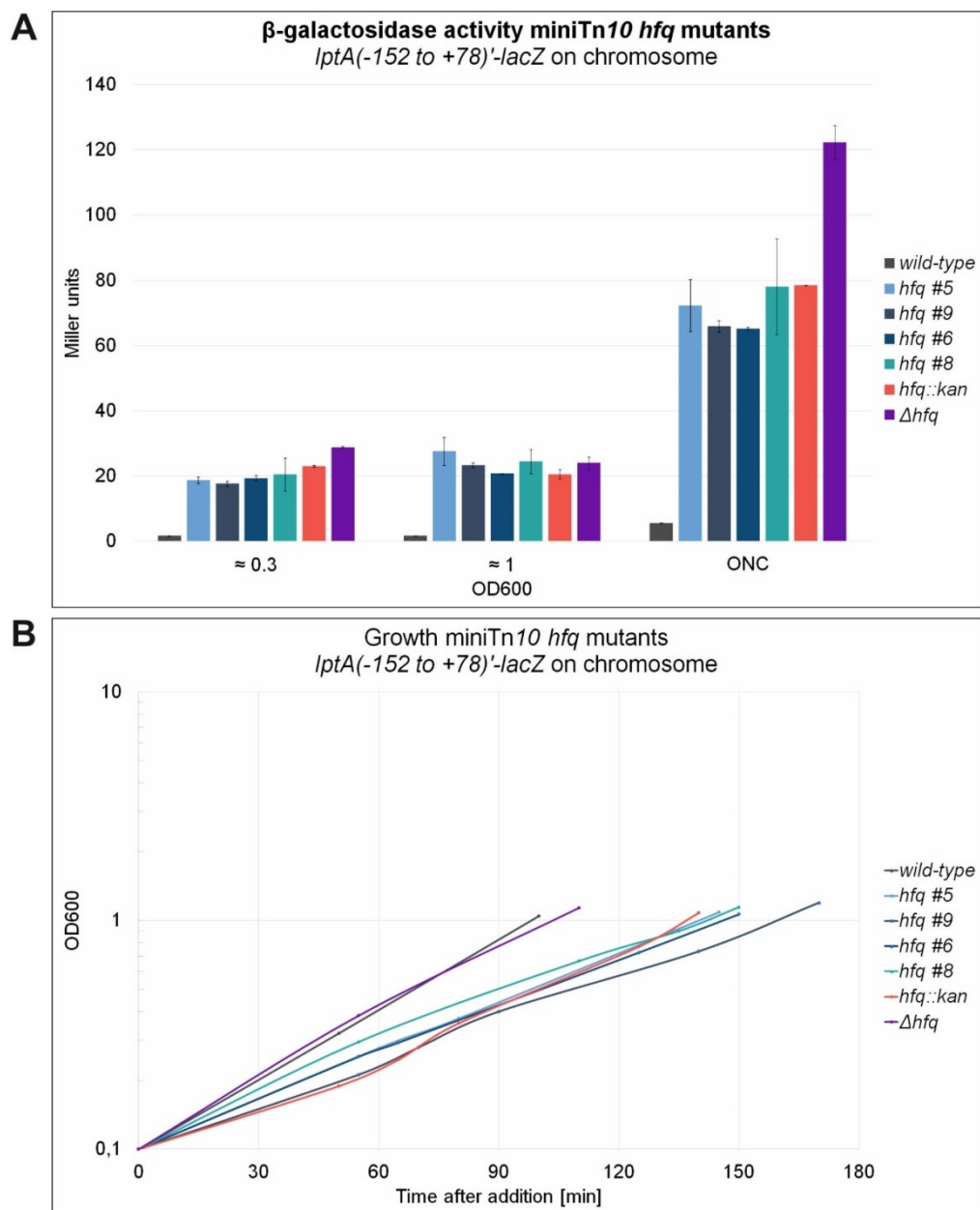


Figure 42. Deletion of *hfq* increases *lptA* expression. **A.** β -galactosidase activity assay addressing the expression of the chromosomal *lptA*(-152 to +78)-*lacZ* reporter fusion in *E. coli* wild-type (Z1303), *hfq* transposon insertion mutants (screening candidates 5, 9, 6 and 8), *hfq*::*kan* (Z1307) and Δ *hfq* (Z1350). Cultures were grown in LB medium, supplemented with appropriate antibiotics. Samples for β -galactosidase activity assay were taken from the overnight cultures (OD_{600} of 2-5) and at an OD_{600} of ~ 0.3 and ~ 1 . Miller unit values are presented as mean $\pm$ SD that derived from at least three independent measurements ($n = 3 - 5$). **B.** The growth of the cultures addressing the expression of the chromosomal *lptA*-*lacZ* reporter fusion in *E. coli*. OD_{600} values are presented as mean ($n = 3-5$).

The deletion of *hfq* drastically increased the activity of the *lptA'-lacZ* reporter fusion in both the exponential and the stationary growth phase as compared to the *wild-type* (Figure 42A, gray, pink and purple columns), although the maximum induction of the *lptA* expression was achieved in the stationary growth phase. The screening candidates with transposon insertions in *hfq* exhibited a similar fold-induction of the fusion in both growth phases (Figure 42A, blue columns). Together, those data confirm that the transposon disruptions of the gene *hfq* phenocopy the Δhfq deletion and indeed increase *lptA* expression. However, based on these results, it cannot be concluded whether this regulation is executed at the transcriptional or post-transcriptional level.

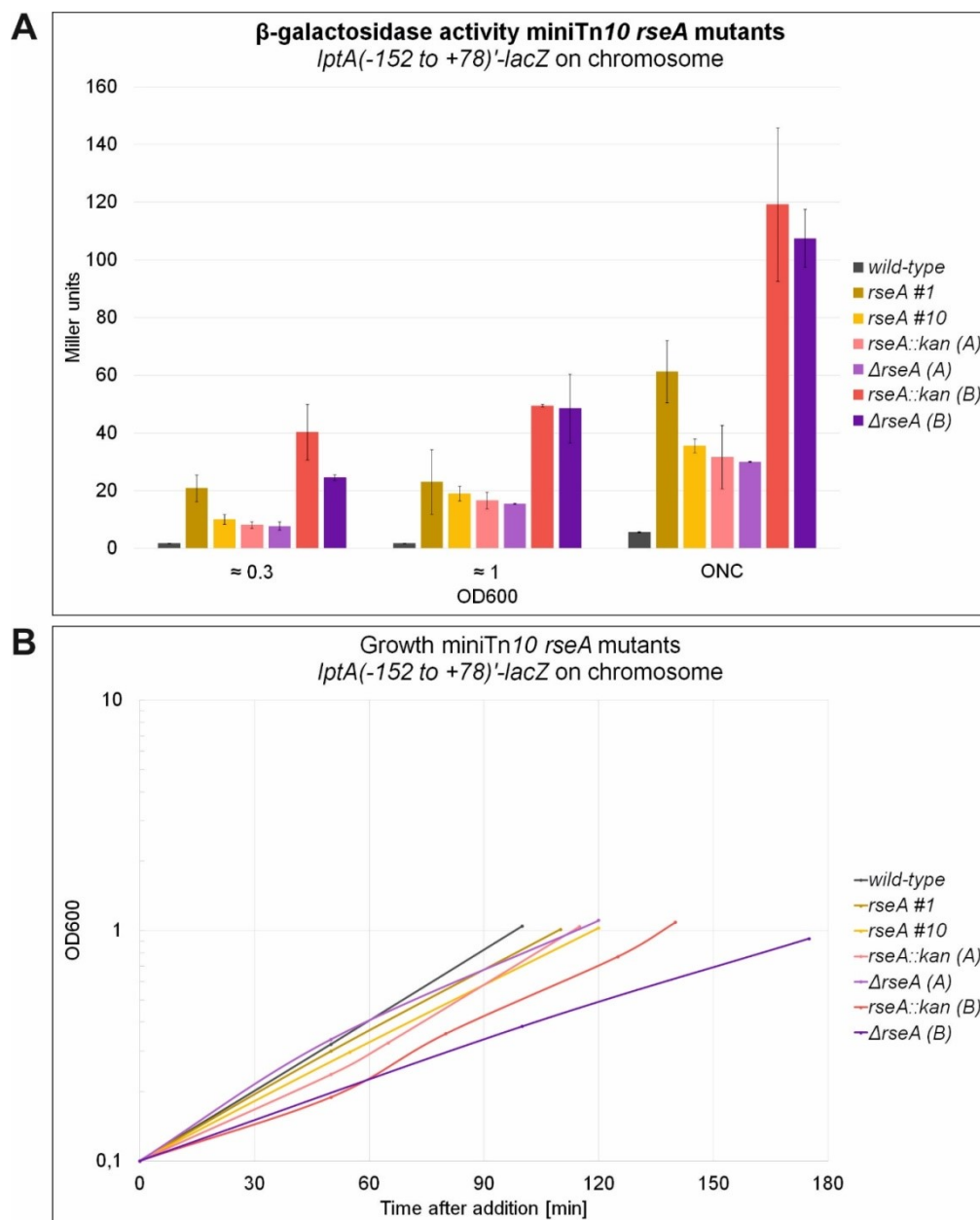


Figure 43. Deletion of *rseA* increases *lptA* expression. **A.** β -galactosidase activity assay addressing the expression of the chromosomal *lptA*(-152 to +78)'-lacZ reporter fusion in *E. coli* *wild-type* (Z1303), *rseA* transposon insertion mutants (screening candidates 1 and 10), *rseA::kan* (Z1324) and $\Delta rseA$ (Z1351).

Cultures were grown in LB medium, supplemented with appropriate antibiotics. Samples for β -galactosidase activity assay were taken from the overnight cultures (OD_{600} of 2-5) and at an OD_{600} of ~ 0.3 and ~ 1 . Miller unit values are presented as mean $\pm$ SD that derived from at least three independent measurements ($n = 3 - 5$). **B.** The growth of the cultures addressing the expression of the chromosomal *lptA'*-*lacZ* reporter fusion in *E. coli*. OD_{600} values are presented as mean ($n = 3-5$).

Similarly, the deletion of *rseA* drastically increased the activity of the *lptA'*-*lacZ* reporter gene fusion in both the exponential and the stationary growth phase as compared to the *wild-type* (Figure 43A, gray, pink and purple columns). The maximum induction of the reporter fusion was achieved in the stationary growth phase as well. Two clones of the *rseA::kan* and $\Delta rseA$ mutants, respectively, were tested since one of them exhibited lower *lptA* expression and less growth retardation. Possibly, this mutant received a suppressor mutation somewhere else in the chromosome that was causing the different phenotype and is therefore responsible for the lower activity of the *lptA'*-*lacZ* reporter fusion. β -Galactosidase activities of the two screening candidates with transposon insertions in *rseA* differ in the stationary growth phase but nevertheless, both transposon mutants exhibited a similar fold-induction of the *lptA* expression in both growth phases (Figure 43A, blue columns). Together, those data confirm that the transposon disruptions of the gene *rseA* phenocopy the *rseA* deletion and indeed increase *lptA* expression and activate the *lptAp1* promoter.

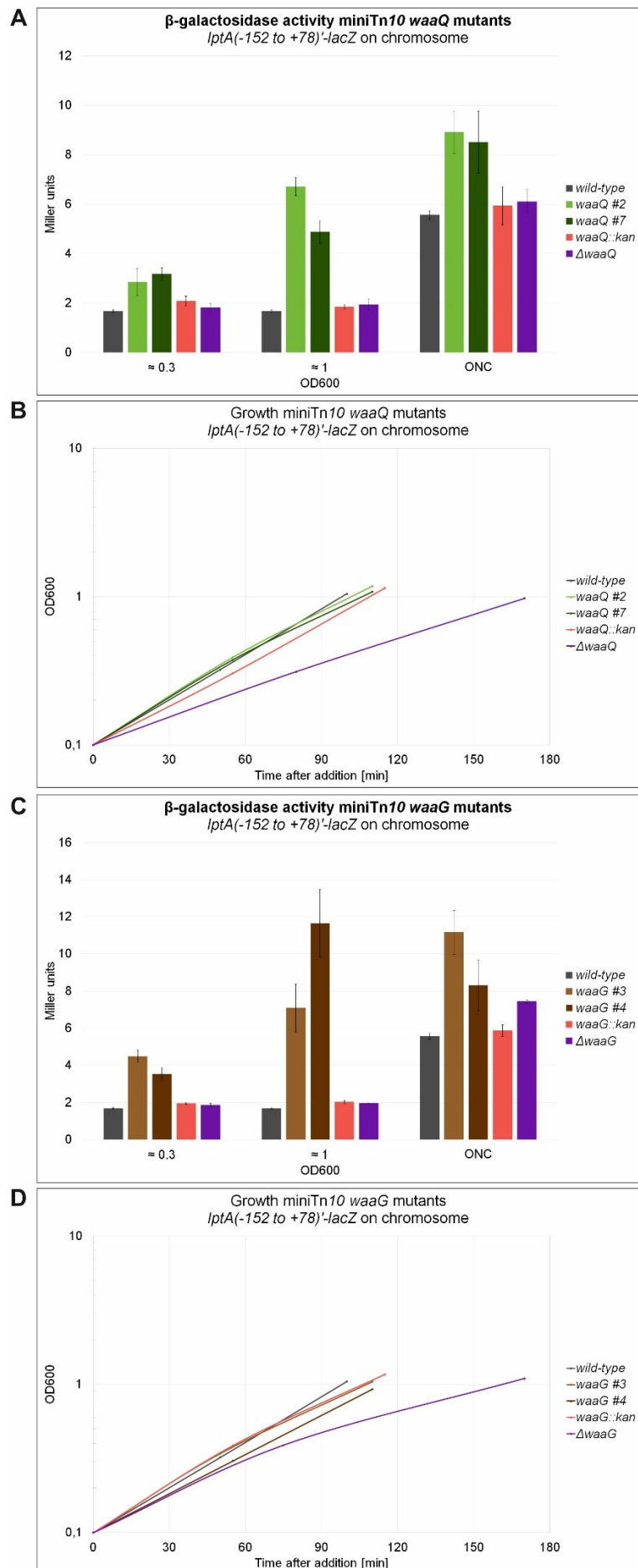


Figure 44. Deletion of *waaQ* or *waaG* does not increase *lptA* expression. **A.** β -galactosidase activity assay addressing the expression of the chromosomal *lptA*(-152 to +78)'-lacZ reporter fusion in *E. coli* wild-type (Z1303), *waaQ* (screening candidates 2 and 7) and *waaG* (screening candidates 3 and 4) transposon insertion mutants, *waaQ::kan* (Z1326), Δ *waaQ* (Z1353), *waaG::kan* (Z1325) and Δ *waaG* (Z1352). Cultures were grown in LB medium, supplemented with appropriate antibiotics. Samples for β -galactosidase activity assay were taken from the overnight cultures (OD₆₀₀ of 2-5) and at an OD₆₀₀ of $\sim$ 0.3 and $\sim$ 1. Miller unit values are presented as mean $\pm$ SD that derived from at least three independent measurements (n = 3 - 5). **B.** The growth of the cultures addressing the expression of the chromosomal *lptA*'-lacZ reporter fusion in *E. coli*. OD₆₀₀ values are presented as mean (n = 3-5).

The deletion of either *waaQ* or *waaG* did not increase the activity of the *lptA*'-lacZ reporter fusion as compared to the *wild-type* (Figure 44A, gray, pink and purple columns). This is in sharp contrast to the original clones isolated in the transposon mutagenesis screen, as the screening candidates clearly show higher expression levels of the *lptA*'-lacZ reporter gene fusion. However, the screening candidates with transposon insertions in *waaQ* or *waaG* exhibited much lower increase in the activity of the reporter fusion as compared to the screening candidates with transposon insertions in *hfq* or *rseA* (Figure 41A, 44A and 44C). Furthermore, the maximum induction of the *lptA* expression of the *waaQ* or *waaG* transposon mutants was achieved in the exponential growth phase at an OD₆₀₀ of 1. The genes *waaQ* and *waaG* are encoded in a large operon, comprising of genes that are involved in LPS core-oligosaccharide assembly. Consequently, the observed increase in *lptA* expression of *waaQ* or *waaG* transposon mutants could be due to polar effect on the downstream genes of the *waaQGPSBOJZU* operon, resulting in an altered expression of the whole operon. Together, those data suggest that deletion of the genes *waaQ* or *waaG* alone does not result in an increased *lptA* expression, but the transposon insertions might cause indirect effects affecting expression of the downstream operon, which in turn alters *lptA* expression.

6.4.4. Validation of the effects caused by *surA* mutations by complementation

The last screening candidate with a transposon insertion in *surA* exhibited a similar increase in the activity of the *lptA*'-lacZ reporter fusion as compared to the screening candidates with transposon insertions in *waaQ* or *waaG*. The *surA::kan* single-gene deletion mutant from the Keio collection could not be cultivated despite several attempts at different temperatures or in medium without antibiotics. Therefore, a complementation experiment was carried out to confirm the involvement of *surA* in the activation of the *lptAp1* promoter. In order to generate an expression plasmid with *surA* under control of an arabinose-inducible promoter, the *surA* gene with its authentic SD was amplified by PCR and cloned via *XbaI*/*HindIII* into the expression plasmid pAraX (Table 4). This is a plasmid with low copy number and a kanamycin resistance cassette which is compatible with the transposon insertions, that render the cells resistant to chloramphenicol. Once constructed, the *wild-type* strain carrying the chromosomal *lptA*(-152 to +78)'-lacZ reporter gene fusion and the screening candidate

with a transposon insertion in *surA* were transformed with the *surA* expression plasmid (pFS38) or the corresponding VC (pAraX), respectively. Pre-cultures were inoculated from the overnight cultures to an OD₆₀₀ of 0.1 and grown at 37°C in 25 mL LB until reaching an OD₆₀₀ of ~ 0.3. Next, each pre-culture was split into two 10 mL cultures, one of which was supplemented with 0,2% arabinose to induce expression of *surA* and the other with dH₂O as mock control. Subsequently, the *lptA* expression levels of the transformants were quantitatively monitored by the β-galactosidase activity assays in 30 min intervals following the addition of arabinose (as described in 5.2.7.1.). For this purpose, the mutants were grown as previously described (in 6.4.2.).

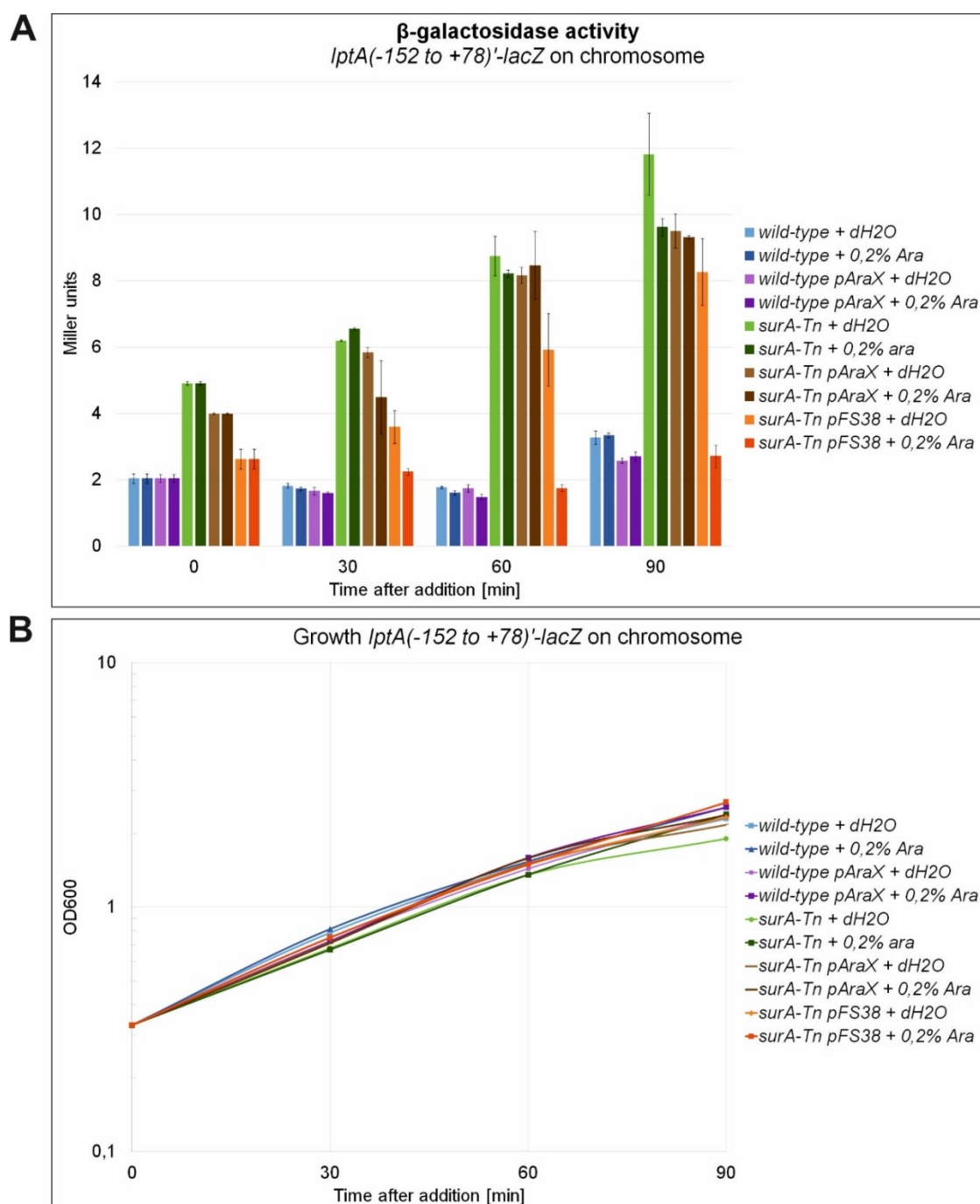


Figure 45. Transposon insertion in *surA* increases *lptA* expression. **A.** β -galactosidase activity assay addressing the expression of the chromosomal *lptA*(-152 to +78)'-lacZ reporter fusion in *E. coli* *wild-type* (Z1303) and *surA* transposon insertion mutant (screening candidate 11) by complementation of *surA* gene. Strain Z1303 (S4197 [*attB*::*lptA*(-152 to +78)'-lacZ]) and the *surA* transposon mutant were transformed with the *surA* expression plasmid (pFS38) or the corresponding VC (pAraX). Transformants were grown in LB supplemented with chloramphenicol +/- 0,2% arabinose. Empty plasmid pAraX provided the VC and the untransformed *wild-type* strain was analyzed in parallel for comparison. Miller unit values are presented as mean $\pm$ SD that derived from at least three independent measurements (n = 3-5). **B.** The growth of the cultures addressing the expression of the chromosomal *lptA*'-lacZ reporter fusion in *E. coli*. OD₆₀₀ values are presented as mean (n = 3-5).

Complementation of the *surA* transposon mutant with the plasmid encoding *surA* decreased the activity of the *lptA*'-lacZ reporter fusion and restored it to the activity of the *wild-type* (Figure 45A, blue, orange and red columns). Presence of the empty plasmid (VC) or the inducer arabinose did not have an effect on the activity of the *lptA* expression (purple, green and brown columns). Together, those data confirm that the transposon disruption of the gene *surA* indeed increases *lptA* expression and activates the *lptAp1* promoter.

6.5. Screening for genes that activate the *sixA'*-*lacZ* reporter gene fusion

The *E. coli rpoN* operon consists of *rpoN*, *hpf*, *ptsN*, *rapZ* and *npr* (Figure 7). All gene products of the *rpoN* operon (except for *rpoN* itself) exert regulatory functions at the post-transcriptional (RapZ) and post-translational level (Hpf, PTS^{Ntr}). Genes *ptsN* and *npr* encode proteins working in the nitrogen-related phosphotransferase system (PTS^{Ntr}), a paralogous phosphotransferase system that is phosphorylated on the expense of phosphoenolpyruvate (PEP) (Figure 9) (Pflüger-Grau and Görke, 2010). The nitrogen-related phosphotransferase system regulates various activities through protein-protein interaction which are predominantly exerted by the non-phosphorylated form of PtsN (EIIA^{Ntr}). A well described role of the PTS^{Ntr} is the regulation of potassium homeostasis. In *E. coli*, PtsN stimulates activation of the high affinity potassium KdpFABC transport system when external potassium levels are low to maintain the cellular homeostasis (Lüttmann *et al.*, 2009; Lüttmann *et al.*, 2012; Lüttmann *et al.*, 2015; Mörk-Mörkenstein *et al.*, 2017).

NPr can be dephosphorylated by phosphohistidine phosphatase SixA (Schulte and Goulian, 2018; Schulte *et al.*, 2020) that was suggested to be part of the σ^E regulon (Rezuchova *et al.*, 2003). Two overlapping promoters, the σ^{70} promoter and the σ^{24} promoter, control expression of the *sixA* gene (Rezuchova *et al.*, 2003; Rhodius *et al.*, 2006; Wade *et al.*, 2006). Data from our lab show that σ^E triggers activity of the PTS^{Ntr} by a dual mechanism: a σ^E -dependent upregulation of PtsN and NPr (through a burst in *rpoN* operon expression) and its further dephosphorylation through employment of SixA which results in accumulation of non-phosphorylated PtsN and consequent expression of *kdpFABC* (Sikora and Görke, unpublished). Beyond that, no physiological conditions or genetic alterations leading to activation of SixA, and thereby up-regulation of the KdpFABC transporter, were known so far. In order to search for mutations and genes that lead to upregulation of *sixA* expression, a random transposon mutagenesis screen was carried out in a strain containing chromosomal *sixA'*-*lacZ* reporter gene fusion. For this purpose, the miniTn10 delivery system was used as before (described in 5.2.3.). To identify gene disruptions that lead to a σ^E -dependent upregulation of *sixA* expression, the expression levels of *sixA*(-300 to +51)-*lacZ* reporter fusion, comprising the σ^{70} *sixAp1* and the σ^E *sixAp2* promoter, were quantitatively monitored.

6.5.1. Construction of a *sixA'*-*lacZ* reporter gene fusion suitable for screening

To screen for mutations that lead to a σ^E -dependent upregulation of *sixA* expression, the general transduction by phage T4-GT7 (Wilson *et al.*, 1979) was carried out to introduce the

spectinomycin resistance tagged *sixA*(-300 to +51)'-lacZ fusion from the strain Z1272, carrying a FLAG-tagged *ptsN* allele, into the *wild-type* strain S4197 (as described in 5.2.2.1.).

In order to check whether the *sixA*(-300 to +51)'-lacZ reporter fusion is suitable for screening, a spotting assay was carried out to evaluate the difference at the level of phenotypes on X-Gal indicator plates. For the purpose of this experiment, the constructed Z1315 strain was transformed with the *rpoE* expression plasmid (pFS28) or the corresponding VC (pBAD33), respectively. Bacteria were grown and spotted onto LB plates supplemented with chloramphenicol and X-Gal either containing or lacking arabinose as previously described (in 6.2.1.).

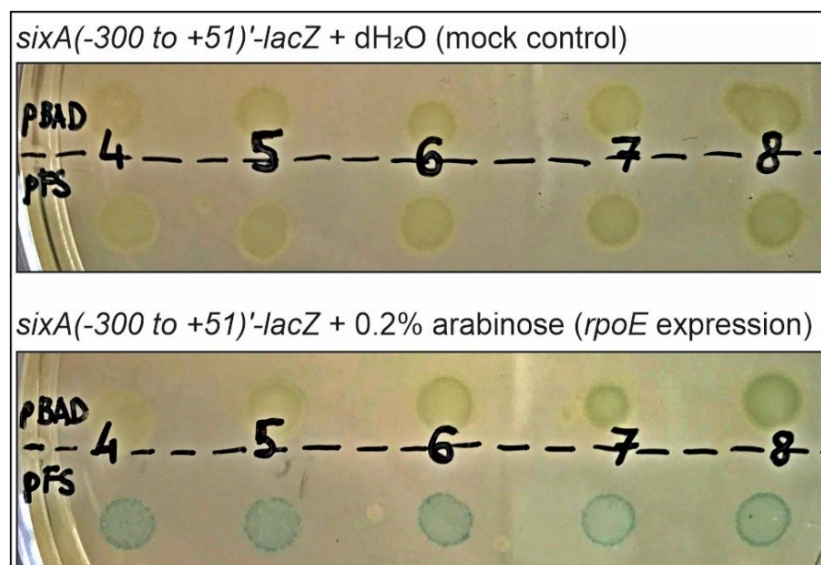


Figure 46. Impact of ectopic *rpoE* expression on the chromosomal *sixA*'-lacZ reporter fusion in *E. coli*. Strain Z1315 (*S4197* [*attB*:: *sixA*(-300 to +51)'-lacZ]) was transformed with the *rpoE* expression plasmid (pFS28) or the corresponding VC (pBAD33). Overnight cultures of transformants were adjusted to 1 OD₆₀₀ = 10⁹ cells/mL and 10-fold serial dilutions were prepared in 1 mL LB medium, ranging from 10⁸ to 10⁴ cells/mL (spotted from right to left). 2 μ L of each serial dilution was spotted onto LB plates supplemented with chloramphenicol and X-Gal either containing or lacking 0,2% arabinose to induce expression of *rpoE*. The plates were incubated overnight at 37°C.

For the screening procedure it is most important to clearly recognize differences at the level of phenotypes which allows specific selection of colonies that are exhibiting increased expression of the *lacZ* reporter fusion amongst hundreds of others on the same plate. The ectopic expression of *rpoE* from an arabinose-inducible promoter on a plasmid, mimicking σ^E activation, led to the σ^E -dependent upregulation of *sixA* expression which resulted in a blue phenotype of the transformants on an LB-X-Gal plate (Figure 46), rendering the *sixA*(-300 to +51)'-lacZ reporter fusion suitable for phenotypic screening. However, the blue phenotypes on the plate were not as strong as compared to the phenotypes caused by the *lptA*'-lacZ fusion in presence of active σ^E (in 6.4.1.).

Additionally, the *sixA* expression levels were quantitatively monitored by the reporter gene assay in the strain carrying the chromosomal *sixA'*-*lacZ* reporter gene fusion. Cells were grown as previously described (in 6.2.2.) and samples for β -galactosidase activity measurements were taken from the cultures over 2 h in 30 min intervals following the addition of arabinose.

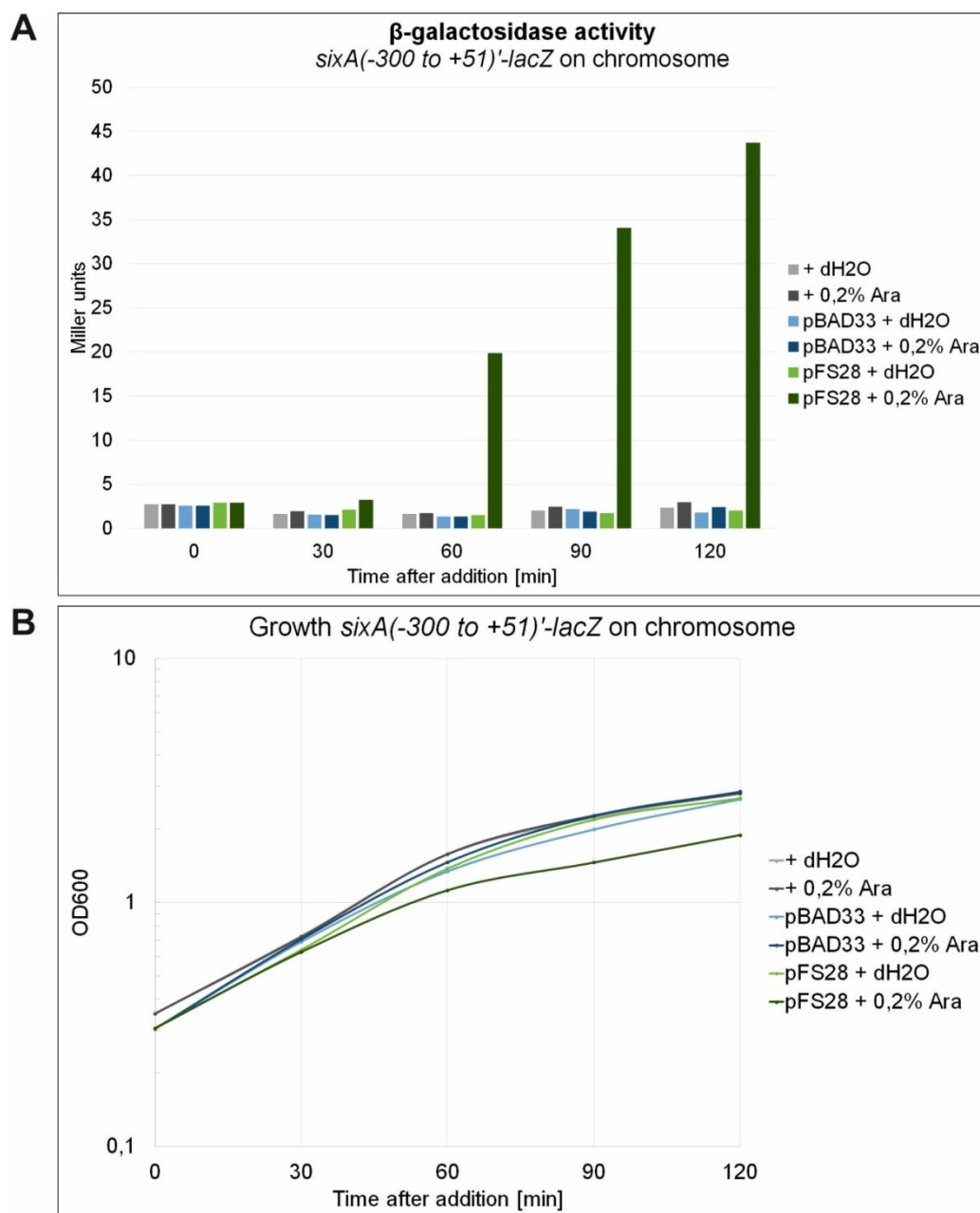


Figure 47. Ectopic *rpoE* expression increases *sixA* expression. **A.** β -galactosidase activity assay addressing the expression of the chromosomal *sixA'*-*lacZ* reporter fusion in *E. coli*. Strain Z1315 (*S4197* [*attB*:: *sixA*(-300 to +51)'-*lacZ*]) was transformed with the *rpoE* expression plasmid (pFS28) or the corresponding VC (pBAD33). Transformants were grown in LB supplemented with chloramphenicol +/- 0,2% arabinose. Empty plasmid pBAD33 provided the VC and the untransformed *wild-type* strain was analyzed in parallel for comparison. Miller unit values derived from one measurement. **B.** The growth of the cultures addressing the expression of the chromosomal *sixA'*-*lacZ* reporter fusion in *E. coli*.

Induction of the plasmid-encoded *rpoE* under the control of an arabinose-inducible promoter in the strain carrying the *sixA'*-*lacZ* reporter fusion led to an increase in activity of the reporter fusion (Figure 47A, dark green columns), demonstrating the σ^E -dependent upregulation of *sixA* expression upon ectopic *rpoE* expression. A maximum induction of the fusion was reached 120 min after the addition of arabinose, resulting in more than 20-fold induction of the β -galactosidase activity. The activities of the controls used in the experiment, the untransformed *wild-type* strain or the VC control grown in the presence or absence of arabinose and the uninduced *rpoE* transformant, are comparable (Figure 47A).

6.5.2. Finding signals affecting activity of the *sixA'*-*lacZ* reporter gene fusion by transposon mutagenesis screen using a miniTn10 delivery system

Subsequently, the strain carrying the chromosomal *sixA*(-300 to +51)-*lacZ* reporter gene fusion was subjected to a random transposon mutagenesis screen (as described in 5.2.3.) using the mTn10 delivery plasmid pKESK18 (Madhusudan *et al.*, 2005). Three screening rounds were performed in total and for each round 100-150 plates were inoculated with 100 μ L of the diluted overnight culture of the strain carrying the chromosomal *iptA'*-*lacZ* that was initially transformed with the plasmid pKESK18. In each round of screening, the selection procedure on average yielded 3 appropriate candidates to investigate further. Subsequently, the *sixA* expression levels of those mutants were quantitatively monitored by the β -galactosidase activity assays (as described in 5.2.7.1.). For this purpose, the mutants were grown as previously described (in 6.4.2.).

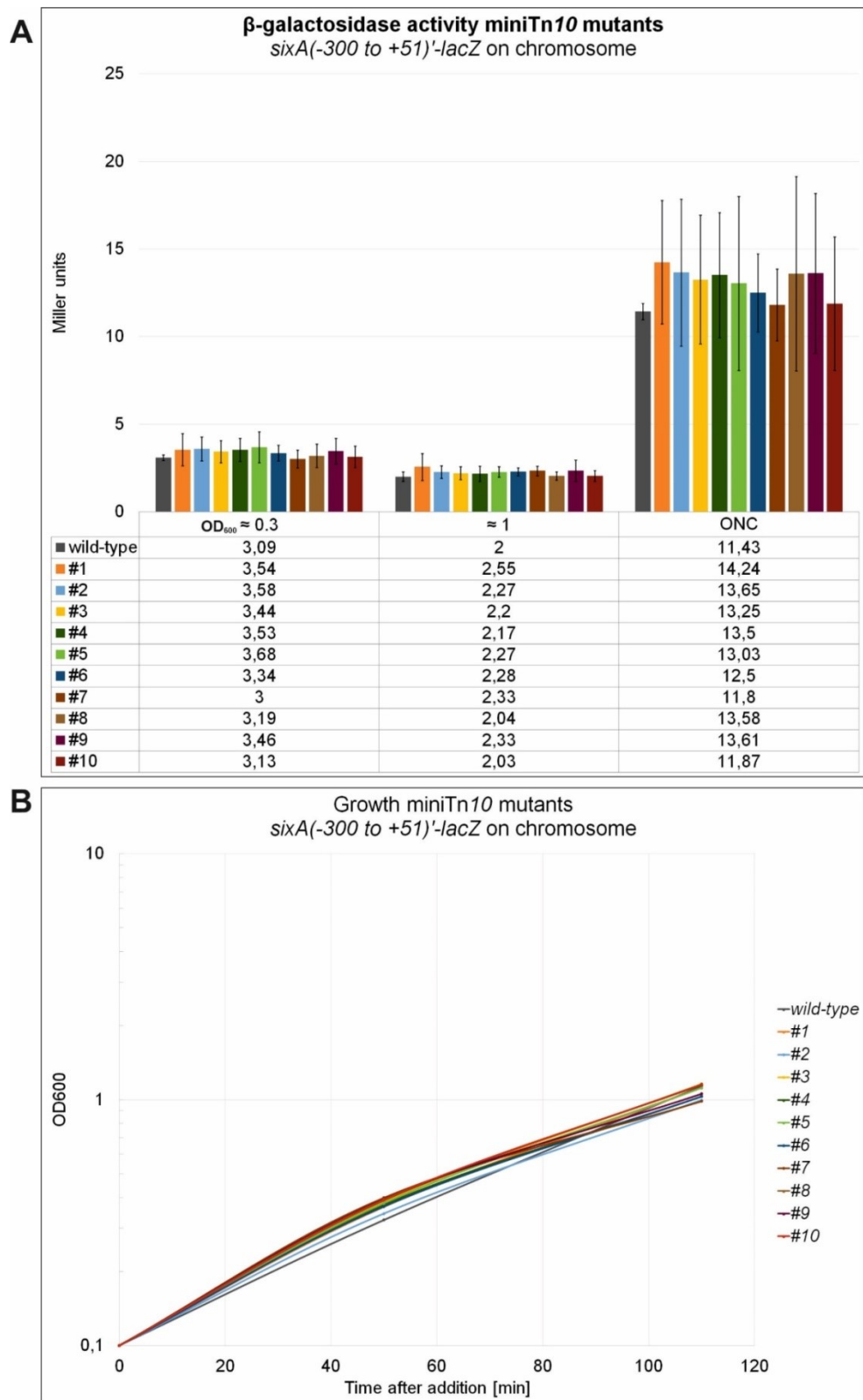


Figure 48. Analysis of *sixA* expression in transposon mutants displaying blue phenotypes on LB-agar with X-Gal. A. β -galactosidase activity assay addressing the expression of the chromosomal *sixA*'-lacZ reporter fusion in strain Z1315 (S4197 [*attB*:: *sixA*(-300 to +51)'-lacZ]) and screening candidates obtained by transposon mutagenesis using pKESK18. Transposon insertion mutants were selected on chloramphenicol plates and the screening candidates exhibiting a lactose⁺ phenotype on LB-X-Gal plates were further analyzed by the quantitative β -galactosidase activity assay. Cultures of the screening candidates were grown in LB supplemented with chloramphenicol. Samples for β -galactosidase activity

assay were taken from the overnight cultures (OD₆₀₀ of 2-5) and at an OD₆₀₀ of ~ 0.3 and ~ 1. Screening candidates are labeled with the number that was assigned to them during the transposon mutagenesis screening procedure. Miller unit values are presented as mean $\pm$ SD that derived from three independent measurements (n = 3). **B.** The growth of the cultures addressing the expression of the chromosomal *sixA'*-*lacZ* reporter fusion in *E. coli*. OD₆₀₀ values are presented as mean (n = 3).

To identify gene disruptions that lead to the σ^E -dependent upregulation of *sixA* expression, the expression levels of the *sixA*(-300 to +51)'-*lacZ* reporter fusion were quantitatively monitored in the exponential and the stationary growth phase of the transposon mutants. Unfortunately, the screening procedure did not yield any transposon mutant that exhibited elevated *sixA* expression levels compared to the *wild-type* after three independent measurements (Figure 48). A maximum induction of the fusion was reached in the overnight cultures but the *sixA* expression levels of the transposon mutants were not significantly higher. The initial experiment with *sixA'*-*lacZ* reporter fusion was performed by transformation of the strain with the *rpoE* expression plasmid which resulted in a 20-fold induction of *sixA'*-*lacZ*. The ectopic expression of *rpoE* is an artificial setup which leads to an extreme, non-physiological, σ^E ESR, and, still, the fold-change measured for the *sixA'*-*lacZ* fusion is just 20-fold (as compared to the much stronger upregulation of *lptA'*-*lacZ*; Supplementary Figure S1A). This extensive fold-change is probably not caused by transposon insertions or disruption of single genes and, thus, the difference in *sixA'*-*lacZ* expression is not increased enough to clearly identify these events on LB-agar with X-Gal. These results led to the conclusion that the *sixA*(-300 to +51)'-*lacZ* reporter fusion was either not suitable for screening or that the *Tn10* insertions activating *sixA* expression are lethal or cause a strong growth retardation.

7. Discussion

7.1. Investigation of a possible role of GlmY/GlmZ and RapZ for the expression of cold-shock related transcripts

In order to explore other RNAs that might be regulated by RapZ, an RNA-seq analysis was recently carried out in the CSH50 (R1279) strain lineage. The bioinformatic analysis revealed upregulation of a couple of cold-shock related transcripts (*cspI*, *cspH*, *cspB*, *cspF*, *cspG*, *ydfK*, *ynaE*) specifically in a $\Delta glmY \Delta glmZ$ double mutant, but not in a $\Delta rapZ$ single mutant and also not in a $\Delta rapZ \Delta glmY \Delta glmZ$ triple mutant (Durica-Mitic *et al.*, 2020). The accumulation of *cspB* and *cspH* mRNAs in the $\Delta glmY \Delta glmZ$ double mutant was additionally confirmed by Northern blotting, validating this result (Durica-Mitic *et al.*, 2020). This suggested that RapZ may only affect the cold shock transcripts when its primary binding partners, GlmZ and GlmY, are absent or present at low levels that are insufficient to sequester RapZ.

Most of the corresponding cold-shock proteins (Csp) are transiently synthesized at low temperature and they typically act as nucleic acid chaperones that bind to mRNA in order to prevent formation of deleterious secondary structures and facilitate translation at low temperatures (Keto-Timonen *et al.*, 2016). CspB and CspH are both members of the large CspA family of cold-shock proteins. In *E. coli*, the CspA family consists of nine *csp* genes which show high sequence similarity to CspA (*cspA*, *cspB*, *cspC*, *cspD*, *cspE*, *cspF*, *cspG*, *cspH*, *cspI*) (Yamanaka *et al.*, 1998). Transcription of *cspB* is induced upon temperature downshift below 20°C, with maximal expression at 15°C (Etchegaray *et al.*, 1996). The expression of *cspB* is highest in the mid-log phase when cells grow at 37°C in the defined rich medium (Czapski and Trun, 2014). In contrast, the *cspH* transcript is reportedly not induced by cold and it is only expressed at very low level in mid-log phase (Czapski and Trun, 2014). The induction pattern of *cspH* as well as the function of the protein is still unknown (Czapski and Trun, 2014). The cold-shock transcripts are regulated by a highly conserved 11 nt long “cold box” in the 5' UTR (Fang *et al.*, 1998), but a common regulator acting *in trans* is yet to be determined. Those observations (Durica-Mitic *et al.*, 2020) suggested that RapZ may have an additional function in regulation of cold-shock transcripts and may somehow affect those transcripts by interfering with the “cold box”. However, it would only be able to do so in the mutant cells lacking GlmY and GlmZ because it is not bound to its primary binding partners then and can bind other RNAs. Considering that, it is also possible that high levels of RapZ, exceeding those of GlmY and GlmZ, allow RapZ to bind other RNAs, including the cold shock transcripts. The latter situation might occur upon activation of the σ^E ESR, which also leads to higher RapZ levels (Figure 24).

One aim of this thesis was to investigate this possible role of RapZ for the expression of cold-shock related transcripts in the MG1655 (S4197) strain lineage through Northern blot analysis of *cspB* and *cspH*. Confirming the observations in both *wild-type* strain derivatives would ensure that the obtained results are valid for a wider range of *E. coli*-K12 strains. In contrast to the results obtained in the work of Durica-Mitic et al. (2020), where *cspB* and *cspH* transcripts could just be observed in the $\Delta glmY \Delta glmZ$ double mutant from the CSH50 (R1279) strain lineage, the transcripts of *cspB* and *cspH* were detectable in all strains of the MG1655 (S4197) strain lineage, the *wild-type* strain S4197, the $\Delta rapZ$ single mutant, the $\Delta glmY \Delta glmZ$ double mutant and the $\Delta rapZ \Delta glmY \Delta glmZ$ triple mutant (Figure 20A, B, C, D). As the result of the first replicate (Figure 20 A, B) deviated very much from previous observations, a replicate experiment was performed in which the result of the first replicate could be reproduced only for the *cspB* transcript but not for *cspH* (Figure 20C, D). High fluctuations in the levels of the *cspB* and *cspH* transcripts from the first and the second replicate experiment (Figure 20E, F) raised the question of the reproducibility of the sampling procedure and how strongly fluctuations in temperature might impact the expression of cold-shock related transcripts. Therefore, the influence of the temperature during handling and centrifugation of the samples was tested in the MG1655 (S4197) strain lineage via Northern blot analysis of *cspB* and *cspH*. This experiment revealed that transcripts were more abundant when cells are incubated on ice prior to RNA isolation (Figure 21A, B). The obtained results indicate that the sampling temperature strongly influences the levels of *cspH* transcript, which are upregulated in all the samples that were incubated on ice prior to RNA isolation (Figure 21B), but has only minimal impact on the levels of *cspB* transcript (Figure 21A). Hence, it can be concluded that the previous results from the CSH50 (R1279) strain lineage (Durica-Mitic et al., 2020) are not reproducible for MG1655 (S4197) derivatives.

However, this discrepancy might not be due to the genetic differences between the two strain lineages, but is perhaps caused by slightly unequal handling of the samples. In this experiment, the aim was to take samples of the different mutants in the exponential growth phase (approximately at an OD₆₀₀ of 0,3). Accordingly, the samples were taken at different times, meaning that the cells were placed on ice to prevent further growth. This can result in unequal times for which the samples were kept at low temperature before centrifugation (or other steps in the sampling procedure) and exactly these conditions may induce expression of the cold shock related transcripts. Therefore, these putative artefacts can hardly be avoided in *in vivo* experiments, such as Northern blot analysis or RNA sequencing, and similarly published results have to be analyzed carefully.

Taken together, the Northern blot analysis of *cspB* and *cspH* transcripts in the independent MG1655 (S4197) strain lineage showed conflicting results and would therefore require

further investigation. In order to clarify whether the expression of cold-shock related transcripts might be influenced by RapZ, other methods could be employed in future studies, whereas *in vitro* approaches should be preferred. An electrophoretic mobility shift assay (EMSA) could be conducted to test whether RapZ directly binds the cold shock transcripts *in vitro*. *In vivo* this could alternatively be tested through a co-purification approach by purification of Strep-tagged RapZ and analysis of the RNA in the eluate under cold-shock conditions.

Furthermore, the recent RNA-seq analysis in the CSH50 (R1279) strain lineage (Durica-Mitic *et al.*, 2020), studying the impact of a *rapZ* deletion on the whole transcriptome, identified additional genes with altered expression in the $\Delta glmY \Delta glmZ$ double mutant. The analysis revealed downregulation of a couple of flagella-related transcripts (*flgB*, *flgD*, *flgF*, *fliF*, *fliL*, *fliA*) in a $\Delta glmY \Delta glmZ$ double mutant as well as in a $\Delta rapZ \Delta glmY \Delta glmZ$ triple mutant (Durica-Mitic *et al.*, 2020), suggesting that GlmY and GlmZ might play a role in motility, supporting the idea of Bak *et al.* (Bak *et al.*, 2015). Another transcript that was upregulated especially in a $\Delta glmY \Delta glmZ$ double mutant is the *nsrR* transcript which is part of the *nsrR-rnr-rlmB-yjfl* operon. Intriguingly, expression of this locus is also induced by cold shock (Cairrão *et al.*, 2003). The gene *nsrR* encodes the nitric oxide sensitive transcriptional repressor NsrR which controls genes involved in protection against reactive nitrogen species (Bodenmiller and Spiro, 2006; Filenko *et al.*, 2007). In addition, NsR regulates σ^E -dependent genes independently of RseA and genes involved in motility (Thompson *et al.*, 2007; Partridge *et al.*, 2009).

7.2. Impact of σ^E on the *rpoN* operon

The sigma factor RpoE (σ^E) represents the major cell ESR system of Gram-negative bacteria, including *Escherichia coli* (Mitchell and Silhavy, 2019). It responds to two major types of stresses, the buildup of misfolded OMPs in the periplasm and to LPS damages (Walsh *et al.*, 2003; Lima *et al.*, 2013). As a response, σ^E regulates extracytoplasmic functions of LPS translocation and some of its modifications (Klein *et al.*, 2016). As the cell envelope consists of more than 1000 different proteins, it is the least understood cellular compartment of Gram-negative bacteria and little is understood about how the σ^E ESR responds and counteracts LPS damages. It is known that different perturbations in LPS biosynthesis (e.g. production of altered forms of LPS) and trafficking problems (e.g. depletion of Lpt machinery components) cause activation of the σ^E ESR (Klein and Raina, 2019) which likely occurs by displacement of RseB through the interaction with the LPS acyl side chains that accumulate in the periplasm (Lima *et al.*, 2013), but little is known beyond. Several

observations suggest that the genes of the *rpoN* operon are connected to the σ^E regulon, but how they contribute to the ESR is unknown.

These observations support the hypothesis that the *rpoN* operon, which is conserved in all *Enterobacteriaceae*, represents an important regulatory layer of the σ^E ESR. As all gene products of the *rpoN* operon (except for *rpoN* itself) exert regulatory functions at the post-transcriptional (RapZ) and post-translational level (Hpf, PTS^{Ntr}), they might help bacteria to counteract envelope stress by interaction with target proteins or small regulatory RNAs. For example, comparative genome analysis, exploring the gene synteny, suggested that RpoN (σ^{54}) is evolutionary connected to LPS and peptidoglycan synthesis (Francke *et al.*, 2011). Additionally, the transcription of genes of the Psp ESR system, which is dedicated to damages of the cytoplasmic membrane, as well as *rpoE* itself can be initiated from σ^{54} -dependent promoters, thus connecting σ^{54} to envelope functions (Flores-Kim and Darwin, 2016; Klein *et al.*, 2016). The protein NPr has been described to inhibit LpxD activity by direct interaction, thus decreasing lipid A biosynthesis (Kim *et al.*, 2011). Additionally, Lee *et al.* (2015) have reported that *E. coli* strains with increased levels of dephosphorylated form of NPr are sensitive to diverse envelope stresses (e.g. osmotic, ethanol and SDS), and these phenotypes are independent of LpxD (Lee *et al.*, 2015). The phosphatase SixA, which is a member of the σ^E regulon (Sikora and Görke, unpublished; Rezuchova *et al.*, 2003), was shown to dephosphorylate NPr (Schulte and Goulian, 2018; Schulte *et al.*, 2020) and in turn leads to dephosphorylation of PtsN (Sikora and Görke, unpublished). Intriguingly, Hayden and Ades (2008) have reported that *ptsN* is a suppressor of σ^E essentiality as the overexpression of *ptsN* lowers multiple ESRs (Rcs, Cpx) and reduces stress in the cell envelope by an unknown mechanism (Hayden and Ades, 2008). Moreover, our lab has extensively studied the multifunctional sRNA-binding protein RapZ and its direct role in modulation of cell envelope precursor synthesis, controlling UDP-GlcNAc levels by post-transcriptionally regulating *glmS* expression in response to the level of glucosamine-6-phosphate (GlcN6P) via sRNAs GlmY and GlmZ (Khan and Görke, 2020). These observations strengthen the hypothesis that the gene products of the *rpoN* operon constitute an important regulatory layer of the σ^E ESR, acting predominantly at the post-transcriptional and post-translational level.

Considering the regulatory functions exerted by NPr, PtsN and RapZ and that the σ^E -dependent *lptAp1* promoter is believed to respond solely to LPS biogenesis defects (Martorana *et al.*, 2011), the *rpoN* operon likely plays a role in the cellular response to LPS perturbations, acting predominantly at the post-transcriptional and post-translational level also through activation of other ESR systems, perhaps mediated by increased σ^{54} levels. Multiple mechanisms exerted by the gene products of the *rpoN* operon might help relieve the

LPS stress. When the cell envelope becomes leaky, the activation of potassium uptake mediated by PtsN and NPr may contribute to the maintenance of cell turgor and the modulation of cell envelope precursor synthesis may be adjusted accordingly to the cell's needs by RapZ and PtsN. Increased Hpf levels may protect ribosomes under stress conditions and increased σ^{54} levels may initiate transcription of genes involved in the ESR and boost *rpoE* expression from the σ^{54} -dependent *rpoE-P2* promoter. Furthermore, additional still unknown targets and mechanisms, regulated by proteins of the *rpoN* operon, may contribute to stress recovery. Clarification of the role of the *rpoN* operon for the σ^E ESR could bring important insights into the functional connections between the two. To address the latter hypothesis, the regulatory elements responding to envelope stress in the *lptAB* and the *rpoN* operon were initially dissected in strains carrying different reporter gene fusions (*lptA'-lacZ* and *lptA→rapZ'-lacZ*) in the current study. Furthermore, the consequences of active σ^E for accumulation of RapZ protein and for the GlmY/GlmZ/*glmS* circuit were studied through the use of ectopic *rpoE* expression from a plasmid. Additionally, as thanatin is one of the few known inhibitors of the LPS transport machinery, its impact on the RapZ/GlmY/GlmZ/*glmS* circuit was addressed as well. Moreover, a global screening approach was employed in order to indentify mutations that cause activation of the *lptAp1* promoter and the σ^E -dependent *sixAp2* promoter.

7.2.1. Dissection of regulatory elements responding to envelope damage in the *lptAB* and *rpoN* operon

The accumulation of all proteins encoded in the *rpoN* operon in response to ectopic expression of *rpoE*, mimicking σ^E activation, was previously confirmed via translational *lacZ* reporter gene fusions and subsequently by Western blot analysis, addressing levels of FLAG-tagged σ^{54} , Hpf, PtsN, RapZ and NPr (Sikora and Görke, unpublished). Intriguingly, Northern Blot analysis indicated that this up-regulation upon ectopic expression of *rpoE* leads to a burst of *rpoN* operon expression at the transcriptional level (Sikora and Görke, unpublished). Further dissection of the responsible regulatory elements through *lacZ* reporter gene fusions revealed a dual regulation of the *rpoN* operon by σ^E . These data are in agreement with the hypothesis, that the *rpoN* operon is a member of the σ^E regulon. The *lptAp1* promoter upstream of *lptA* that is activated by specific stress conditions which impair LPS biogenesis (Martorana *et al.*, 2011) is the first σ^E -responsive element in genetic proximity and likely mediates a ~10-fold increase of the *rpoN* operon expression via read-through transcription (Figure 22; Sikora and Görke, unpublished). Rhodius *et al.* (2006) already observed increased abundance of some transcripts of the *rpoN* operon in a global transcriptome study, but with lower fold-changes (Rhodius *et al.*, 2006). A second σ^E -responsive element within the *rpoN* operon is located in the region comprising *hpf* and the

intergenic region separating *hpf* and *ptsN* and can also be activated by diverse envelope stressors (e.g. 5% ethanol, a combination of SDS and EDTA), mediating a ~2 to 3-fold upregulation of the *ptsN-rapZ-ptsO* gene cluster (Figure 22; Sikora and Görke, unpublished).

7.2.1.1. Activation of the *lptAp1* promoter

The *E. coli rpoN* operon is preceded by *lptA* and *lptB* that are part of the *yrbG-lptB* locus, which encodes genes for OM biogenesis (Figure 22). The genes *lptA* and *lptB* encode components of the Lpt machinery that is dedicated to LPS transport from the IM to the OM (Sperandeo *et al.*, 2011). In addition to the σ^{70} -dependent *lptAp2* promoter, there are two previously described σ^E -dependent promoters preceding the *rpoN* operon, the σ^E -dependent promoter upstream of gene *lptA*, *lptAp1* (Martorana *et al.*, 2011) and the σ^E -dependent promoter within the gene *lptA*, *lptBp* (Rhodius *et al.*, 2006). The *lptAp1* was shown to be selectively activated upon depletion of the LPS transport machinery, suggesting that it responds to LPS biogenesis defects and trafficking problems (Martorana *et al.*, 2011) and the *lptBp* was shown to not actually respond to σ^E (Martorana *et al.*, 2011) and is more or less inactive *in vitro* and *in vivo* (Mutalik *et al.*, 2009; Rhodius and Mutalik, 2010).

The activation of the *lptAp1* promoter upon ectopic *rpoE* expression was initially confirmed by a reporter gene assay in the strain carrying the chromosomal *lptA'-lacZ* fusion, resulting in more than 400-fold induction of the β -galactosidase activity as a maximum induction of the fusion (Supplementary Figure S1A; Sikora and Görke, unpublished). This reporter gene fusion also contains the *lptBp*, which was reported to be inactive (Martorana *et al.*, 2011), therefore it can be argued that the σ^E -dependent effects are likely regulating the *lptAp1* promoter. The current literature suggests that this promoter specifically mediates the response to LPS trafficking problems, for example upon depletion of *lptA* or *lptC*, two components of the Lpt machinery, or upon the addition of Thanatin, an antimicrobial compound targeting this Lpt machinery (Martorana *et al.*, 2011; Vetterli *et al.*, 2018; Moura *et al.*, 2020). In contrast, *lptA* expression is not affected by other conditions (e.g. ethanol or overexpression of *ompC*) leading to up-regulation of σ^E -dependent genes, such as the gene of the sRNA MicA (Martorana *et al.*, 2011). Taken together, these data imply that there are additional levels of regulation of σ^E -dependent genes, likely mediated by specific transcription factors or taking place on the post-transcriptional level. In order to identify putative further regulators of *lptA* expression, a global screening approach was employed in order to identify mutations that cause activation of the promoters in the *lptAB* locus. The affected genes were divided into four distinct classes; proteins involved in the assembly of the core region of the LPS (WaaQ, WaaG), periplasmic chaperons (SurA), proteins involved in the σ^E ESR (RseA) and RNA binding proteins (Hfq) (Table 34). The results revealed different

mutants with the transposon insertions in the same gene, confirming their involvement in activation of the *lptAp1* promoter.

The analysis of the mutants yielding increased *lptA'-lacZ* expression revealed that two insertions mapped in the coding region of gene *rseA*, encoding the anti-sigma factor that sequesters σ^E at the IM and consequently represses its transcriptional activity (Walsh *et al.*, 2003). The disruption of *rseA* drastically increased the activity of the *lptA'-lacZ* reporter gene fusion. Mutants with a clean deletion of *rseA* were comparable to the mutants with transposon insertions in *rseA*, confirming that non-functional RseA leads to an increase of *lptA* expression (Figure 43A). These results can be explained by previous reports, showing that mutations of *rseA* result in constitutive, high σ^E activity (De Las Penas *et al.*, 1997; Missiakas *et al.*, 1997), which in turn can also activate the σ^E -dependent *lptAp1* promoter. Deleterious over-activation of σ^E is exemplified by the toxicity of *rseA* deletion that causes constitutive full activation of the σ^E ESR (Missiakas *et al.*, 1997), resulting in cell lysis during the stationary phase (Nitta *et al.*, 2000). Similar conclusions can be drawn from the growth curves of the *rseA* deletion strains or cells overproducing σ^E (Figure 43B, Figure 23A, Figure 31B, C). This is primarily caused by defects in OM integrity, arising from the σ^E -dependent overexpression of sRNAs MicA and RybB that largely decrease production of OMPs (Nicoloff *et al.*, 2017).

The most transposon insertions (4 of 11), were mapped in different genome positions in the coding region of the *hfq* gene. Furthermore, the mutants with transposon insertions in *hfq* exhibited the highest induction of the reporter gene fusion (approximately 14-fold; Figure 41), which implies the possibility that *lptA* is likely regulated on the post-transcriptional level. The RNA-binding chaperone Hfq is an important global regulator of gene expression in *E. coli* that stimulates the formation of cognate sRNA/mRNA pairs and stabilizes the resulting duplexes (Vogel and Luisi, 2011), consequently affecting many cellular processes (Moller *et al.*, 2002). The clean deletion of *hfq* resulted in a drastic increase of the activity of the *lptA'-lacZ* reporter fusion, which was comparable to the induction of the fusion in mutants with transposon insertions in *hfq* (Figure 42A, blue columns). These observations confirm that the transposons disrupting the *hfq* gene phenocopy the Δhfq deletion and indeed increase *lptA* expression. However, the data of this current study do not allow clear cut conclusions about the mechanism of the Hfq-dependent regulation of *lptA*. Since a translational reporter gene fusion, containing the promoter regions as well as the genetic features required for translation, was used, it is not clear whether the promoters are affected. An alternative explanation could be that Hfq mediates sRNA-mRNA interaction to regulate *lptA* expression on the post-transcriptional level for example by recruiting a regulatory RNA to block the Shine-Dalgarno sequence and thereby inhibit *lptA* translation. Upon *hfq* deletion, this sRNA-

mRNA pairing would be lost and translation of *lptA* would be initiated, reflected by an increase of the translational *lptA'*-*lacZ* reporter gene fusion. However, the Hfq-dependent regulation of *lptA* translation would suggest that *E. coli* possibly regulates this component of the Lpt machinery independent of the other *lpt* genes.

In order to determine, whether Hfq regulates *lptA* on the post-transcriptional level, transcriptional reporter gene fusions could be used, including the promoter regions but omitting the Shine Dalgarno sequence of *lptA*. If the transcriptional fusions are still differentially regulated in the absence of Hfq, these results would contradict the hypothesis, that *lptA* is regulated on the post-transcriptional level and would rather indicate, that the deletion of *hfq* affects the promoters in the *lptAB* locus.

A microarray analysis employed by Guisbert et al. (2007) revealed various potential new targets for Hfq regulation which also included genes encoding OMPs (Guisbert et al., 2007). They reported that Hfq regulates the activity of σ^E by indirect downregulation of the expression of seven mRNAs encoding OMPs (*lpp*, *ompA*, *ompC*, *ompF*, *ompX*, *fiu*, and *tsx*) (Guisbert et al., 2007). In the Δhfq mutant, the latter mRNAs are upregulated as they are no longer subjected to Hfq-dependent sRNA-mediated degradation and overproduction of those proteins induces the σ^E ESR (Guisbert et al., 2007). Hfq binds the σ^E -dependent sRNA MicA-mRNA *ompA* duplex which results in *ompA* mRNA degradation in *E. coli* (Vytvytska et al., 1998; Vytvytska et al., 2000; Udekwu et al., 2005). Another σ^E -dependent sRNA RybB, that controls ongoing synthesis of abundant OMPs by targeting *ompA*, *ompC*, *tsx* mRNAs for degradation, was reported to interact with Hfq (Wassarman et al., 2001; Guisbert et al., 2007; Thompson et al., 2007). Besides, *rybB* is expressed from the σ^E -dependent *rybB* promoter upon cell envelope stress (Johansen et al., 2006), but RybB also indirectly represses the synthesis of σ^E , creating an autoregulatory loop (Thompson et al., 2007). Furthermore, RseA, a central regulator of the σ^E ESR (Missiakas et al., 1997; Ades et al., 1999; Campbell et al., 2003) is regulated on the post-transcriptional level by sRNAs RyhB and FnrS (London et al., 2021). Melamed et al. (2016) already identified RyhB as RseA binding partner through RIL-seq (RNA interaction by ligation and sequencing) approach (Melamed et al., 2016). The σ^E regulon comprises genes encoding periplasmic chaperones, proteases and sRNAs (MicA, RybB and MicL) which in turn assist in correct OMP folding and degradation of misfolded OMPs (Rhodius et al., 2006; Klein and Raina, 2017). Thus, the absence of Hfq indirectly activates the σ^E ESR in response to misregulation of OMPs, which is a consequence of abolished regulation of the Hfq-dependent sRNA interaction partners. This indirect activation of σ^E could then in turn activate the *lptAp1* promoter and therefore the *lptA'*-*lacZ* reporter gene fusion.

Furthermore, two insertions each were mapped in the coding region of genes *waaQ* and *waaG*, encoding for the proteins that are involved in the assembly of the oligosaccharide region of the LPS. Upon the completion of Kdo₂-lipid A synthesis, the rough-form LPS (lipid A-core oligosaccharide) is synthesized by a conserved pathway involving sequential glycosyl transfer from nucleotide sugar precursors. At the inner (cytoplasmic) leaflet of the membrane, the coordinated complex of membrane-associated glycosyltransferases (Waa proteins) assembles the core oligosaccharide chains to the Kdo₂-lipid A (Whitfield *et al.*, 2003). The LPS heptosyltransferase WaaQ adds a heptose moiety to the side-branch of the inner core of LPS (Yethon *et al.*, 1998) and the LPS glucosyltransferase WaaG carries out the next step, the addition of a glucose residue to a second heptose moiety of the inner core of LPS which is also the first step in the synthesis of the outer core of LPS (Whitfield *et al.*, 2003). However, the deletion of either *waaQ* or *waaG* did not increase the activity of the *lptA'-lacZ* reporter gene fusion as compared to the original clones isolated in the transposon mutagenesis screen (Figure 44A). Those mutants clearly exhibited higher expression levels of the *lptA'-lacZ* reporter gene fusion, but to a much lower extent as compared to the mutants with transposon insertions in *hfq* or *rseA* (Figure 41A, 44A and 44C). Since both *waaQ* and *waaG* genes are encoded in a large *waaQGPSBOJZU* operon, comprising genes that are involved in LPS core-oligosaccharide assembly, the observed increase in *lptA* expression of *waaQ* or *waaG* transposon mutants could be due to polar effects on the downstream genes of the *waaQGPSBOJZU* operon, resulting in an altered expression of the whole operon. The transcription of the *waaQGPSBOJZU* operon is controlled by the transcriptional antiterminator RfaH (Klein and Raina, 2019). RfaH is an important regulator that controls the transcription of a subset of operons which are encoding proteins required for the production of LPS (Beutin *et al.*, 1981; Bailey *et al.*, 1996; Wang *et al.*, 2022). It specifically recognizes operons containing a short 8-nt conserved sequence in the 5'-UTR that is called the *ops* (operon polarity suppressor) pause site which recruits RfaH into a paused RNAP complex (Bailey *et al.*, 2000; Artsimovitch and Landick, 2002). By stabilizing the upstream edge of the transcription bubble, RfaH prevents the Rho-dependent termination of the *waa* operon and subsequently suppresses pausing downstream (Artsimovitch and Landick, 2002; Svetlov and Nudler, 2018). Furthermore, the sRNA RirA (RfaH interacting RNA), binds RfaH in the presence of RNAP and this interaction depends on intact *ops* sites within RirA (Klein *et al.*, 2016). RirA is encoded at the beginning of the *waa* operon in the 5'-UTR of *waaQ* mRNA and consequently has the same start site as the *waaQ* mRNA and may be produced by processing of the 5'-UTR (Klein *et al.*, 2016). Moreover, overexpression of *rirA* activates *rpoE* expression from the *rpoEP3* promoter which is σ^{70} (RpoD)-dependent and directly controlled by the Rcs system in response to LPS core biosynthesis defects and overproduction of certain lipoproteins, resulting in abrogated synthesis of the O-antigen, a truncated LPS core

and reduction of total amount of LPS, an effect that is also observed in the $\Delta rfaH$ mutant (Klein *et al.*, 2016; Klein and Raina, 2019). Thus, this phenotype might be due to loss of the specificity of RfaH for recognition of *ops*-containing operons, which are needed for LPS biosynthesis due to sequestration of RfaH by RirA (Klein *et al.*, 2016; Klein and Raina, 2019). It was already previously reported that mutations of *waaQ* result in truncated form of LPS, in turn destabilizing the OM of *E. coli* (Yethon *et al.*, 2000). As clean deletion of the genes *waaQ* or *waaG* alone does not result in an increased *lptA* expression, the transposon insertions might cause indirect effects affecting expression of the downstream operon, which in turn alters *lptA* expression. The transposon insertions in *waaQ* might as well influence the expression of sRNA *rirA* which in turn leads to altered expression of the whole *waa* operon due to loss of the specificity of RfaH for recognition of the *ops* pause site in the 5'-UTR. In future studies it could be tested whether the deletion of *rfaH* activates the *lptAp1* promoter to conclude more about this regulation.

Additionally, one insertion was mapped in the coding region of gene *surA*, encoding the periplasmic chaperone that is involved in the biogenesis of the β -barrel OMPs. SurA assists in a proper folding of the OMPs OmpA, OmpF and LamB (Lazar and Kolter, 1996). The mutant with a transposon insertion in *surA* exhibited a similar increase in the activity of the *lptA'-lacZ* reporter fusion as compared to the mutants with transposon insertions in *waaQ* or *waaG* (Figure 41). As the *surA::kan* single-gene deletion mutant from the Keio collection could not be cultivated, a complementation experiment was carried out to confirm the involvement of *surA* in the activation of the *lptA'-lacZ* reporter gene fusion. Complementation of the *surA* transposon mutant with the plasmid containing the *surA* coding sequence decreased the activity of the *lptA'-lacZ* reporter gene fusion and restored it to the activity of the *wild-type* strain (Figure 45A). Those observations confirm that the transposon disruption of the gene *surA* indeed increases *lptA* expression and likely activates the *lptAp1* promoter. Upon secretion of proteins through the Sec complex into the periplasm, the periplasmic chaperone SurA maintains the OMPs in an unfolded state prior to the assembly into the OM by the Bam complex (Mitchell and Silhavy, 2019). The periplasmic protease DegP degrades the unfolded or misfolded OMPs (Strauch and Beckwith, 1988). When these processes are compromised, unfolded OMPs accumulate in the periplasm and activate the σ^E ESR (Walsh *et al.*, 2003). Furthermore, the loss of SurA results in increased σ^E activity and OM defects (Lazar and Kolter, 1996; Missiakas *et al.*, 1996; Rouvière and Gross, 1996). It decreases the abundance of some β -barrel OMPs in the outer membrane, including OmpA, OmpF, LamB and, interestingly, also LptD, a component of the Lpt machinery, as the assembly of those OMPs is defective (Lazar and Kolter, 1996; Sklar *et al.*, 2007; Vertommen *et al.*, 2009). Thus, the defective assembly of OMPs and unfolded OMPs that accumulate in the periplasm due to

the transposon insertion in *surA* are likely responsible for the observed activation of the *lptAp1* promoter. Especially the putative mis-regulation of LptD in the *surA* mutant provides an interesting connection to the regulation of *lptA*, that is reportedly up-regulated upon depletion of the Lpt machinery components (Martorana *et al.*, 2011). Taken together, transposon insertions in the genes which are involved in LPS biogenesis and composition, *waa* and *surA* respectively, further strengthen the observations that the *lptAp1* promoter is indeed activated upon LPS or OM defects.

As mentioned before, the σ^E -dependent *lptAp1* promoter is not activated by each envelope stress condition that usually induces the σ^E ESR, but it was shown to be selectively activated upon depletion or disruption of the LPS transport machinery, suggesting that it responds to LPS biogenesis defects and trafficking problems (Martorana *et al.*, 2011). For a functional Lpt machinery, the interaction between the Lpt proteins is of critical importance and the depletion of any component results in the accumulation of a colanic-acid modified LPS at the outer leaflet of the IM (Sperandeo *et al.*, 2011). Upon incorrect assembly of the Lpt bridge, LptA undergoes degradation (Sperandeo *et al.*, 2011). Therefore, destabilization of LptA and appearance of modified forms of LPS are considered as the signals of Lpt defects (Sperandeo *et al.*, 2011). Due to its relevance in Gram-negative bacteria, LPS and proteins involved in its transport and biosynthesis represent potential antibiotic targets. A lot of focus has been put in identification and development of novel inhibitors of the enzymes involved in LPS biogenesis and modifications. In the past studies, potent inhibitors that either block the expression or directly inhibit lipid A modifying enzymes were identified (Simpson and Trent, 2019). Some mutations known to impair LPS biogenesis and transport have been identified as well. The conditional expression of *lpt* genes, an internal *lptD* Δ 330-352 deletion or mutation of *dsbA*, which is required for disulfide bonds in LptD, all impair the activity of the Lpt machinery (Lima *et al.*, 2013). The deletions of genes encoding enzymes LpxL and LpxM, involved in synthesis of Kdo2-lipid A, and of Waa enzymes, involved in the assembly of the oligosaccharide region of the LPS, are known to induce σ^E via LPS perturbation (Lima *et al.*, 2013; Klein *et al.*, 2016). Recently, Zhang *et al.* (2019) have developed a yeast-two hybrid (YTH)-based screening system in *E. coli* which enables identification of compounds that disrupt the interaction between the Lpt components LptA and LptC (Zhang *et al.*, 2019). LptA and LptC interact with each other to form a complex that enables LPS transports from the IM to the OM (Okuda *et al.*, 2012; Moura *et al.*, 2020). In the latter study, Zhang *et al.* (2019) have identified a compound (IMB-881) that blocks LPS transport by inhibiting LptA-LptC interaction, resulting in accumulation of LPS in the periplasm and in turn disrupting OM biosynthesis (Zhang *et al.*, 2019). The same system was later employed by Dai *et al.* (2022) and they identified another compound (IMB-0042) which binds to both LptA and LptC and

blocks LPS transport, efficiently inhibiting the growth of Gram-negative bacteria (Dai *et al.*, 2022). Another selective inhibitor of LptD in *Pseudomonas aeruginosa*, Murepavadin, has shown promising antibacterial properties, but has been suspended from Phase III clinical trials due to nephrotoxicity (Srinivas *et al.*, 2010; Lehman and Grabowicz, 2019). Furthermore, compound CHR-90 is another identified inhibitor of LPS biogenesis that inhibits LpxC (Erwin, 2016). There are also some cationic antimicrobial peptides (CAMPs) (e.g. spermine, Colistin, Polymyxin B, C18G and LL-37) which are known to perturb the OM and peptidoglycan (Farris *et al.*, 2010; Page and Walker, 2021).

Thanatin is a naturally occurring cationic antimicrobial peptide (CAMP) and one of the few known inhibitors of the LPS transport machinery (Moura *et al.*, 2020). It exhibits antimicrobial activity against a broad spectrum of organisms, including *E. coli* (Fehlbaum *et al.*, 1996). By binding LptA and LptD, it disrupts the interactions required for the correct Lpt-complex assembly (Vetterli *et al.*, 2018), which results in LptA degradation as well as the accumulation of colanic-acid modified LPS (Moura *et al.*, 2020). The disassembly of the Lpt machinery leads to the inhibition of LPS transport to the OM and defects in membrane assembly (Moura *et al.*, 2020). Since thanatin inhibits the LPS transport machinery, it could elicit activation of the *lptAp1* promoter, which was shown to selectively respond to LPS biogenesis defects and trafficking problems. It was previously shown, that even the level of the sRNA MicA, which is controlled by σ^E , is up-regulated upon depletion of LptA or LptC (Martorana *et al.*, 2011). In addition, primer extension analysis supports that the increased expression of *lptA* under these conditions is indeed initiated at the σ^E -dependent *lptAp1* promoter (Martorana *et al.*, 2011). Nevertheless, the *lptAp1* promoter is an interesting example of σ^E -dependent promoters, that are mostly considered to respond to σ^E alone. The *lptAp1* promoter, however, only responds to a subset of stress conditions, which would require an additional level of regulation. The defects in LPS or its transport could activate another activating regulator, such as transcription factors, or inactivate a repressor, for example by proteolysis.

In this study, the activation of the *lptAp1* promoter upon the addition of thanatin was confirmed by the reporter gene assay in the strain carrying the chromosomal *lptA'-lacZ* reporter fusion, resulting in 4,5-fold increase of the β -galactosidase activity (Figure 35A). These data strengthen the observations that LPS transport is a critical signal for the activation of the *lptAp1* promoter and confirm the hypothesis that thanatin likely elicits the appropriate response via the latter promoter. The further characterization of *lptAp1* is an important step in better understanding of its regulation. This study has identified various conditions leading to transcription from *lptAp1*, such as the *surA* mutation or the addition of thanatin. These conditions can now be used to analyze mutants with defective transcription

factors, proteases or other major regulators of gene expression with regard to activation of *lptAp1*. If there are mutants showing no up-regulation of *lptA* under these conditions, these findings would suggest that the affected gene plays a role in expression of *lptA* in response to LPS defects.

7.2.1.2. Read-through transcription from *lptAp1* into the *rpoN* operon

As mentioned above, the RNase E adaptor protein RapZ itself contributes to the cell envelope precursor homeostasis by post-transcriptionally regulating *glmS* expression and perhaps also UMP synthesis in response to the level of GlcN6P via sRNAs GlmY and GlmZ (Khan and Görke, 2020). Thus, it might help to counteract envelope stress by adjusting cell envelope precursor synthesis accordingly through the interaction with small regulatory RNAs, acting at the post-transcriptional level. This seems a reasonable hypothesis considering the unpublished preliminary work in which the accumulation of all proteins of the *rpoN* operon in response to ectopic expression of *rpoE* was initially confirmed by Western blot analysis, addressing levels of FLAG-tagged σ^{54} , Hpf, PtsN, RapZ and NPr (Sikora and Görke, unpublished). In order to check whether the observed accumulation of FLAG-tagged RapZ upon ectopic *rpoE* expression is reproducible with RapZ antiserum, the RapZ protein levels were monitored directly. Additionally, a strain with untagged RapZ protein allows better conclusions about the downstream effects that might be caused by altered RapZ levels. RapZ forms a tetrameric structure in the cell, which requires interactions including the N-terminal domain as well as the C-terminal domain (Gonzalez *et al.*, 2017). Therefore, the FLAG-peptide tag on RapZ might perturb the tetramerization or RNA-binding and, thus, the functionality of RapZ, since the sRNAs GlmY and GlmZ bind to several residues of the RapZ tetramer, including the C-terminus (Durica-Mitic *et al.*, 2020; Islam *et al.*, 2023).

Indeed, the Western blot analysis of RapZ levels upon ectopic expression of *rpoE* from the arabinose-inducible promoter on a plasmid in the *wild-type* strain confirmed the latter (Figure 24). Furthermore, elevated RapZ levels in cells ectopically expressing σ^E are comparable to the amounts that were detected in the strain overexpressing *rapZ* from the arabinose-inducible promoter on a plasmid. Thus, ectopic expression of *rpoE*, resulting in over-activation of the σ^E ESR, leads to substantial accumulation of RapZ. This observation further strengthens the hypothesis that the *rpoN* operon represents an important layer of the σ^E ESR as the observed elevated levels of RapZ may activate downstream processes contributing to stress recovery.

In addition, the effect of strong activation of *lptAp1* on the whole *rpoN* operon by read-through transcription was analyzed in a strain carrying the chromosomal *lptA* $\rightarrow$ *rapZ'*-*lacZ* translational reporter gene fusion. As expected, the reporter gene assay confirmed that *rapZ*

expression increases upon σ^E induction, which was indicated by almost 35-fold induction of the β -galactosidase activity as a maximum induction of the fusion (Figure 26A). The same strain was used to check if the activation of the *lptAp1* promoter upon the addition of thanatin also results in read-through transcription into the *rapZ* gene as observed in the previous experiments with ectopic *rpoE* expression. The reporter gene assay again confirmed the latter which was indicated by a 2-fold induction of the β -galactosidase activity upon the addition of thanatin (Figure 35C). This is most likely mediated by read-through transcription into the *rapZ* gene from the upstream located σ^E -dependent *lptAp1* promoter. However, the employed fusion also contains an additional σ^E -responsive element located within the *rpoN* operon as well, that yields a 2-3-fold change in reporter gene measurements upon overexpression of *rpoE*. The weaker fold-change of the long reporter gene fusion that translates all the way into the *rapZ* gene as compared to the short translational fusion, comprising only the *lptAp1* and the *lptB* promoter, indicates that RNA processing events by endoribonucleases might occur, leading to different expression levels of the genes of the long primary pranscript. In bacteria, all the generated primary RNA transcripts (including rRNAs, tRNAs, mRNAs and sRNAs) are regulated on the level of stability and are in most cases short lived as they are rapidly subject to turnover by endoribonucleases (Hui *et al.*, 2014; Chen *et al.*, 2015). The RNase E is a major essential endoribonuclease in *Enterobacteriaceae*, initiating RNA turnover by internal cleavages in most mRNAs (Carpousis *et al.*, 2009; Clarke *et al.*, 2014). It associates with additional proteins into a large complex called the RNA degradosome which promotes degradation of bulk RNA (Miczak *et al.*, 1996; Mackie, 2013). Furthermore, the coupled degradation of some sRNA/mRNA complexes can occur when RNase E associates with other proteins like Hfq, which confer cleavage specificity towards selected targets (De Lay *et al.*, 2013). Chao *et al.* (2017) have reported that a uridine at 2 nt downstream of the cleavage site (U_{+2}) is the RNase E consensus motif which is crucial for RNase E cleavage of many coding and non-coding transcripts at the 3' end (Chao *et al.*, 2017). Indeed, several RNase E cleavage sites have been mapped upstream of *rpoN* and within the *rpoN* operon in the closely related species *Salmonella enterica* (Chao *et al.*, 2017), so these processing sites are likely conserved in *E. coli*. Furthermore, Martorana *et al.* (2011) also concluded that the mRNAs started upstream of *rpoN* are subjected to Rnase E-mediated decay (Martorana *et al.*, 2011). This could explain higher expression levels of *lptA* and *lptB*, but a weaker induction of *rpoN* operon genes. Indeed, there is initial data from our lab showing RNase E-dependent degradation of the long *lptA-lptB-rpoN-hpf-ptsN-rapZ-ptsO* transcript, that might thus be regulated on the post-transcriptional level.

In future studies it could be tested whether the σ^E -dependent regulation of the *rpoN* operon is lost in the Δhfq mutant as the σ^E regulon comprises several Hfq-dependent sRNAs. In case regulation is lost, an involvement of σ^E dependent candidate sRNAs could be tested through deletion and overexpression approaches and in case regulation persists, a processing event leading to a more stable *lptA-lptB-rpoN-hpf-ptsN-rapZ* transcript would be a plausible option. Furthermore, a 5' RACE could be carried out in the *wild-type* strain ectopically expressing *rpoE* to map the 5' end of the corresponding different transcripts in order to search for major endoribonuclease RNase E cleavage sites that were previously mapped in *Salmonella* according to Chao et al. (2017). Taken together, these data indicate that increased translation of RapZ upon σ^E expression likely results from the read-through transcription of the σ^E -dependent *lptAp1* promoter upstream of the *rpoN* operon (Martorana et al., 2011) or by another σ^E -responsive element within the *rpoN* operon, or a combination of these regulatory elements, strengthening the hypothesis that the *rpoN* operon is a member of the σ^E regulon.

7.2.2. The consequences of the σ^E -dependent accumulation of protein RapZ for the GlmY/GlmZ/*glmS* circuit

The multifunctional sRNA-binding protein RapZ controls the synthesis of GlcN6P synthase GlmS at the post-transcriptional level (Göpel et al., 2014; Khan and Görke, 2020). The enzyme GlmS synthesizes GlcN6P *de novo*, initiating the amino sugar pathway which generates UDP-GlcNAc required for biosynthesis of peptidoglycan and LPS. To achieve GlcN6P homeostasis, RapZ regulates *glmS* expression by binding two homologous small RNAs GlmY and GlmZ (Khan and Görke, 2020). The sRNA GlmZ positively regulates *glmS* expression by base-pairing with the 5' UTR of the *glmS* mRNA in an Hfq-dependent manner, which stabilizes the *glmS* mRNA and activates *glmS* translation (Kalamorz et al., 2007; Urban and Vogel, 2008; Göpel et al., 2013). The stability of sRNA GlmZ is controlled by the RNase E adaptor protein RapZ, which in turn adjusts cell envelope precursor synthesis according to the cell's needs. Under normal growth conditions when GlcN6P levels in the cell are sufficient, high levels of GlmS are not required. Hence, RapZ is mainly dedicated to recruit GlmZ to processing by RNase E, adjusting a basal GlmS level (Durica-Mitic et al., 2020). While the processed form of GlmZ can no longer base-pair with the *glmS* mRNA, it is still able to bind and sequester RapZ from the full-length GlmZ to inhibit the cleavage of the latter and prevent a complete GlmZ turnover in order to maintain *glmS* translation at a basal level (Durica-Mitic and Görke, 2019). The activity of RapZ is primarily regulated through interaction with the metabolite GlcN6P (Khan et al., 2020), but GlcN6P has no impact on RapZ amounts (Göpel et al., 2013). When RapZ is not bound to GlcN6P, it activates the QseE/QseF TCS by direct interaction and thereby *glmY* expression from the σ^{54} -dependent

promoter in a QseF-dependent manner (Göpel and Görke, 2018). The sRNA GlmY is a homologous sRNA which mimics GlmZ and sequesters RapZ, counteracting GlmZ decay to achieve upregulation of *glmS* in response to decreasing GlcN6P (Göpel *et al.*, 2013). Consequently, GlmZ remains unprocessed and is able to base-pair with the *glmS* mRNA, increasing *glmS* translation and in turn *de novo* GlcN6P synthesis to maintain GlcN6P homeostasis (Göpel *et al.*, 2013; Göpel *et al.*, 2014). Furthermore, when RapZ is bound to GlmY, it is unable to interact with the QseE/QseF TCS, providing a negative feedback loop to limit *glmY* expression (Khan *et al.*, 2020).

The main focus of this thesis is to reveal the consequences of the σ^E -driven upregulation for downstream processes regulated by RapZ as the role of RapZ for regulation of *glmS* has so far been studied under the assumption that *rapZ* is constitutively expressed and RapZ amounts do not significantly vary (Göpel *et al.*, 2013; Gonzalez *et al.*, 2017; Durica-Mitic *et al.*, 2020; Khan *et al.*, 2020; Khan and Görke, 2020). Up to this point, the data confirm that ectopic expression of *rpoE* from a plasmid, resulting in over-activation of the σ^E ESR, triggers accumulation of RapZ as observed by the Western blot analysis (Figure 24). Thus, the consequences of active σ^E for accumulation of RapZ protein and for the GlmY/GlmZ/*glmS* circuit were studied through the use of ectopic *rpoE* expression from a plasmid.

The upregulation of *rapZ* expression by σ^E should increase both RapZ fractions, the GlcN6P-bound as well as the GlcN6P-free RapZ which should activate the QseE/QseF TCS to upregulate *glmY* expression from the σ^{54} -dependent promoter, resulting in sequestration of RapZ by GlmY into long-lasting complexes and increased *glmS* translation by full-length GlmZ. On the other hand, in case of RapZ amounts exceeding GlmY amounts, part of the RapZ population is left un-sequestered and thereby should be free to recruit RNase E to process GlmZ and prevent activation of *glmS* translation. A transient downregulation of GlcN6P synthesis would be in line with the general observation that ESR systems downregulate synthesis of envelope precursors until repair of the damage in the envelope has been achieved (Mitchell and Silhavy, 2019). To elucidate whether the σ^E -driven upregulation of RapZ activates *glmS* expression (via upregulation of GlmY which subsequently sequesters RapZ) or prevents *glmS* expression (in case of RapZ amounts exceeding GlmY amounts, leaving part of the RapZ population un-sequestered and thereby free to recruit RNase E to GlmZ), σ^E was ectopically expressed from a plasmid and the levels of RapZ and GlmS proteins as well as the levels of GlmY and GlmZ sRNAs were addressed by Western and Northern Blot analysis.

Initially, the pFS28 plasmid carrying *rpoE* under control of an arabinose-inducible promoter (P_{ara}) was used to achieve ectopic *rpoE* expression as it was already used in previous

experiments dissecting the σ^E -dependent regulatory elements in the *lptAB* and the *rpoN* and subsequently confirming the accumulation of RapZ by Western blot analysis. The latter plasmid was not well suited to study the consequences of σ^E activation for the regulatory functions controlled by RapZ, because there was an obvious effect of the inducer on the system reflected by enhanced GlmZ processing (Figure 32B, C). Nevertheless, ectopic expression of *rpoE* from the arabinose-inducible promoter on plasmid led to important observations. The levels of GlmY did not significantly vary regardless of the *rpoE* overexpression, but the processed form of GlmZ was present in much higher amounts when *rpoE* was overexpressed (Figure 32B, C). In contrast, the level of the regulatory active full-length GlmZ was decreased, but an effect of this regulation on the target GlmS was not observed (Figure 32A, B, C). That is because GlmS is already only present at the basal level in a rich growth medium that was used for this experiment. Even though the GlmZ cleavage by RapZ was strongly enhanced upon *rpoE* overexpression, the processed form of GlmZ still retains the ability to bind and sequester RapZ from the full-length GlmZ in order to prevent a complete GlmZ turnover (Durica-Mitic and Görke, 2019) and thus maintain *glmS* translation at a basal level that was observed throughout the experiment (Figure 32A). The enhanced GlmZ processing that was observed in P_{ara} VC upon the addition of arabinose is likely the consequence of activation of a *cyaA*-dependent element, which is located upstream of the *ptsN* gene. The effect of this element was already observed in previously mentioned experiments as the *wild-type* and P_{ara} VC strains, carrying the *lptA*→*rapZ'*-*lacZ* reporter fusion, grown in the presence of arabinose exhibited slightly higher activities compared to the control strains grown in the absence of arabinose (Figure 26B). To overcome this interference with the inducer, the change of inducer and expression plasmid was necessary and thus, the arabinose-inducible P_{ara} promoter cassette was replaced by the anhydrotetracycline-inducible promoter cassette, generating the expression plasmid pFS37, allowing to express *rpoE* upon the addition of anhydrotetracycline.

The arabinose-inducible promoter cassette carries the P_{ara} promoter, the P_c promoter and the regulatory gene *araC* (Guzman *et al.*, 1995). The AraC protein regulates the activity of both promoters. The transcription of both promoters occurs only when the cAMP bound CAP protein is bound to the sequence between the promoters which are adjacent to each other. In the absence of an inducer, the AraC protein dimerizes at the O_2 and I_1 operator sites, thereby looping the DNA and tightly repressing both, the P_{ara} promoter and the P_c promoter, which in turn prevents expression of the gene that is under control of the P_{ara} promoter. When L-arabinose is present, it binds to the arabinose-binding pocket of AraC protein which allows it to dimerize at the I_1 and I_2 operator sites, thereby enabling the cAMP bound CAP protein to bind to its binding sites between both promoters, recruiting RNAP and in turn activating the

expression of the gene that is under control of the P_{ara} promoter. The advantages of the P_{ara} promoter are a tight repression in the absence of an inducer and a linearly tunable expression level by an inducer (Guzman *et al.*, 1995). The anhydrotetracycline-inducible promoter cassette carries the Tet^R repressor and the P_{tet} promoter which also contains the $tetO$ operator sequence that is recognized by the TetR repressor protein. In the absence of an inducer, the TetR repressor protein binds the tet operator sequence in the promoter and prevents expression of the gene that is under control of the P_{tet} promoter. When tetracycline or one of its derivatives like anhydrotetracycline is present, it binds to the TetR repressor protein, which is in turn released from the tet operator sequence. Consequently, the P_{tet} promoter is induced and expression of the gene that is under control of the P_{tet} promoter can occur. Anhydrotetracycline is a less-toxic derivative of tetracycline and an efficient inducer of the P_{tet} promoter but it still exhibits a lower antibiotic activity towards *E. coli* (Gossen and Bujard, 1993). Nevertheless, it is a more suitable plasmid system to study the σ^E -driven upregulation for downstream processes regulated by RapZ as the effect of the inducer on the system, reflected by enhanced GlmZ processing, is abolished. In order to determine the optimal anhydrotetracycline concentration for induction of *rpoE* expression from the newly constructed plasmid, various concentrations of anhydrotetracycline as reported in the literature (Ehrt *et al.*, 2005; Lim *et al.*, 2017; Burke *et al.*, 2020), ranging from 25 – 200 ng/mL, were tested. At first, the RapZ protein levels were directly addressed by Western blot analysis in the $P_{ara}::rpoE$ and $P_{tet}::rpoE$ wild-type transformants in order to monitor the growth and the σ^E -mediated accumulation of RapZ depending on the presence of different *rpoE* expression plasmids and various concentrations of anhydrotetracycline. The observed fold-upregulation of RapZ levels, achieved by ectopic expression of *rpoE* from either the arabinose-inducible or the anhydrotetracycline-inducible promoter on a plasmid, appeared comparable regardless of the *rpoE* expression plasmid and the anhydrotetracycline concentration (Figure 28B). However, there was an obvious difference in growth-defect of the cultures depending on the presence of either arabinose-controlled or anhydrotetracycline-controlled *rpoE* expression plasmid during the whole experiment. In comparison to the $P_{ara}::rpoE$ transformant which reached an OD₆₀₀ of ~ 1.5 after 2 h of ectopic *rpoE* expression, the $P_{tet}::rpoE$ transformants were in an OD₆₀₀ range of ~ 0.75, independent of the concentration of anhydrotetracycline (Figure 28A). Since the growth rate of either the P_{ara} or P_{tet} VC controls grown in the presence or absence of the inducer was comparable, the growth disadvantage of the $P_{tet}::rpoE$ transformants was not due to the presence of the inducer anhydrotetracycline, but due to ectopic expression of *rpoE* from the tetracycline-inducible promoter on plasmid pFS37, resulting in over-activation of the σ^E ESR. Therefore, a lower concentration of anhydrotetracycline that results in a somehow similar growth rate of the $P_{tet}::rpoE$ transformant compared to the $P_{ara}::rpoE$ transformant and a similar range of

activation of the *lptA*→*rapZ*'-*lacZ* reporter gene fusion upon ectopic *rpoE* expression had to be determined. Therefore, lower concentrations, ranging from 12,5 to 0,25 ng/mL, were subsequently tested in the strain carrying the chromosomal *lptA*(-152)→*rapZ*(+78)'-*lacZ* fusion to exclude growth-stage dependent differences. The concentration of anhydrotetracycline that led to somehow similar growth rates as observed in the previous experiments with *P_{ara}::rpoE* transformant was 1 ng/mL (Figure 30B). However, the *P_{tet}::rpoE* transformants still did not reach the same OD₆₀₀ as compared to the *P_{ara}::rpoE* transformants after 2 h of ectopic *rpoE* expression, as the OD₆₀₀ of the *P_{tet}::rpoE* transformants was in a range of ~ 1.1. Nevertheless, the maximum induction of the reporter gene fusion, resulting in a 20- and 25-fold induction of the β-galactosidase activity 60 and 90 min after the addition of anhydrotetracycline (Figure 30A), was comparable to the fold-induction observed in the *P_{ara}::rpoE* transformants, which resulted in a 20- and 30-fold induction of the β-galactosidase activity 60 and 90 min after the addition of arabinose (Figure 26A). Furthermore, the uninduced *P_{tet}::rpoE* transformants exerted a minor activation of the reporter gene fusion due to leaky expression of *rpoE* in the absence of an inducer which was negligibly small compared to the activation achieved by induction of the plasmid-encoded *rpoE* (Figure 30A). Based on these results, 1 ng/mL of anhydrotetracycline was used in subsequent experiments to achieve ectopic *rpoE* expression and analyze the consequences of active σ^E for accumulation of RapZ protein and for the GlmY/GlmZ/*glmS* circuit.

Subsequently, the pFS37 plasmid carrying *rpoE* under control of an anhydrotetracycline-inducible promoter (*P_{tet}*) was used to achieve ectopic *rpoE* expression and the levels of RapZ and GlmS proteins as well as the levels of GlmY and GlmZ sRNAs were addressed by Western and Northern Blot analysis (Figure 33). The ectopic expression of *rpoE* from the anhydrotetracycline-inducible promoter, mimicking σ^E activation, strongly elevated the levels of RapZ protein which started accumulating already 20 min after induction of *rpoE* expression (Figure 33A). The levels of GlmY were strongly elevated when *rpoE* was overexpressed from the plasmid (Figure 33B, C), as compared to the plasmid system with *P_{ara}* where GlmY was present in slightly higher amounts only at latest points in time (Figure 32B, C). Interestingly, upon ectopic expression of *rpoE*, an additional GlmY signal was observed on top of the membrane (Figure 32B, Figure 33B). The latter RNA started accumulating already 20 min after induction of *rpoE* expression and is estimated to be ~ 300 bp longer than the processed form of GlmY RNA. The sRNA *glmY* is expressed from two perfectly overlapping σ⁷⁰ and QseF-dependent σ⁵⁴ promoters (Reichenbach *et al.*, 2009). The processing of GlmY primary sRNA in its 3' end results in the 5' cleavage product which is the active form of GlmY (labeled as GlmY*) that sequesters RapZ (Reichenbach *et al.*, 2009; Göpel *et al.*, 2013). The additional ~ 500 bp GlmY RNA product that is observed only when

rpoE is expressed ectopically could potentially result from a read-through transcription of the *glmY* gene into the *qseE* gene which is located further downstream. If that is the case, the RNA product should be as well detectable using the RNA probe specific for *qseE*, which could be tested in future experiments. Furthermore, the GlmZ cleavage by RapZ was strongly enhanced when *rpoE* was overexpressed from the plasmid as it was already observed in the previous experiments using the arabinose-controlled *rpoE* expression plasmid pFS28 (Figure 33B, Figure 32B). Consequently, the processed form of GlmZ is present in much higher amounts and the level of full-length GlmZ is decreased as a consequence of the σ^E -mediated upregulation of RapZ (Figure 33B, left middle panel). As observed previously, the levels of GlmS protein did not differ regardless of the *rpoE* ectopic expression (Figure 33A) because under normal conditions in a rich growth medium (LB), GlmS is already only present at the basal level and cannot be down-regulated further. Since the processed form of GlmZ still retains the ability to bind and sequester RapZ to prevent a complete GlmZ turnover and maintain *glmS* translation at a basal level, further downregulation of GlmS is not possible even though there is more of GlmZ processing due to higher RapZ amounts as a consequence of σ^E overproduction. So far, the obtained results confirmed that the σ^E -mediated upregulation of RapZ, achieved by ectopic expression of *rpoE* from a plasmid, elevates the levels of GlmY and leads to enhanced GlmZ processing but does not affect the GlmS, which is already at a basal level.

Based on these results, the impact of σ^E -mediated upregulation of RapZ on GlmS levels and in turn synthesis of LPS and peptidoglycan precursors was addressed under conditions of GlcN6P depletion. Nva-FMDP is a synthetic derivative of modified dipeptide antibiotics that selectively inhibit GlmS enzymatic activity (Milewski *et al.*, 1986). Once Nva-FMDP is hydrolyzed in the cytoplasm, its active moiety FMDP, which acts as glutamine analogue, inhibits GlmS by covalently binding to a residue in the glutamine binding pocket, resulting in an irreversible inhibition of GlmS that in turn leads to GlcN6P depletion (Milewski *et al.*, 1986; Kucharczyk *et al.*, 1990). GlcN6P depletion results in upregulation of sRNAs GlmY and GlmZ to achieve increased *glmS* expression in order to overcome antibiotic inhibition (Khan *et al.*, 2016). The GlmY molecules that accumulate in response to GlcN6P depletion bind and sequester RapZ from processing GlmZ through molecular mimicry and thus, full-length GlmZ can activate translation of *glmS* mRNA in an Hfq-dependent manner in order to replenish intracellular GlcN6P pools and achieve GlcN6P homeostasis (Khan *et al.*, 2016). In order to achieve combined conditions of GlcN6P depletion and cell envelope stress, Nva-FMDP was used in combination with ectopic *rpoE* expression from the anhydrotetracycline-controlled plasmid. In order to just inhibit GlmS enzymatic while not impairing the growth of the cultures, the sub-inhibitory concentration of Nva-FMDP was used (60 $\mu\text{g/mL}$). Under combined

conditions of GlcN6P depletion and cell envelope stress, a transient downregulation of GlcN6P synthesis until the damage in the envelope has been repaired seems like a more probable outcome based on the previous observations obtained with ectopic *rpoE* expression. To determine which of these conditions is more dominant, the upregulation of GlmS due to GlcN6P depletion or the enhanced GlmZ processing and no upregulation of GlmS due to σ^E -mediated upregulation of RapZ, the levels of RapZ and GlmS proteins as well as the levels of GlmY and GlmZ sRNAs were addressed by Western and Northern Blot analysis (Figure 34). Indeed, the obtained results confirmed the hypothesis and led to following observations. The addition of Nva-FMDP alone strongly elevated the levels of both GlmY and full-length GlmZ as previously reported (Figure 34B) (Khan *et al.*, 2016). Consequently, RapZ is sequestered from GlmZ processing by GlmY and the increased levels of full-length GlmZ in turn activate translation of the *glmS* mRNA. The effect of this regulation resulted in strong accumulation of protein GlmS (Figure 34A) to overcome GlmS enzymatic inhibition, caused by Nva-FMDP, and to replenish intracellular GlcN6P pools. The addition of Nva-FMDP at the same time when σ^E was ectopically expressed from a plasmid elevated the levels of protein RapZ (Figure 34A). Furthermore, GlmY accumulated to a comparable extent as observed when Nva-FMDP was added alone (Figure 34B). The cleavage of full-length GlmZ was enhanced by the higher levels of RapZ that were achieved by ectopic *rpoE* expression. As a result, the level of the processed form of GlmZ was increased and the level of full-length GlmZ was decreased, contrary to the condition when Nva-FMDP is added alone (Figure 34B). Consequently, the accumulation of GlmS was not observed despite the GlcN6P depletion that leads to the lack of the cell envelope precursors (Figure 34A). Taken together, these data confirmed the hypothesis that the σ^E -dependent accumulation of RapZ enhances the cleavage of the full-length GlmZ and prevents upregulation of GlmS even under conditions of GlcN6P depletion. Thus, during cell envelope stress, which induces a σ^E -response, *de novo* synthesis of the cell envelope precursors is inhibited. This mechanism likely allows the bacteria to first repair the damage in the envelope without activating its biosynthesis pathway from the very initial steps. Furthermore, this result is in correspondence with the general observation that ESR systems downregulate synthesis of envelope precursors until repair of the damage in the envelope has been achieved (Mitchell and Silhavy, 2019).

The increased *rapZ* expression as a consequence of activation of the ESR, elicited by thanatin, could result in elevated RapZ protein levels and account for activation of the *glmY* expression from the σ^{54} -dependent promoter in a QseF-dependent manner. To test this hypothesis and further analyze the impact that thanatin might have on the GlmY/GlmZ/*glmS* circuit, RapZ and GlmS protein levels and GlmY and GlmZ RNA levels were monitored

directly. In addition, *glmY* expression was quantitatively monitored by the reporter gene assay in the strain carrying the chromosomal *glmY'-lacZ* reporter gene fusion. The reporter gene assay indicated that the addition of thanatin does not result in activation of *glmY* expression from the σ^{54} -dependent promoter as the *glmY'-lacZ* reporter fusion was induced to a similar extent in both the *wild-type* and the $\Delta rapZ$ mutant (Figure 37A). The Western blot analysis revealed that RapZ accumulates to a minor extent upon the addition of thanatin (Figure 37C, E). The processed form of GlmZ was accumulating upon the addition of thanatin which would be in accordance with the slightly higher RapZ amount (Figure 37D, F). Again, no effect of this regulation on the target GlmS was observed (Figure 37C, E). Those observations confirm the hypothesis that thanatin likely elicits the ESR, leading to higher RapZ amounts, which in turn trigger processing of full-length GlmZ by recruitment of RNase E. Based on these results, the impact of thanatin on GlmS levels and in turn synthesis of LPS and peptidoglycan precursors was also addressed under conditions of GlcN6P depletion. The sub-inhibitory concentration of Nva-FMDP (60 μ g/mL) was used in combination with thanatin to determine which of these conditions is more dominant, the upregulation of GlmS due to GlcN6P depletion, achieved by Nva-FMDP, or the enhanced GlmZ processing due to upregulation of RapZ, caused by thanatin. The addition of Nva-FMDP alone or in combination with thanatin resulted in a similar increase in the activity of the *glmY'-lacZ* reporter gene fusion as compared to the untreated cells (Figure 38A). This is in agreement with previous results (Khan *et al.*, 2020) as Nva-FMDP triggers accumulation of sRNA GlmY. The addition of thanatin on top of Nva-FMDP had a weaker impact on the levels of RapZ compared to the previous experiments with ectopic *rpoE* expression (Figure 38C). Furthermore, GlmY amounts did not differ much from those in the untreated cells but were lower than the amounts achieved by the addition of Nva-FMDP alone (Figure 38D). The processing of GlmZ was enhanced due to the addition of thanatin, but simultaneously, the level of full-length GlmZ was increased as well to activate the translation of *glmS* mRNA and counteract GlcN6P depletion, achieved by Nva-FMDP (Figure 38D). Consequently, GlmS levels were highly elevated to overcome GlmS enzymatic inhibition and did not differ much from the levels achieved by the addition of Nva-FMDP alone (Figure 38C). Hence, the effect of thanatin on the system was not strong enough to prevent GlmS upregulation under conditions of GlcN6P starvation even though the processing of GlmZ was enhanced due to slightly higher RapZ amounts. This is the contrary to what was observed previously in experiments with ectopic *rpoE* expression, which leads to artificial over-activation of the *lptAp1* promoter and saturated amounts of RapZ that are exceeding the physiological requirements. Perhaps, different concentrations of thanatin and Nva-FMDP must be used to gain the same effect. Those observations demonstrate that the upregulation of GlmS due to GlcN6P depletion is a more dominant cue under combined conditions of cell envelope stress,

triggered by thanatin, and GlcN6P depletion. However, both of these antimicrobial compounds are not yet commercially available, so more explorative experiments were not possible to alternate the concentrations of thanatin and Nva-FMDP. It would have been interesting to analyze, whether the thanatin concentration used would have led to more prominent effects in the presence of lower Nva-FMDP concentrations. *Escherichia coli* might be able to regulate its envelope stress response in accordance with the availability of GlcN6P and the disruption of the Lpt machinery via regulation of RapZ and thereby coordinate its metabolism with the repair of the outer membrane in the presence of multiple stressors.

7.3. Impact of σ^E on the PTS^{Ntr}

Genes *ptsN* and *npr* of the *rpoN* operon encode proteins working in the nitrogen-related phosphotransferase system (PTS^{Ntr}), a paralogous phosphotransferase system that is phosphorylated on the expense of phosphoenolpyruvate (PEP) (Figure 9) (Pflüger-Grau and Görke, 2010). Various activities are regulated by the PTS^{Ntr} through protein-protein interaction which are predominantly exerted by the non-phosphorylated form of PtsN (EIIA^{Ntr}). In *E. coli*, PtsN stimulates activation of the high affinity potassium KdpFABC transport system when potassium levels are low to maintain the cellular homeostasis (Lüttmann *et al.*, 2009; Lüttmann *et al.*, 2012; Lüttmann *et al.*, 2015; Mörk-Mörkenstein *et al.*, 2017).

Osmotic stress can result in a leaky envelope and consequently, regulation of potassium homeostasis is essential for the maintenance of cell turgor and adaptation of bacteria to osmotic stress (Epstein, 2003). In Gram-positive bacteria, potassium homeostasis is carried out by the second messenger cyclic-di-AMP and kinase KdpD is directly regulated by cyclic-di-AMP in some Gram-positive species (Moscoso *et al.*, 2016; Gibhardt *et al.*, 2019). Thus, it can be speculated the PTS^{Ntr} takes over this function in Gram-negative bacteria. Additionally, the preliminary unpublished work suggests that σ^E activates *kdpFABC* expression through the employment of PTS^{Ntr} and SixA to achieve potassium homeostasis (Sikora and Görke, unpublished). Intriguingly, Hayden and Ades (2008) have reported that *ptsN* is a suppressor of σ^E essentiality as the overexpression of *ptsN* lowers multiple ESRs (Rcs, Cpx) and reduces stress in the cell envelope by an unknown mechanism (Hayden and Ades, 2008).

It was recently discovered that NPr can be dephosphorylated by phosphohistidine phosphatase SixA (Schulte and Goulian, 2018; Schulte *et al.*, 2020) which was suggested to be part of the σ^E regulon (Rezuchova *et al.*, 2003). The expression of *sixA* is regulated by two overlapping promoters, the σ^{70} -dependent *sixAp1* and the σ^E -dependent *sixAp2* promoter (Rezuchova *et al.*, 2003; Rhodius *et al.*, 2006; Wade *et al.*, 2006). According to the unpublished preliminary work, σ^E triggers activity of the PTS^{Ntr} by a dual mechanism: a σ^E -

dependent upregulation of PtsN and NPr amounts (through a burst in *rpoN* operon expression) and its further dephosphorylation through employment of SixA, which results in accumulation of non-phosphorylated PtsN and consequently expression of *kdpFABC* (Sikora and Görke, unpublished). The activation of the *sixAp2* promoter upon ectopic *rpoE* expression was initially confirmed by the reporter gene assay in the strain carrying the chromosomal *sixA'-lacZ* reporter fusion which resulted in more than 20-fold induction of the β -galactosidase activity as a maximum induction of the fusion (Figure 47), confirming that SixA is indeed upregulated by σ^E . Since no other physiological conditions or genetic alterations leading to activation of SixA, and in turn up-regulation of the KdpFABC transporter, are known so far, a global screening approach was employed in order to indentify mutations that lead to upregulation of *sixA* expression. Therefore, a random mutagenesis screen using the *mTn10* delivery plasmid pKESK18 (Madhusudan *et al.*, 2005) was carried out in the strain carrying the *sixA'-lacZ* reporter gene fusion, comprising the σ^{70} *sixAp1* and the σ^E *sixAp2* promoter, to search for genes that lead to upregulation of *sixA* expression. Unfortunately, the screening procedure did not yield any transposon mutants that exhibited elevated *sixA* expression levels compared to the *wild-type* (Figure 48) which led to the conclusion that the *sixA'-lacZ* reporter fusion is not well suitable for screening. The initial transformation of the strain with the *rpoE* expression plasmid creates an artificial setup which leads to an extreme, non-physiological, σ^E ESR. Upon ectopic *rpoE* expression, the fold-change measured for the *sixA'-lacZ* fusion was just 20-fold which demonstrates a much lower upregulation of the fusion as compared to the *lptA'-lacZ* reporter gene fusion that was also used for screening and resulted in more than 400-fold induction of the β -galactosidase activity (Supplementary Figure S1A). Thus, the screening approach did not yield any results as the difference in *sixA'-lacZ* expression was not distinct enough to clearly identify any transposon mutants on LB-agar with X-Gal. One explanation could be that the *Tn10* insertions activating *sixA* expression are lethal or mutations upregulating SixA do not exist. Possibly, an activator protein is needed to express *sixA* from the σ^E -dependent *sixAp2* promoter. Furthermore, genetic alterations that could lead to activation of SixA could bring important insights into the functional connections between PtsN and the regulated potassium transporters, since the physiological conditions leading to dephosphorylation of PtsN are so far not completely clear.

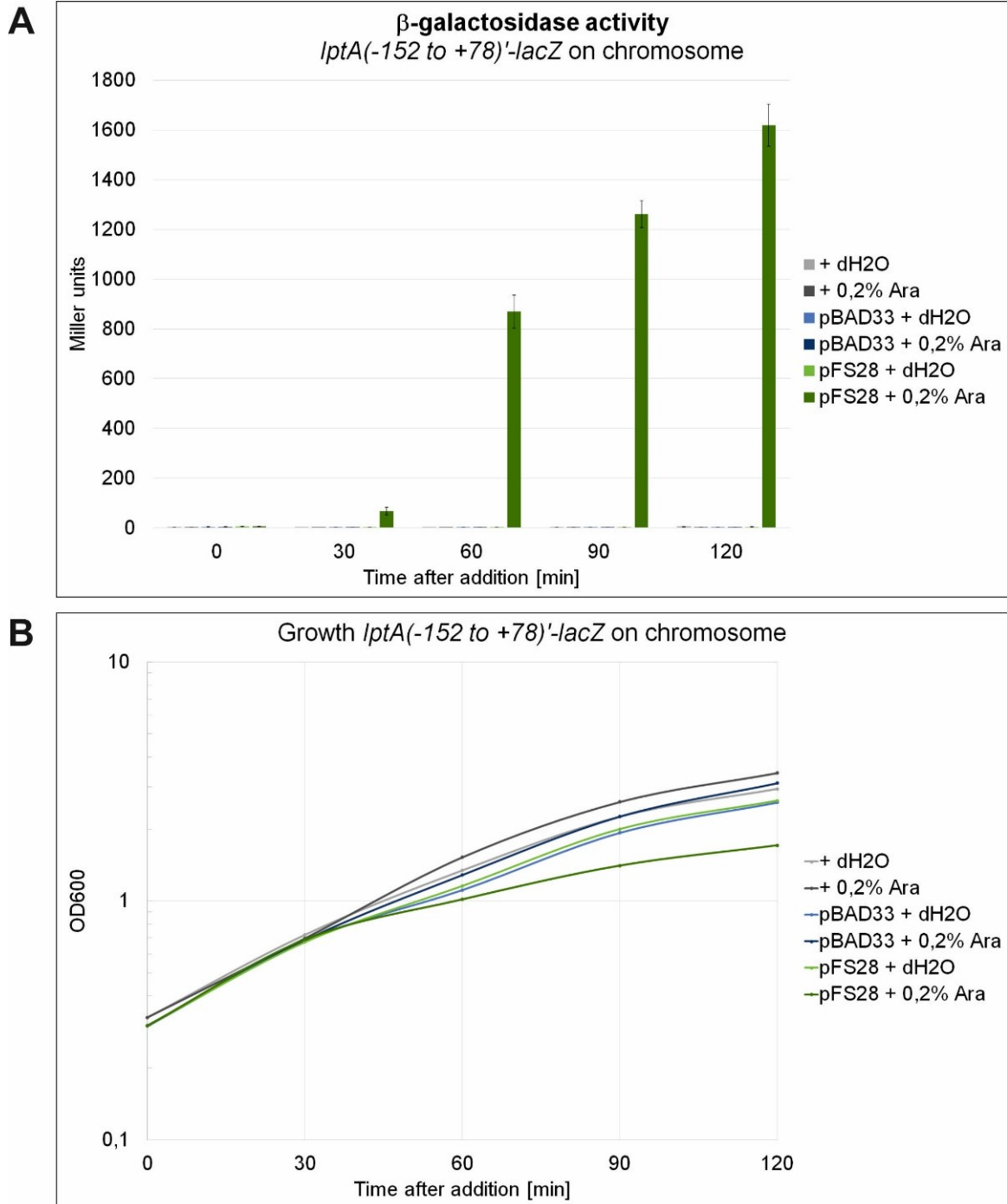
7.4. Final conclusions and outlook

The unpublished preliminary work indicates that the *rpoN* operon is a member of the σ^E regulon as all proteins of the *rpoN* operon accumulate enormously in response to σ^E over production. We hypothesize that the *rpoN* operon represents an important layer of σ^E ESR since all gene products of the *rpoN* operon (except for *rpoN* itself) exert regulatory functions

at the post-transcriptional (RapZ) and post-translational level (Hpf, PTSNtr). Thus, they might help bacteria to counteract envelope stress by interaction with target proteins or small regulatory RNAs through multiple mechanisms. Higher levels of proteins of the *rpoN* operon may activate downstream processes contributing to stress recovery, like activation of potassium uptake by PtsN and NPr, modulation of cell envelope precursor synthesis by RapZ, protection of ribosomes under stress conditions by Hpf and activation of the σ^{54} regulon. The co-localization of genes encoded in the *rpoN* operon reflects their involvement in regulation of processes maintaining the cell envelope integrity. PtsN and NPr are part of the nitrogen-related phosphotransferase system which regulates potassium homeostasis, RapZ contributes to the cell envelope precursor homeostasis by regulating *glmS* expression via sRNAs GlmY and GlmZ, and NPr and Hpf protects ribosomes under stress conditions.

The results obtained in this study revealed that σ^E overexpression triggers accumulation of protein RapZ, likely mediated by read-through transcription from the upstream located *lptAp1* promoter or by another σ^E -responsive element within the *rpoN* operon, or a combination of these regulatory elements. The σ^E -dependent accumulation of RapZ enhances the cleavage of the full-length GlmZ and prevents upregulation of GlmS even under conditions of GlcN6P depletion. Thus, during cell envelope stress, which induces a σ^E -response, *de novo* synthesis of the cell envelope precursors is inhibited until repair of the damage in the envelope has been achieved. Furthermore, the *lptAp1* promoter is activated by the CAMP thanatin which is one of the few known inhibitors of the LPS transport machinery. The mutations in genes that were identified to activate the *lptAp1* promoter are involved in LPS biogenesis and composition and further strengthen the observations that *lptAp1* promoter is indeed activated upon LPS or outer membrane defects.

8. Supplementary data



Supplementary Figure S1. Ectopic *rpoE* expression activates *lptAp1* promoter. **A.** β -galactosidase activity assay addressing the expression of the chromosomal *lptA*'-*lacZ* reporter fusion in *E. coli*. Strain Z1297 (*S4197 ptsN-3xFLAG [attB::lptA(-152 to +78)']-lacZ*) was transformed with the *rpoE* expression plasmid (pFS28) or the corresponding VC (pBAD33). Transformants were grown in LB supplemented with chloramphenicol +/- 0,2% arabinose. Empty plasmid pBAD33 provided the VC and the untransformed *wild-type* strain was analyzed in parallel for comparison. Miller unit values are presented as mean $\pm$ SD that derived from three independent measurements ($n = 3$). **B.** The growth of the cultures addressing the expression of the chromosomal *lptA*'-*lacZ* reporter fusion in *E. coli*. OD₆₀₀ values are presented as mean ($n = 3$). Measurements were performed and data were provided by Florian Sikora (unpublished).

Supplementary Table S2. Raw data for Figure 23 (n = 5).

Average OD₆₀₀ (SD)	S4197 + dH₂O	S4197 + 0,2% Ara	S4197 + pBAD33 + dH₂O	S4197 + pBAD33 + 0,2% Ara	S4197 + pFS28 + dH₂O	S4197 + pFS28 + 0,2% Ara
Time after addition [min]						
0	0,31 (0,02)	0,31 (0,02)	0,33 (0,03)	0,33 (0,03)	0,33 (0,03)	0,33 (0,03)
30	0,67 (0,01)	0,67 (0,02)	0,71 (0,04)	0,71 (0,05)	0,67 (0,06)	0,67 (0,06)
60	1,27 (0,2)	1,45 (0,32)	1,3 (0,24)	1,43 (0,4)	1,2 (0,2)	1,05 (0,17)
90	1,99 (0,06)	2,25 (0,14)	2,1 (0,25)	2,36 (0,26)	1,99 (0,19)	1,36 (0,13)
120	2,64 (0,01)	3,25 (0,14)	2,74 (0,14)	3,16 (0,15)	2,69 (0,23)	1,6 (0,09)

Supplementary Table S3. Raw data for Figure 25 (n = 3).

Average Miller units (SD)	Z741 + dH₂O	Z741 + 0,2% Ara	Z741 + pBAD33 + dH₂O	Z741 + pBAD33 + 0,2% Ara	Z741 + pFS28 + dH₂O	Z741 + pFS28 + 0,2% Ara
Average OD₆₀₀ (SD)						
Time after addition [min]						
0	1417,88 (276,08)	1417,88 (276,08)	1714,89 (113,95)	1714,89 (113,95)	1769,30 (260,98)	1769,30 (260,98)
	0,35 (0,04)	0,35 (0,04)	0,3 (0,05)	0,3 (0,05)	0,35 (0,04)	0,35 (0,04)
30	880,91 (95,91)	919,7 (97,5)	1034,37 (150,8)	976,67 (176,54)	1085,39 (103,63)	1068,39 (157,02)
	0,72 (0,03)	0,72 (0,03)	0,64 (0,05)	0,64 (0,05)	0,71 (0,06)	0,67 (0,05)
60	839,97 (107,14)	971,51 (172,51)	819,98 (108,01)	953,25 (151,79)	904,9 (52,4)	813,49 (160,24)
	1,37 (0,13)	1,47 (0,17)	1,02 (0,13)	1,24 (0,21)	1,26 (0,19)	1,05 (0,08)
90	995,57 (174,2)	1199,98 (171,5)	784,68 (141,5)	1104,39 (127,81)	850,82 (193,93)	868,01 (375,53)
	2,2 (0,12)	2,6 (0,12)	1,86 (0,12)	2,26 (0,24)	2,11 (0,22)	1,47 (0,07)
120	1134,75 (227,17)	1616,47 (244,67)	828,65 (61,22)	1412,03 (82,07)	1002,42 (67,79)	597,39 (65,46)
	3,04 (0,12)	3,55 (0,13)	2,74 (0,11)	3,22 (0,1)	2,86 (0,16)	1,78 (0,03)

Supplementary Table S4. Raw data for Figure 25 (n = 3).

Average Miller units (SD)	Z854 + dH ₂ O	Z854 + 0,2% Ara	Z854 + pBAD33 + dH ₂ O	Z854 + pBAD33 + 0,2% Ara	Z854 + pFS28 + dH ₂ O	Z854 + pFS28 + 0,2% Ara
Average OD ₆₀₀ (SD)						
Time after addition [min]						
0	8,89 (1,68)	8,89 (1,68)	4,73 (1,55)	4,73 (1,55)	4,48 (0,96)	4,48 (0,96)
	0,31 (0,02)	0,31 (0,02)	0,33 (0,03)	0,33 (0,03)	0,31 (0,03)	0,31 (0,03)
30	7,9 (1,25)	7,73 (1,42)	4,2 (1,27)	4,61 (1,13)	4,16 (0,94)	4,03 (0,71)
	0,67 (0,01)	0,67 (0,02)	0,71 (0,04)	0,71 (0,05)	0,67 (0,06)	0,67 (0,06)
60	5,13 (0,71)	6,37 (0,51)	2,98 (0,83)	3,38 (0,73)	2,76 (0,39)	5,03 (1,84)
	1,27 (0,2)	1,45 (0,32)	1,3 (0,24)	1,43 (0,4)	1,2 (0,2)	1,05 (0,17)
90	10,67 (2,56)	15,0 (1,25)	3,8 (1,3)	8,23 (2,02)	3,56 (0,94)	8,68 (1,95)
	1,99 (0,06)	2,25 (0,14)	2,1 (0,25)	2,36 (0,26)	1,99 (0,19)	1,36 (0,13)
120	9,49 (1,2)	12,4 (0,83)	4,19 (0,6)	7,42 (1,78)	4,55 (1,21)	10,24 (0,75)
	2,64 (0,01)	3,25 (0,14)	2,74 (0,14)	3,16 (0,15)	2,69 (0,23)	1,6 (0,09)

Supplementary Table S5. Raw data for Supplementary Figure S1 (n = 3). Measurements were performed and data were provided by Florian Sikora (unpublished).

Average Miller units (SD)	Z1297 + dH ₂ O	Z1297 + 0,2% Ara	Z1297 + pBAD33 + dH ₂ O	Z1297 + pBAD33 + 0,2% Ara	Z1297 + pFS28 + dH ₂ O	Z1297 + pFS28 + 0,2% Ara
Average OD ₆₀₀ (SD)						
Time after addition [min]						
0	2,25 (0,08)	2,25 (0,08)	3,36 (0,86)	3,36 (0,86)	5,16 (1,09)	5,16 (1,09)
	0,33 (0,03)	0,33 (0,03)	0,3 (0,00)	0,3 (0,00)	0,30 (0,01)	0,30 (0,01)
30	1,64 (0,01)	1,66 (0,06)	1,82 (0,31)	1,76 (0,33)	2,62 (0,38)	67,09 (15,58)
	0,72 (0,03)	0,7 (0,01)	0,68 (0,01)	0,69 (0,02)	0,67 (0,01)	0,69 (0,01)
60	1,65 (0,11)	1,47 (0,12)	1,95 (0,4)	1,91 (0,43)	2,43 (0,48)	869,5 (66,64)
	1,34	1,52	1,11	1,29	1,16	1,015

	(0,03)	(0,01)	(0,04)	(0,04)	(0,05)	(0,03)
90	3,69 (0,26)	2,8 (0,25)	1,82 (0,37)	3,18 (0,31)	2,31 (0,31)	1260,7 (54,5)
	2,25 (0,05)	2,6 (0,02)	1,93 (0,06)	2,26 (0,06)	2 (0,07)	1,41 (0,04)
120	4,17 (0,29)	3,33 (0,49)	2,72 (0,19)	3,57 (0,41)	4,03 (0,59)	1618,33 (84,98)
	2,94 (0,06)	3,43 (0,1)	2,59 (0,03)	3,11 (0,05)	2,63 (0,11)	1,71 (0,06)

Supplementary Table S6. Raw data for Figure 31 (n = 3).

Average Miller units (SD)	Z1377 + dH₂O	Z1377 + 0,2% Ara	Z1377 + pBAD33 + dH₂O	Z1377 + pBAD33 + 0,2% Ara	Z1377 + pFS28 + dH₂O	Z1377 + pFS28 + 0,2% Ara
Average OD₆₀₀ (SD)						
Time after addition [min]						
0	17,69 (1,81)	17,69 (1,81)	18,39 (1,6)	18,39 (1,6)	21,48 (0,23)	21,48 (0,23)
	0,33 (0,04)	0,33 (0,04)	0,31 (0,01)	0,31 (0,01)	0,32 (0,02)	0,32 (0,02)
30	20,49 (1,3)	18,65 (2,06)	18,743 (1,4)	18,23 (0,93)	20,56 (0,46)	112,12 (28,57)
	0,69 (0,05)	0,71 (0,03)	0,69 (0,01)	0,67 (0,00)	0,69 (0,05)	0,67 (0,01)
60	22,26 (1,11)	24,13 (0,77)	19,82 (1,24)	22,43 (1,72)	21,97 (0,96)	422,84 (58,76)
	1,34 (0,07)	1,47 (0,04)	1,16 (0,1)	1,3 (0,13)	1,23 (0,12)	0,89 (0,02)
90	24,61 (1,23)	32,53 (3,58)	20,66 (1,73)	25,86 (1,82)	20,86 (1,5)	663,65 (56,99)
	2,38 (0,14)	2,66 (0,22)	2,01 (0,12)	2,37 (0,17)	2,16 (0,3)	1,31 (0,12)
120	33,45 (0,65)	41,77 (2,03)	24,95 (1,99)	43,36 (0,81)	27,25 (2,6)	924,07 (187,03)
	2,96 (0,02)	3,45 (0,14)	2,76 (0,09)	3,08 (0,29)	2,85 (0,14)	1,63 (0,32)

Supplementary Table S7. Raw data for Figure 32 (n = 1).

Time after addition [min]	0	30	60	90	120
OD₆₀₀					
S4197 + pBAD33 + dH₂O	0,34	0,72	1,28	2,03	2,69
S4197 + pBAD33 + 0,2% Ara	0,34	0,71	1,46	2,53	2,83
S4197 + pFS28 + dH₂O	0,34	0,69	1,19	1,89	2,47
S4197 + pFS28 + 0,2% Ara	0,34	0,63	0,95	1,19	1,49
S4197 + pFS36 + dH₂O	0,36	0,7	1,38	2,1	2,73

S4197 + pFS36 + 100 ng/mL ATc	0,36	0,74	1,11	1,79	2,77
S4197 + pFS37 + dH₂O	0,37	0,66	1,34	2,15	2,86
S4197 + pFS36 + 25 ng/mL ATc	0,37	0,53	0,65	0,83	0,72
S4197 + pFS36 + 50 ng/mL ATc	0,37	0,56	0,65	0,74	0,76
S4197 + pFS36 + 100 ng/mL ATc	0,37	0,53	0,7	0,78	0,73
S4197 + pFS36 + 200 ng/mL ATc	0,37	0,53	0,6	0,75	0,76

Supplementary Table S8. Raw data for Figure 33 (n = 1).

Miller units	Z1303 + dH₂O	Z1303 + 25 ng/mL ATc	Z1303 + pFS36 + dH₂O	Z1303 + pFS36 + 25 ng/mL ATc	Z1303 + pFS37 + dH₂O	Z1303 + pFS37 + 25 ng/mL ATc
OD₆₀₀						
Time after addition [min]						
0	1,92	1,92	2	2	23,92	23,92
	0,36	0,36	0,34	0,34	0,38	0,38
30	1,54	1,65	1,45	1,57	15,98	373,45
	0,74	0,7	0,73	0,7	0,65	0,52
60	1,7	1,65	1,71	1,5	11,73	999,9
	1,44	1,37	1,17	1,26	1,23	0,59
90	3,3	3,32	2,65	2,39	11,56	1537,07
	2,25	2,07	2	2,13	2,16	0,66
120	3,98	3,75	3,23	3,26	14,6	1535,6
	3,2	2,95	3,03	3,05	2,83	0,81

Supplementary Table S9. Raw data for Figure 34 (n = 1-3).

Strain S4197	ATc conc. [ng/mL]	Time after addition [min]	OD₆₀₀ (SD)	Average β-galactosidase activities (SD)
pFS36 + dH₂O	/	0	0,34	16,13
	/	30	0,79	
	/	60	1,35	20,03
	/	90	2,2	22,18
	/	120	3,02	
pFS36 + ATc	0,5	0	0,34	16,13
		30	0,75	
		60	1,39	20,08
		90	2,48	19,73
		120	3,19	
pFS37 + dH₂O	/	0	0,35 (0,05)	51,89 (8,84)
	/	30	0,73 (0,07)	
	/	60	1,44 (0,16)	33,95 (2,19)
	/	90	2,3 (0,11)	33,86 (3,32)

	/	120	2,87 (0,17)	
pFS37 + ATc	12,5	0	0,3	62,07
		30	0,54	
		60	0,59	434,2
		90	0,74	611,67
		120	0,79	
pFS37 + ATc	10	0	0,3	62,07
		30	0,55	
		60	0,59	442,82
		90	0,73	621,49
		120	0,8	
pFS37 + ATc	7,5	0	0,3	62,07
		30	0,54	
		60	0,59	448,13
		90	0,74	642,35
		120	0,82	
pFS37 + ATc	5	0	0,3	62,07
		30	0,55	
		60	0,61	460,74
		90	0,75	672,61
		120	0,82	
pFS37 + ATc	2,5	0	0,3	62,07
		30	0,56	
		60	0,67	589,62
		90	0,86	846,51
		120	0,94	
pFS37 + ATc	2	0	0,3	62,07
		30	0,58	
		60	0,67	573,9
		90	0,94	717,49
		120	0,95	
pFS37 + ATc	1,5	0	0,3	62,07
		30	0,57	
		60	0,68	699,81
		90	0,9	726,42
		120	0,94	
pFS37 + ATc	1	0	0,35 (0,07)	54,76 (10,33)
		30	0,62 (0,05)	
		60	0,74 (0,06)	643,16 (86,32)
		90	0,94 (0,03)	801,03 (71,86)
		120	1 (0,03)	
pFS37 + ATc	0,9	0	0,39	47,46
		30	0,67	
		60	0,79	614,73

		90	0,98	619,69
		120	1,06	
pFS37 + ATc	0,8	0	0,39	47,46
		30	0,69	
		60	0,84	557,85
		90	0,99	527,38
		120	1,16	
pFS37 + ATc	0,75	0	0,39	47,46
		30	0,69	
		60	0,86	495,77
		90	1,09	412,35
		120	1,2	
pFS37 + ATc	0,7	0	0,39	47,46
		30	0,69	
		60	0,89	483,38
		90	1,12	431
		120	1,28	
pFS37 + ATc	0,6	0	0,39	47,46
		30	0,71	
		60	0,9	400,36
		90	1,17	361,34
		120	1,28	
pFS37 + ATc	0,5	0	0,35 (0,05)	54,1 (11,26)
		30	0,66 (0,06)	
		60	0,87 (0,07)	383,13 (35,99)
		90	1,21 (0,11)	340,99 (46,45)
		120	1,42 (0,06)	
pFS37 + ATc	0,4	0	0,34	46,15
		30	0,69	
		60	0,98	382,52
		90	1,55	301,02
		120	2,07	
pFS37 + ATc	0,3	0	0,34	46,15
		30	0,71	
		60	1,05	248,51
		90	1,84	184,4
		120	2,21	
pFS37 + ATc	0,25	0	0,34	46,15
		30	0,72	
		60	1,1	194,43
		90	1,91	137,13
		120	2,34	

Supplementary Table S10. Raw data for Figure 35 (n = 1).

Miller units	Z1303 + dH ₂ O	Z1303 + 5,25 µg/mL Thanatin	Z1378 + dH ₂ O	Z1378 + 5,25 µg/mL Thanatin
OD ₆₀₀				
Time after addition [min]				
0	1,77	1,77	17,01	17,01
	0,1	0,1	0,12	0,12
30	1,62	2,64	13,84	14,22
	0,26	0,26	0,21	0,22
45	1,22	2,73	15,95	21,58
	0,44	0,29	0,41	0,31
60	1,11	5,12	18,52	28,42
	0,56	0,32	0,65	0,32
90	1,45	6,36	26,03	52,56
	0,96	0,25	1,13	0,22
120	3,06	9,24	23,65	49,4
	1,62	0,17	2,23	0,17

Supplementary Table S11. Raw data for Figure 37 (n = 1-2).

Strain	Average OD ₆₀₀ (SD)	Average Miller units	SD
Z741 + dH ₂ O 0'	0,12 (0,01)	2155,63	296,91
Z741 + dH ₂ O 90'	1,64 (0,12)	931,27	18,42
Z741 + 5,25 µg/mL Thanatin 90'	0,23 (0,05)	2116,85	307,92
Z833 + dH ₂ O 0'	0,12	1369,38	/
Z833 + dH ₂ O 90'	1,56	1073,57	/
Z833 + 5,25 µg/mL Thanatin 90'	0,29	1681,99	/

Supplementary Table S12. Raw data for Figure 38 (n = 1).

Strain	OD ₆₀₀	Miller units
Z741 + dH ₂ O 0'	0,14	2435,36
Z741 + dH ₂ O 90'	1,5	1066,61
Z741 + 60 µg/mL Nva-FMDP 90'	1,43	1452,32
Z741 + 60 µg/mL Nva-FMDP + 5,25 µg/mL Thanatin 90'	0,45	1397,44

Supplementary Table S13. Raw data for Figure 41 (n = 3-5).

Strain	Average Miller units	SD
Z1303 <i>wild-type</i> OD ₆₀₀ ~ 0,3	1,67	0,04
Z1303 <i>wild-type</i> OD ₆₀₀ ~ 1	1,67	0,05
Z1303 <i>wild-type</i> ONC	5,56	0,17
SC #2 <i>waaQ</i> OD ₆₀₀ ~ 0,3	2,85	0,54
SC #2 <i>waaQ</i> OD ₆₀₀ ~ 1	6,71	0,37
SC #2 <i>waaQ</i> ONC	8,91	0,86
SC #7 <i>waaQ</i> OD ₆₀₀ ~ 0,3	3,17	0,26

SC #7 <i>waaQ</i> OD ₆₀₀ ~ 1	4,87	0,46
SC #7 <i>waaQ</i> ONC	8,51	1,25
SC #3 <i>waaG</i> OD ₆₀₀ ~ 0,3	4,48	0,33
SC #3 <i>waaG</i> OD ₆₀₀ ~ 1	7,09	1,3
SC #3 <i>waaG</i> ONC	11,17	1,18
SC #4 <i>waaG</i> OD ₆₀₀ ~ 0,3	3,52	0,34
SC #4 <i>waaG</i> OD ₆₀₀ ~ 1	11,66	1,82
SC #4 <i>waaG</i> ONC	8,3	1,39
SC #11 <i>surA</i> OD ₆₀₀ ~ 0,3	4,74	0,36
SC #11 <i>surA</i> OD ₆₀₀ ~ 1	7,99	1
SC #11 <i>surA</i> ONC	10,09	0,43
SC #1 <i>rseA</i> OD ₆₀₀ ~ 0,3	20,75	4,69
SC #1 <i>rseA</i> OD ₆₀₀ ~ 1	23,02	11,25
SC #1 <i>rseA</i> ONC	61,16	10,82
SC #10 <i>rseA</i> OD ₆₀₀ ~ 0,3	10,03	1,65
SC #10 <i>rseA</i> OD ₆₀₀ ~ 1	18,93	2,54
SC #10 <i>rseA</i> ONC	35,5	2,35
SC #5 <i>hfq</i> OD ₆₀₀ ~ 0,3	18,68	1,03
SC #5 <i>hfq</i> OD ₆₀₀ ~ 1	27,59	4,33
SC #5 <i>hfq</i> ONC	72,2	7,97
SC #9 <i>hfq</i> OD ₆₀₀ ~ 0,3	17,6	0,85
SC #9 <i>hfq</i> OD ₆₀₀ ~ 1	23,32	0,72
SC #9 <i>hfq</i> ONC	65,9	1,79
SC #6 <i>hfq</i> OD ₆₀₀ ~ 0,3	19,33	0,8
SC #6 <i>hfq</i> OD ₆₀₀ ~ 1	20,73	0,09
SC #6 <i>hfq</i> ONC	65,16	0,47
SC #8 <i>hfq</i> OD ₆₀₀ ~ 0,3	20,42	5,13
SC #8 <i>hfq</i> OD ₆₀₀ ~ 1	24,39	3,7
SC #8 <i>hfq</i> ONC	78,09	14,72

Supplementary Table S14. Raw data for Figures 42, 43 and 44 (n = 3-5).

Strain	Average Miller units	SD
Z1303 <i>wild-type</i> OD ₆₀₀ ~ 0,3	1,67	0,04
Z1303 <i>wild-type</i> OD ₆₀₀ ~ 1	1,67	0,05
Z1303 <i>wild-type</i> ONC	5,56	0,17
SC #5 <i>hfq</i> OD ₆₀₀ ~ 0,3	18,68	1,03
SC #5 <i>hfq</i> OD ₆₀₀ ~ 1	27,59	4,33
SC #5 <i>hfq</i> ONC	72,2	7,97
SC #9 <i>hfq</i> OD ₆₀₀ ~ 0,3	17,6	0,85
SC #9 <i>hfq</i> OD ₆₀₀ ~ 1	23,32	0,72
SC #9 <i>hfq</i> ONC	65,9	1,79
SC #6 <i>hfq</i> OD ₆₀₀ ~ 0,3	19,33	0,8
SC #6 <i>hfq</i> OD ₆₀₀ ~ 1	20,73	0,09
SC #6 <i>hfq</i> ONC	65,16	0,47
SC #8 <i>hfq</i> OD ₆₀₀ ~ 0,3	20,42	5,13
SC #8 <i>hfq</i> OD ₆₀₀ ~ 1	24,39	3,7
SC #8 <i>hfq</i> ONC	78,09	14,72

Z1307 <i>hfq::kan</i> OD₆₀₀ ~ 0,3	22,94	0,33
Z1307 <i>hfq::kan</i> OD₆₀₀ ~ 1	20,53	1,37
Z1307 <i>hfq::kan</i> ONC	78,49	0,00
Z1350 Δhfq OD₆₀₀ ~ 0,3	28,76	0,29
Z1350 Δhfq OD₆₀₀ ~ 1	23,96	1,85
Z1350 Δhfq ONC	122,29	5,16
SC #1 <i>rseA</i> OD₆₀₀ ~ 0,3	20,75	4,69
SC #1 <i>rseA</i> OD₆₀₀ ~ 1	23,02	11,25
SC #1 <i>rseA</i> ONC	61,16	10,82
SC #10 <i>rseA</i> OD₆₀₀ ~ 0,3	10,03	1,65
SC #10 <i>rseA</i> OD₆₀₀ ~ 1	18,93	2,54
SC #10 <i>rseA</i> ONC	35,5	2,35
Z1324A <i>rseA::kan</i> OD₆₀₀ ~ 0,3	8,06	1,02
Z1324A <i>rseA::kan</i> OD₆₀₀ ~ 1	16,58	2,93
Z1324A <i>rseA::kan</i> ONC	31,66	10,99
Z1351A $\Delta rseA$ OD₆₀₀ ~ 0,3	7,72	1,36
Z1351A $\Delta rseA$ OD₆₀₀ ~ 1	15,45	0,2
Z1351A $\Delta rseA$ ONC	29,96	0,19
Z1324B <i>rseA::kan</i> OD₆₀₀ ~ 0,3	40,28	9,59
Z1324B <i>rseA::kan</i> OD₆₀₀ ~ 1	49,48	0,45
Z1324B <i>rseA::kan</i> ONC	119,16	26,63
Z1351B $\Delta rseA$ OD₆₀₀ ~ 0,3	24,49	1,04
Z1351B $\Delta rseA$ OD₆₀₀ ~ 1	48,47	12
Z1351B $\Delta rseA$ ONC	107,46	10,12
SC #2 <i>waaQ</i> OD₆₀₀ ~ 0,3	2,85	0,54
SC #2 <i>waaQ</i> OD₆₀₀ ~ 1	6,71	0,37
SC #2 <i>waaQ</i> ONC	8,91	0,86
SC #7 <i>waaQ</i> OD₆₀₀ ~ 0,3	3,17	0,26
SC #7 <i>waaQ</i> OD₆₀₀ ~ 1	4,87	0,46
SC #7 <i>waaQ</i> ONC	8,51	1,3
Z1326 <i>waaQ::kan</i> OD₆₀₀ ~ 0,3	2,08	0,2
Z1326 <i>waaQ::kan</i> OD₆₀₀ ~ 1	1,85	0,09
Z1326 <i>waaQ::kan</i> ONC	5,93	0,77
Z1353 $\Delta waaQ$ OD₆₀₀ ~ 0,3	1,8	0,16
Z1353 $\Delta waaQ$ OD₆₀₀ ~ 1	1,93	0,22
Z1353 $\Delta waaQ$ ONC	6,1	0,49
SC #3 <i>waaG</i> OD₆₀₀ ~ 0,3	4,48	0,33
SC #3 <i>waaG</i> OD₆₀₀ ~ 1	7,09	1,3
SC #3 <i>waaG</i> ONC	11,17	1,18
SC #4 <i>waaG</i> OD₆₀₀ ~ 0,3	3,52	0,34
SC #4 <i>waaG</i> OD₆₀₀ ~ 1	11,66	1,82
SC #4 <i>waaG</i> ONC	8,3	1,39
Z1325 <i>waaG::kan</i> OD₆₀₀ ~ 0,3	1,94	0,03
Z1325 <i>waaG::kan</i> OD₆₀₀ ~ 1	2,02	0,09
Z1325 <i>waaG::kan</i> ONC	5,87	0,31
Z1352 $\Delta waaG$ OD₆₀₀ ~ 0,3	1,86	0,08
Z1352 $\Delta waaG$ OD₆₀₀ ~ 1	1,95	0,01
Z1352 $\Delta waaG$ ONC	7,43	0,07

Supplementary Table S15. Raw data for Figure 45 (n = 3-5).

Strain	Average OD ₆₀₀ (SD)	Average Miller units (SD)
Z1303 <i>wild-type</i> 0'	0,35 (0,02)	2,03 (0,15)
Z1303 <i>wild-type</i> + dH₂O 30'	0,77 (0,03)	1,81 (0,07)
Z1303 <i>wild-type</i> + dH₂O 60'	1,43 (0,09)	1,77 (0,03)
Z1303 <i>wild-type</i> + dH₂O 90'	2,28 (0,03)	3,27 (0,2)
Z1303 <i>wild-type</i> + 0,2% Ara 30'	0,79 (0,03)	1,72 (0,06)
Z1303 <i>wild-type</i> + 0,2% Ara 60'	1,54 (0,01)	1,6 (0,07)
Z1303 <i>wild-type</i> + 0,2% Ara 90'	2,59 (0,03)	3,33 (0,07)
Z1303 <i>wild-type</i> + pAraX 0'	0,36 (0,03)	2,03 (0,12)
Z1303 <i>wild-type</i> + pAraX + dH₂O 30'	0,76 (0,04)	1,65 (0,11)
Z1303 <i>wild-type</i> + pAraX + dH₂O 60'	1,51 (0,11)	1,74 (0,12)
Z1303 <i>wild-type</i> + pAraX + dH₂O 90'	2,32 (0,07)	2,57 (0,08)
Z1303 <i>wild-type</i> + pAraX + 0,2% Ara 30'	0,77 (0,06)	1,6 (0,04)
Z1303 <i>wild-type</i> + pAraX + 0,2% Ara 60'	1,68 (0,08)	1,47 (0,09)
Z1303 <i>wild-type</i> + pAraX + 0,2% Ara 90'	2,72 (0,17)	2,7 (0,13)
SC #11 <i>surA</i> 0'	0,33 (0,04)	4,9 (0,06)
SC #11 <i>surA</i> + dH₂O 30'	0,71 (0,06)	6,19 (0,03)
SC #11 <i>surA</i> + dH₂O 60'	1,37 (0,18)	8,74 (0,6)
SC #11 <i>surA</i> + dH₂O 90'	2,04 (0,36)	11,81 (1,24)
SC #11 <i>surA</i> + 0,2% Ara 30'	0,71 (0,08)	6,54 (0,04)
SC #11 <i>surA</i> + 0,2% Ara 60'	1,41 (0,09)	8,21 (0,11)
SC #11 <i>surA</i> + 0,2% Ara 90'	2,47 (0,21)	9,61 (0,27)
SC #11 <i>surA</i> + pAraX 0'	0,31 (0,02)	3,99 (0,02)
SC #11 <i>surA</i> + pAraX + dH₂O 30'	0,69 (0,03)	5,83 (0,14)

SC #11 <i>surA</i> + pAraX + dH₂O 60'	1,39 (0,13)	8,15 (0,25)
SC #11 <i>surA</i> + pAraX + dH₂O 90'	2,14 (0,06)	9,49 (0,51)
SC #11 <i>surA</i> + pAraX + 0,2% Ara 30'	0,7 (0,05)	4,48 (1,1)
SC #11 <i>surA</i> + pAraX + 0,2% Ara 60'	1,49 (0,12)	8,46 (1,02)
SC #11 <i>surA</i> + pAraX + 0,2% Ara 90'	2,36 (0,02)	9,3 (0,06)
SC #11 <i>surA</i> + pFS38 0'	0,33 (0,06)	2,61 (0,3)
SC #11 <i>surA</i> + pFS38 + dH₂O 30'	0,69 (0,09)	3,59 (0,49)
SC #11 <i>surA</i> + pFS38 + dH₂O 60'	1,3 (0,19)	5,92 (1,1)
SC #11 <i>surA</i> + pFS38 + dH₂O 90'	2,1 (0,21)	8,26 (1)
SC #11 <i>surA</i> + pFS38 + 0,2% Ara 30'	0,72 (0,07)	2,24 (0,1)
SC #11 <i>surA</i> + pFS38 + 0,2% Ara 60'	1,41 (0,21)	1,74 (0,1)
SC #11 <i>surA</i> + pFS38 + 0,2% Ara 90'	2,66 (0,17)	2,71 (0,33)

Supplementary Table S16. Raw data for Figure 47 (n = 1).

Miller units	Z1315 + dH ₂ O	Z1315 + 0,2% Ara	Z1315 + pBAD33 + dH ₂ O	Z1315 + pBAD33 + 0,2% Ara	Z1315 + pFS28 + dH ₂ O	Z1315 + pFS28 + 0,2% Ara
OD ₆₀₀						
Time after addition [min]						
0	2,7	2,7	2,55	2,55	2,87	2,87
	0,35	0,35	0,3	0,3	0,3	0,3
30	1,6	1,94	1,56	1,49	2,08	3,21
	0,73	0,72	0,69	0,705	0,64	0,63
60	1,6	1,72	1,34	1,31	1,51	19,84
	1,58	1,58	1,34	1,46	1,38	1,12
90	2,02	2,42	2,14	1,86	1,74	34,03
	2,24	2,27	1,99	2,26	2,18	1,46
120	2,31	2,92	1,77	2,4	2	43,678
	2,82	2,79	2,65	2,84	2,67	1,885

Supplementary Table S17. Raw data for Figure 48 (n = 3).

Strain	Average Miller units	SD
Z1315 <i>wild-type</i> OD₆₀₀ ~ 0,3	3,09	0,16
Z1315 <i>wild-type</i> OD₆₀₀ ~ 1	2	0,27
Z1315 <i>wild-type</i> ONC	11,43	0,46
SC #1 OD₆₀₀ ~ 0,3	3,54	0,91
SC #1 OD₆₀₀ ~ 1	2,55	0,76
SC #1 ONC	14,24	3,53
SC #2 OD₆₀₀ ~ 0,3	3,58	0,69
SC #2 OD₆₀₀ ~ 1	2,27	0,36
SC #2 ONC	13,65	4,19
SC #3 OD₆₀₀ ~ 0,3	3,44	0,63
SC #3 OD₆₀₀ ~ 1	2,2	0,38
SC #3 ONC	13,25	3,68
SC #4 OD₆₀₀ ~ 0,3	3,53	0,65
SC #4 OD₆₀₀ ~ 1	2,17	0,43
SC #4 ONC	13,5	3,59
SC #5 OD₆₀₀ ~ 0,3	3,68	0,88
SC #5 OD₆₀₀ ~ 1	2,27	0,29
SC #5 ONC	13,03	4,97
SC #6 OD₆₀₀ ~ 0,3	3,34	0,45
SC #6 OD₆₀₀ ~ 1	2,28	0,22
SC #6 ONC	12,51	2,23
SC #7 OD₆₀₀ ~ 0,3	3	0,51
SC #7 OD₆₀₀ ~ 1	2,33	0,27
SC #7 ONC	11,8	2,04
SC #8 OD₆₀₀ ~ 0,3	3,19	0,66
SC #8 OD₆₀₀ ~ 1	2,04	0,23
SC #8 ONC	13,58	5,54
SC #9 OD₆₀₀ ~ 0,3	3,46	0,73
SC #9 OD₆₀₀ ~ 1	2,33	0,61

SC #9 ONC	13,61	4,55
SC #10 OD₆₀₀ ~ 0,3	3,13	0,61
SC #10 OD₆₀₀ ~ 1	2,03	0,31
SC #10 ONC	11,87	3,81

9. Zusammenfassung

Die Zellhülle von Gram-negativen Bakterien ist eine wichtige Barriere, die die Zellen vor Veränderungen in ihrer Umgebung und schädlichen Chemikalien, wie zum Beispiel Antibiotika, schützt. Um die Schäden an ihrer Zellhülle zu überstehen, evolvierten Bakterien komplexe Signaltransduktionswege. In *Escherichia coli* nimmt der Sigma-Faktor σ^E eine Schlüsselrolle in der Zellhüllstressantwort ein, aber die von σ^E vermittelte Antwort ist noch nicht vollständig aufgeklärt.

Vor kurzem wurde entdeckt, dass die Induktion der σ^E -Antwort die Expression des *rpoN* Operons über den oberhalb liegenden *lptAp1* Promoter stark hochreguliert. In dieser Arbeit wurde der *lptAp1* Promoter durch Transposonmutagenese und die Zugabe des antimikrobiellen Peptids Thanatin charakterisiert. Dabei stellte sich heraus, dass dieser Promoter spezifisch auf Schäden im Lipopolysaccharid reagiert.

Darüber hinaus weisen die Ergebnisse dieser Arbeit darauf hin, dass die Transkription vom *lptAp1* Promoter weit in das *rpoN* Operon hinein durchliest. Dadurch akkumulieren alle Proteine, die in dem Operon kodiert sind, inklusive RapZ, wenn der Sigma-Faktor σ^E aktiviert wird. Das RNA-bindende Protein RapZ kontrolliert die Biosynthese der Zellhüllenbestandteile über die Steuerung der GlmS Mengen unter Berücksichtigung der Glucosamin-6-Phosphat Konzentration und den kleinen RNAs GlmY und GlmZ. Die Ergebnisse zeigten, dass Zellhüllstress die RapZ-Mengen erhöht und dadurch auch die Prozessierung der sRNA GlmZ beeinträchtigt. Somit wird auch die komplexe Regulation von GlmY/GlmZ/*glmS* beeinflusst, wodurch wiederum die Biosynthese von Zellhüllbestandteilen verhindert werden könnte, solange die Zellhülle noch nicht repariert wurde. Des Weiteren wurde eine vermeintliche Funktion von RapZ in der Regulation von Transkripten für Kälte-Schock-Proteine analysiert, aber Ergebnisse einer früheren Transkriptom-Analyse konnten nicht bestätigt werden.

10. References

- Ades, S.E., Connolly, L.E., Alba, B.M., and Gross, C.A. (1999) The *Escherichia coli* σ (E)-dependent extracytoplasmic stress response is controlled by the regulated proteolysis of an anti- σ factor. *Genes Dev* **13**: 2449–2461.
- Aiba, H., and Mizuno, T. (1990) Phosphorylation of a bacterial activator protein, OmpR, by a protein kinase, EnvZ, stimulates the transcription of the *ompF* and *ompC* genes in *Escherichia coli*. *FEBS Lett* **261**: 19–22.
- Akiyama, K., Mizuno, S., Hizukuri, Y., Mori, H., Nogi, T., and Akiyama, Y. (2015) Roles of the membrane-reentrant β -hairpin-like loop of RseP protease in selective substrate cleavage. *Elife* **4**:e08928.
- Akiyama, Y., Kanehara, K., and Ito, K. (2004) RseP (YaeL), an *Escherichia coli* RIP protease, cleaves transmembrane sequences. *EMBO J* **23**: 4434–4442.
- Alphen, W. V., and Lugtenberg, B. (1977) Influence of osmolarity of the growth medium on the outer membrane protein pattern of *Escherichia coli*. *J Bacteriol* **131**: 623–630.
- Anderson, M.S., Bulawa, C.E., and Raetz, C.R.H. (1985) The biosynthesis of gram-negative endotoxin. Formation of lipid A precursors from UDP-GlcNAc in extracts of *Escherichia coli*. *J Biol Chem* **260**: 15536–15541.
- Aravind, L., and Ponting, C.P. (1997) The GAF domain: an evolutionary link between diverse phototransducing proteins. *Trends Biochem Sci* **22**: 458–459.
- Arts, I.S., Gennaris, A., and Collet, J.F. (2015) Reducing systems protecting the bacterial cell envelope from oxidative damage. *FEBS Lett* **589**: 1559–1568.
- Artsimovitch, I., and Landick, R. (2002) The transcriptional regulator RfaH stimulates RNA chain synthesis after recruitment to elongation complexes by the exposed nontemplate DNA strand. *Cell* **109**: 193–203.
- Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., *et al.* (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: The Keio collection. *Mol Syst Biol* **2**:2006.0008.
- Bahr, T., Lüttmann, D., März, W., Rak, B., and Görke, B. (2011) Insight into bacterial phosphotransferase system-mediated signaling by interspecies transplantation of a transcriptional regulator. *J Bacteriol* **193**: 2013–2026.
- Bailey, M.J.A., Hughes, C., and Koronakis, V. (1996) Increased distal gene transcription by

the elongation factor RfaH, a specialized homologue of NusG. *Mol Microbiol* **22**: 729–737.

Bailey, M.J.A., Hughes, C., and Koronakis, V. (2000) *In vitro* recruitment of the RfaH regulatory protein into a specialised transcription complex, directed by the nucleic acid ops element. *Mol Gen Genet* **262**: 1052–1059.

Bak, G., Lee, J., Suk, S., Kim, D., Lee, J.Y., Kim, K.S., *et al.* (2015) Identification of novel sRNAs involved in biofilm formation, motility, and fimbriae formation in *Escherichia coli*. *Sci Rep* **5**: 1–19.

Barabote, R.D., and Saier, M.H. (2005) Comparative genomic analyses of the bacterial phosphotransferase system. *Microbiol Mol Biol Rev* **69**: 608–634.

Belunis, C.J., and Raetz, C.R.H. (1992) Biosynthesis of endotoxins. Purification and catalytic properties of 3- deoxy-D-manno-octulosonic acid transferase from *Escherichia coli*. *J Biol Chem* **267**: 9988–9997.

Berg, B. Van Den, Clemons Jr, W.M., Clemons, W.M., Collinson, I., Modis, Y., Hartmann, E., *et al.* (2004) X-ray structure of a protein-conducting channel. *Nature* **427**: 36–44.

Berks, B.C. (1996) A common export pathway for proteins binding complex redox cofactors? *Mol Microbiol* **22**: 393–404.

Berks, B.C., Palmer, T., and Sargent, F. (2005) Protein targeting by the bacterial twin-arginine translocation (Tat) pathway. *Curr Opin Microbiol* **8**: 174–181.

Bertani, B., and Ruiz, N. (2018) Function and Biogenesis of Lipopolysaccharides. *EcoSal Plus* **8**: 1–33.

Bettenbrock, K., Sauter, T., Jahreis, K., Kremling, A., Lengeler, J.W., and Gilles, E.D. (2007) Correlation between growth rates, EliACrr phosphorylation, and intracellular cyclic AMP levels in *Escherichia coli* K-12. *J Bacteriol* **189**: 6891–6900.

Beutin, L., Manning, P.A., Achtman, M., and Willetts, N. (1981) sfrA and sfrB products of *Escherichia coli* K-12 are transcriptional control factors. *J Bacteriol* **145**: 840–844.

Blattner, F.R., Plunkett, G., Bloch, C.A., Perna, N.T., Burland, V., Riley, M., *et al.* (1997) The complete genome sequence of *Escherichia coli* K-12. *Science* **277**: 1453–1462.

Bodenmiller, D.M., and Spiro, S. (2006) The *yjeB* (*nsrR*) gene of *Escherichia coli* encodes a nitric oxide-sensitive transcriptional regulator. *J Bacteriol* **188**: 874–881.

Bonocora, R.P., Smith, C., Lapierre, P., and Wade, J.T. (2015) Genome-Scale Mapping of *Escherichia coli* σ 54 Reveals Widespread, Conserved Intragenic Binding. *PLoS Genet* **11**: 1–

30.

Botsford, J.L., and Harman, J.G. (1992) Cyclic AMP in prokaryotes. *Microbiol Rev* **56**: 100–122.

Braun, V. (1975) Covalent lipoprotein from the outer membrane of *Escherichia coli*. *BBA - Rev Biomembr* **415**: 335–377.

Brozek, K.A., and Raetz, C.R.H. (1990) Biosynthesis of lipid A in *Escherichia coli*. Acyl carrier protein-dependent incorporation of laurate and myristate. *J Biol Chem* **265**: 15410–15417.

Bullock, W. (1987) XL1-Blue: a high efficiency plasmid transforming *recA Escherichia coli* strain with beta-galactosidase selection. *Biotechniques* **5**: 376–379.

Burke, C., Jankute, M., Moynihan, P., Gonzalez del Rio, R., Li, X., Esquivias, J., *et al.* (2020) Development of a novel secondary phenotypic screen to identify hits within the mycobacterial protein synthesis pipeline. *FASEB BioAdvances* **2**: 600–612.

Bury-Moné, S., Nomane, Y., Reymond, N., Barbet, R., Jacquet, E., Imbeaud, S., *et al.* (2009) Global analysis of extracytoplasmic stress signaling in *Escherichia coli*. *PLoS Genet* **5**: e1000651.

Buurman, E.T., Kim, K.T., and Epstein, W. (1995) Genetic evidence for two sequentially occupied K⁺ binding sites in the Kdp transport ATPase. *J Biol Chem* **270**: 6678–6685.

Cairrão, F., Cruz, A., Mori, H., and Arraiano, C.M. (2003) Cold shock induction of RNase R and its role in the maturation of the quality control mediator SsrA/tmRNA. *Mol Microbiol* **50**: 1349–1360.

Campbell, E.A., Tupy, J.L., Gruber, T.M., Wang, S., Sharp, M.M., and Gross, C.A. (2003) Crystal Structure of *Escherichia coli* sE with the Cytoplasmic Domain of Its Anti-RseA and temperature and chemical stress. *Mol Cell* **11**: 1067–1078.

Carpousis, A.J., Luisi, B.F., and McDowall, K.J. (2009) Endonucleolytic Initiation of mRNA Decay in *Escherichia coli*. *Prog Mol Biol Transl Sci.* **85**: 91–135.

Cezairliyan, B.O., and Sauer, R.T. (2007) Inhibition of regulated proteolysis by RseB. *Proc Natl Acad Sci U S A* **104**: 3771–3776.

Chaba, R., Alba, B.M., Guo, M.S., Sohn, J., Ahuja, N., Sauer, R.T., and Gross, C.A. (2011) Signal integration by DegS and RseB governs the σ^E -mediated envelope stress response in *Escherichia coli*. *Proc Natl Acad Sci U S A* **108**: 2106–2111.

- Chao, Y., Li, L., Girodat, D., Förstner, K.U., Said, N., Corcoran, C., *et al.* (2017) *In vivo* Cleavage Map Illuminates the Central Role of RNase E in Coding and Non-coding RNA Pathways. *Mol Cell* **65**: 39–51.
- Chen, H., Shiroguchi, K., Ge, H., and Xie, X.S. (2015) Genome-wide study of mRNA degradation and transcript elongation in *Escherichia coli*. *Mol Syst Biol* **11**(5):808.
- Chen, H.D., and Groisman, E.A. (2013) The biology of the PmrA/PmrB two-component system: the major regulator of lipopolysaccharide modifications. *Annu Rev Microbiol* **67**: 83–112.
- Chmara, H., Milewski, S., Andruszkiewicz, R., Mignini, F., and Borowski, E. (1998) Antibacterial action of dipeptides containing an inhibitor of glucosamine-6-phosphate isomerase. *Microbiology* **144**: 1349–1358.
- Cho, S.H., Szewczyk, J., Pesavento, C., Zietek, M., Banzhaf, M., Roszczenko, P., *et al.* (2014) Detecting envelope stress by monitoring β -barrel assembly. *Cell* **159**: 1652–1664.
- Chun, K.T., Edenberg, H.J., Kelley, M.R., and Goebel, M.G. (1997) Rapid amplification of uncharacterized transposon-tagged DNA sequences from genomic DNA. *Yeast* **13**: 233–240.
- Clarke, J.E., Kime, L., Romero A., D., and McDowall, K.J. (2014) Direct entry by RNase E is a major pathway for the degradation and processing of RNA in *Escherichia coli*. *Nucleic Acids Res* **42**: 11733–11751.
- Cleveland, D.W., Fischer, S.G., Kirschner M.W., and Laemmli, U.K. (1977) Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J Biol Chem* **252**: 1102–1106.
- Cozzzone, A.J. (1998) Post-translational modification of proteins by reversible phosphorylation in prokaryotes. *Biochimie* **80**: 43–48.
- Czapski, T.R., and Trun, N. (2014) Expression of *csp* genes in *E. Coli* K-12 in defined rich and defined minimal media during normal growth, and after cold-shock. *Gene* **547**: 91–97.
- Dai, X., Yuan, M., Lu, Y., Zhu, X., Liu, C., Zheng, Y., *et al.* (2022) Identification of a Small Molecule That Inhibits the Interaction of LPS Transporters LptA and LptC. *Antibiotics* **11**(10):1385.
- Dartigalongue, C., Missiakas, D., and Raina, S. (2001) Characterization of the *Escherichia coli* σ E Regulon. *J Biol Chem* **276**: 20866–20875.
- Datsenko, K.A., and Wanner, B.L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* **97**: 6640–6645.

- Deuschle, M., Limbrunner, S., Rother, D., Wahler, S., Chavarría, M., Lorenzo, V. de, *et al.* (2015) Interplay of the PtsN (EIIANtr) protein of *Pseudomonas putida* with its target sensor kinase KdpD. *Environ Microbiol Rep* **7**: 899–907.
- Deutscher, J., Aké, F.M.D., Derkaoui, M., Zébré, A.C., Cao, T.N., Bouraoui, H., *et al.* (2014) The bacterial phosphoenolpyruvate:carbohydrate phosphotransferase system: regulation by protein phosphorylation and phosphorylation-dependent protein-protein interactions. *Microbiol Mol Biol Rev* **78**: 231–256.
- Deutscher, J., Francke, C., and Postma, P.W. (2006) How Phosphotransferase System-Related Protein Phosphorylation Regulates Carbohydrate Metabolism in Bacteria. *Microbiol Mol Biol Rev* **70**: 939–1031.
- Diederich, L., Rasmussen, L.J., and Messer, W. (1992) New cloning vectors for integration in the lambda attachment site attB of the *Escherichia coli* chromosome. *Plasmid* **28**: 14–24.
- Dorel, C., Lejeune, P., and Rodrigue, A. (2006) The Cpx system of *Escherichia coli*, a strategic signaling pathway for confronting adverse conditions and for settling biofilm communities? *Res Microbiol* **157**: 306–314.
- Doucette, C.D., Schwab, D.J., Wingreen, N.S., and Rabinowitz, J.D. (2011) A-Ketoglutarate Coordinates Carbon and Nitrogen Utilization Via Enzyme I Inhibition. *Nat Chem Biol* **7**: 894–901.
- Driessen, A.J.M., and Nouwen, N. (2008) Protein translocation across the bacterial cytoplasmic membrane. *Annu Rev Biochem* **77**: 643–667.
- Durica-Mitic, S., Göpel, Y., Amman, F., and Görke, B. (2020) Adaptor protein RapZ activates endoribonuclease RNase E by protein-protein interaction to cleave a small regulatory RNA. *RNA* **26**: 1198–1215.
- Durica-Mitic, S., and Görke, B. (2019) Feedback regulation of small RNA processing by the cleavage product. *RNA Biol* **16**: 1055–1065.
- Dworkin, J., Jovanovic, G., Model, P., and Typically, E. (2000) The PspA Protein of *Escherichia coli* is a negative regulator of sigma(54)-dependent transcription. *J Bacteriol.* **182**(2):311-9.
- Ebbensgaard, A., Mordhorst, H., Aarestrup, F.M., and Hansen, E.B. (2018) The role of outer membrane proteins and lipopolysaccharides for the sensitivity of *Escherichia coli* to antimicrobial peptides. *Front Microbiol* **9**: 1–13.
- Ehrt, S., Guo, X. V., Hickey, C.M., Ryou, M., Monteleone, M., Riley, L.W., and Schnappinger,

- D. (2005) Controlling gene expression in Mycobacteria with anhydrotetracycline and Tet repressor. *Nucleic Acids Res* **33**: 1–11.
- Epstein, W. (2003) The Roles and Regulation of Potassium in Bacteria. *Prog Nucleic Acid Res Mol Biol* **75**: 293–320.
- Erwin, A.L. (2016) Antibacterial drug discovery targeting the lipopolysaccharide biosynthetic enzyme LpxC. *Cold Spring Harb Perspect Med* **6**: 1–14.
- Etchegaray, J.P., Jones, P.G., and Inouye, M. (1996) Differential thermoregulation of two highly homologous cold-shock genes, *cspA* and *cspB*, of *Escherichia coli*. *Genes Cells* **1**: 171–178.
- Fang, L., Hou, Y., and Inouye, M. (1998) Role of the cold-box region in the 5' untranslated region of the *cspA* mRNA in its transient expression at low temperature in *Escherichia coli*. *Mol. Microbiol.* **27**: 247–255.
- Farris, C., Sanowar, S., Bader, M.W., Pfuetzner, R., and Miller, S.I. (2010) Antimicrobial peptides activate the Rcs regulon through the outer membrane lipoprotein RcsF. *J Bacteriol* **192**: 4894–4903.
- Feaga, H.A., Kopylov, M., Kim, J.K., Jovanovic, M., and Dworkin, J. (2020) Ribosome dimerization protects the small subunit. *J Bacteriol* **202**: 1–9.
- Fehlbaum, P., Bulet, P., Chernysh, S., Briand, J.P., Roussel, J.P., Letellier, L., *et al.* (1996) Structure-activity analysis of thanatin, a 21-residue inducible insect defense peptide with sequence homology to frog skin antimicrobial peptides. *Proc Natl Acad Sci U S A* **93**: 1221–1225.
- Feucht, B.U., and Saier, M.H. (1980) Fine control of adenylate cyclase by the phosphoenolpyruvate:sugar phosphotransferase systems in *Escherichia coli* and *Salmonella typhimurium*. *J Bacteriol* **141**: 603–610.
- Filenko, N., Spiro, S., Browning, D.F., Squire, D., Overton, T.W., Cole, J., and Constantinidou, C. (2007) The NsrR regulon of *Escherichia coli* K-12 includes genes encoding the hybrid cluster protein and the periplasmic, respiratory nitrite reductase. *J Bacteriol* **189**: 4410–4417.
- Flores-Kim, J., and Darwin, A.J. (2016) The Phage Shock Protein Response. *Annu Rev Microbiol* **70**: 83–101.
- Flynn, J.M., Levchenko, I., Sauer, R.T., and Baker, T.A. (2004) Modulating substrate choice: The SspB adaptor delivers a regulator of the extracytoplasmic-stress response to the AAA+

- protease ClpXP for degradation. *Genes Dev* **18**: 2292–2301.
- Forst, S., Delgado, J., and Inouye, M. (1989) Phosphorylation of OmpR by the osmosensor EnvZ modulates expression of the *ompF* and *ompC* genes in *Escherichia coli*. *Proc Natl Acad Sci U S A* **86**: 6052–6056.
- Francke, C., Groot Kormelink, T., Hagemeijer, Y., Overmars, L., Sluijter, V., Moezelaar, R., and Siezen, R.J. (2011) Comparative analyses imply that the enigmatic sigma factor 54 is a central controller of the bacterial exterior. *BMC Genomics* **12**: 385.
- Freinkman, E., Chng, S.S., and Kahne, D. (2011) The complex that inserts lipopolysaccharide into the bacterial outer membrane forms a two-protein plug-and-barrel. *Proc Natl Acad Sci U S A* **108**: 2486–2491.
- Froelich, J.M., Tran, K., and Wall, D. (2006) A *pmrA* constitutive mutant sensitizes *Escherichia coli* to deoxycholic acid. *J Bacteriol* **188**: 1180–1183.
- Gassel, M., Möllenkamp, T., Puppe, W., and Altendorf, K. (1999) The KdpF subunit is part of the K⁺-translocating Kdp complex of *Escherichia coli* and is responsible for stabilization of the complex *in vitro*. *J Biol Chem* **274**: 37901–37907.
- Gerken, H., Charlson, E.S., Cicirelli, E.M., Kenney, L.J., and Misra, R. (2009) MzrA: A novel modulator of the EnvZ/OmpR two-component regulon. *Mol Microbiol* **72**: 1408–1422.
- Gerken, H., and Misra, R. (2010) MzrA-EnvZ interactions in the periplasm influence the EnvZ/OmpR two-component regulon. *J Bacteriol* **192**: 6271–6278.
- Gibhardt, J., Hoffmann, G., Turdiev, A., Wang, M., Lee, V.T., and Commichau, F.M. (2019) C-di-AMP assists osmoadaptation by regulating the *Listeria monocytogenes* potassium transporters KimA and KtrCD. *J Biol Chem* **294**: 16020–16033.
- Gogol, E.B., Rhodius, V.A., Papenfort, K., Vogel, J., and Gross, C.A. (2011) Small RNAs endow a transcriptional activator with essential repressor functions for single-tier control of a global stress regulon. *Proc Natl Acad Sci U S A* **108**: 12875–12880.
- Gonzalez, G.M., Durica-Mitic, S., Hardwick, S.W., Moncrieffe, M.C., Resch, M., Neumann, P., *et al.* (2017) Structural insights into RapZ-mediated regulation of bacterial amino-sugar metabolism. *Nucleic Acids Res* **45**: 10845–10860.
- Göpel, Y., and Görke, B. (2018) Interaction of lipoprotein QseG with sensor kinase QseE in the periplasm controls the phosphorylation state of the two-component system QseE/QseF in *Escherichia coli*. *PLoS Genet.* **14**: e1007547.
- Göpel, Y., Khan, M.A., and Görke, B. (2014) Ménage à trois: Post-transcriptional control of

the key enzyme for cell envelope synthesis by a base-pairing small RNA, an RNase adaptor protein, and a small RNA mimic. *RNA Biol* **11**: 433–442.

Göpel, Y., Khan, M.A., and Görke, B. (2016) Domain swapping between homologous bacterial small RNAs dissects processing and Hfq binding determinants and uncovers an aptamer for conditional RNase E cleavage. *Nucleic Acids Res* **44**: 824–837.

Göpel, Y., Lüttmann, D., Heroven, A.K., Reichenbach, B., Dersch, P., and Görke, B. (2011) Common and divergent features in transcriptional control of the homologous small RNAs GlmY and GlmZ in *Enterobacteriaceae*. *Nucleic Acids Res* **39**: 1294–1309.

Göpel, Y., Papenfort, K., Reichenbach, B., Vogel, J., and Görke, B. (2013) Targeted decay of a regulatory small RNA by an adaptor protein for RNase E and counteraction by an anti-adaptor RNA. *Genes Dev* **27**: 552–564.

Görke, B., and Rak, B. (1999) Catabolite control of *Escherichia coli* regulatory protein BglG activity by antagonistically acting phosphorylations. *EMBO J* **18**: 3370–3379.

Görke, B., and Stülke, J. (2008) Carbon catabolite repression in bacteria: Many ways to make the most out of nutrients. *Nat Rev Microbiol* **6**: 613–624.

Gossen, M., and Bujard, H. (1993) Anhydrotetracycline, a novel effector for tetracycline controlled gene expression systems in eukaryotic cells. *Nucleic Acids Res* **21**: 4411–4412.

Grabowicz, M., and Silhavy, T.J. (2017) Envelope Stress Responses: An Interconnected Safety Net. *Trends Biochem Sci* **42**: 232–242.

Gravina, F., Degaut, F.L., Gerhardt, E.C.M., Pedrosa, F.O., Souza, E.M., Antônio de Souza, G., and Huergo, L.F. (2021) The protein-protein interaction network of the *Escherichia coli* EIIANtr regulatory protein reveals a role in cell motility and metabolic control. *Res Microbiol* **172**(7-8):103882.

Gronow, S., Brabetz, W., and Brade, H. (2000) Comparative functional characterization *in vitro* of heptosyltransferase I (WaaC) and II (WaaF) from *Escherichia coli*. *Eur J Biochem* **267**: 6602–6611.

Guillier, M., and Gottesman, S. (2006) Remodelling of the *Escherichia coli* outer membrane by two small regulatory RNAs. *Mol Microbiol* **59**: 231–247.

Guisbert, E., Rhodius, V.A., Ahuja, N., Witkin, E., and Gross, C.A. (2007) Hfq modulates the σ E-mediated envelope stress response and the σ 32-mediated cytoplasmic stress response in *Escherichia coli*. *J Bacteriol* **189**: 1963–1973.

Guzman, L.M., Belin, D., Carson, M.J., and Beckwith, J. (1995) Tight regulation, modulation,

and high-level expression by vectors containing the arabinose PBAD promoter. *J Bacteriol* **177**: 4121–4130.

Hagiwara, D., Yamashino, T., and Mizuno, T. (2004) A genome-wide view of the *Escherichia coli* BasS-BasR two-component system implicated in iron-responses. *Biosci Biotechnol Biochem* **68**: 1758–1767.

Hamda, K., Kato, M., Shimizu, T., Ihara, K., Mizuno, T., and Hakoshima, T. (2005) Crystal structure of the protein histidine phosphatase SixA in the multistep His-Asp phosphorelay. *Genes Cells* **10**: 1–11.

Harwood, J.P., Gazdar, C., Prasad, C., Peterkofsky, A., Curtis, S.J., and Epstein, W. (1976) Involvement of the glucose enzymes II of the sugar phosphotransferase system in the regulation of adenylate cyclase by glucose in *Escherichia coli*. *J Biol Chem* **251**: 2462–2468.

Haupt, M., Bramkamp, M., Coles, M., Kessler, H., and Altendorf, K. (2006) Prokaryotic Kdp-ATPase: Recent insights into the structure and function of KdpB. *J Mol Microbiol Biotechnol* **10**: 120–131.

Hayden, J.D., and Ades, S.E. (2008) The extracytoplasmic stress factor, σ^E , is required to maintain cell envelope integrity in *Escherichia coli*. *PLoS One* **3**(2):e1573.

Heermann, R., Fohrmann, A., Altendorf, K., and Jung, K. (2003) The transmembrane domains of the sensor kinase KdpD of *Escherichia coli* are not essential for sensing K⁺ limitation. *Mol Microbiol* **47**: 839–848.

Heermann, R., and Jung, K. (2010) The complexity of the “simple” two-component system KdpD/KdpE in *Escherichia coli*. *FEMS Microbiol Lett* **304**: 97–106.

Ho, H., Miu, A., Alexander, M.K., Garcia, N.K., Oh, A., Zilberleyb, I., *et al.* (2018) Structural basis for dual-mode inhibition of the ABC transporter MsbA. *Nature* **557**: 196–201.

Hogema, B.M., Arents, J.C., Bader, R., Eijkemans, K., Yoshida, H., Takahashi, H., *et al.* (1998) Inducer exclusion in *Escherichia coli* by non-PTS substrates: The role of the PEP to pyruvate ratio in determining the phosphorylation state of enzyme IIA(Glc). *Mol Microbiol* **30**: 487–498.

Hsieh, Y.J., and Wanner, B.L. (2010) Global regulation by the seven-component Pi signaling system. *Curr Opin Microbiol* **13**: 198–203.

Hui, M.P., Foley, P.L., and Belasco, J.G. (2014) Messenger RNA degradation in bacterial cells. *Annu Rev Genet* **48**: 537–559.

Islam, M.S., Hardwick, S.W., Quell, L., Durica-Mitic, S., Chirgadze, D.Y., Görke, B., and Luisi,

B.F. (2023) Structure of a bacterial ribonucleoprotein complex central to the control of cell envelope biogenesis. *EMBO J* **42**(2):e112574.

Jahn, S., Haverkorn van Rijsewijk, B.R., Sauer, U., and Bettenbrock, K. (2013) A role for EIIANtr in controlling fluxes in the central metabolism of *E. coli* K12. *Biochim Biophys Acta - Mol Cell Res* **1833**: 2879–2889.

Johansen, J., Rasmussen, A.A., Overgaard, M., and Valentin-Hansen, P. (2006) Conserved Small Non-coding RNAs that belong to the σ E Regulon: Role in Down-regulation of Outer Membrane Proteins. *J Mol Biol* **364**: 1–8.

Johnson, J.W., Fisher, J.F., and Mobashery, S. (2014) Bacterial cell-wall recycling. *Ann N Y Acad Sci* **1277**: 54–75.

Jones, D.H.A., Franklin, F.C.H., and Thomas, C.M. (1994) Molecular analysis of the operon which encodes the RNA polymerase sigma factor σ 54 of *Escherichia coli*. *Microbiology* **140**: 1035–1043.

Jovanovic, G., Weiner, L., and Model, P. (1996) Identification, Nucleotide Sequence, and Characterization of PspF, the Transcriptional Activator of the *Escherichia coli* stress-induced *psp* operon. *J Bacteriol* **178**: 1936–1945.

Jung, K., Tjaden, B., and Altendorf, K. (1997) Purification, reconstitution, and characterization of KdpD, the turgor sensor of *Escherichia coli*. *J Biol Chem* **272**: 10847–10852.

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

Kalynychn, S., Morona, R., and Cygler, M. (2014) Progress in understanding the assembly process of bacterial O-antigen. *FEMS Microbiol Rev* **38**: 1048–1065.

Kato, A., Tanabe, H., and Utsumi, R. (1999) Molecular characterization of the PhoP-PhoQ two-component system in *Escherichia coli* K-12: Identification of extracellular Mg²⁺-responsive promoters. *J Bacteriol* **181**: 5516–5520.

Keto-Timonen, R., Hietala, N., Palonen, E., Hakakorpi, A., Lindström, M., and Korkeala, H. (2016) Cold Shock Proteins: A Minireview with Special Emphasis on Csp-family of Enteropathogenic Yersinia. *Front Microbiol* **7**:1151.

Khan, M.A., Durica-Mitic, S., Göpel, Y., Heermann, R., and Görke, B. (2020) Small RNA-binding protein RapZ mediates cell envelope precursor sensing and signaling in *Escherichia*

coli. *EMBO J* **39**: 1–16.

Khan, M.A., Göpel, Y., Milewski, S., and Görke, B. (2016) Two small RNAs conserved in enterobacteriaceae provide intrinsic resistance to antibiotics targeting the cell wall biosynthesis enzyme glucosamine-6-phosphate synthase. *Front Microbiol* **7**: 1–13.

Khan, M.A., and Görke, B. (2020) A multifunctional small RNA binding protein for sensing and signaling cell envelope precursor availability in bacteria. *Microb Cell* **7**: 139–142.

Kim, H.J., Lee, C.R., Kim, M., Peterkofsky, A., and Seok, Y.J. (2011) Dephosphorylated NPr of the nitrogen PTS regulates lipid A biosynthesis by direct interaction with LpxD. *Biochem Biophys Res Commun* **409**: 556–561.

Kim, K., Jeong, J.H., Lim, D., Hong, Y., Yun, M., Min, J.J., *et al.* (2013) A Novel Balanced-Lethal Host-Vector System Based on *glmS*. *PLoS One* **8**: 1–11.

Klein, G., and Raina, S. (2017) Small regulatory bacterial RNAs regulating the envelope stress response. *Biochem Soc Trans* **45**: 417–425.

Klein, G., and Raina, S. (2019) Regulated assembly of LPS, its structural alterations and cellular response to LPS defects. *Int J Mol Sci* **20**(2):356.

Klein, G., Stupak, A., Biernacka, D., Wojtkiewicz, P., Lindner, B., and Raina, S. (2016) Multiple transcriptional factors regulate transcription of the *rpoE* Gene in *Escherichia coli* under different growth conditions and when the lipopolysaccharide biosynthesis is defective. *J Biol Chem* **291**: 22999–23019.

Kobayashi, R., Suzuki, T., and Yoshida, M. (2007) *Escherichia coli* phage-shock protein A (PspA) binds to membrane phospholipids and repairs proton leakage of the damaged membranes. *Mol Microbiol* **66**: 100–109.

Konovalova, A., Kahne, D.E., and Silhavy, T.J. (2017) Outer Membrane Biogenesis. *Annu Rev Microbiol* **71**: 539–556.

Kucharczyk, N., Denisot, M.A., Goffic, F. Le, and Badet, B. (1990) Glucosamine-6-phosphate Synthase from *Escherichia coli* Determination of the Mechanism of Inactivation by N3-Fumaroyl-L-2,3-diaminopropionic Derivatives. *Biochemistry* **29**: 3668–3676.

Laermann, V., Ćudić, E., Kipschull, K., Zimmann, P., and Altendorf, K. (2013) The sensor kinase KdpD of *Escherichia coli* senses external K⁺. *Mol Microbiol* **88**: 1194–1204.

Laloux, G., and Collet, J.F. (2017) Major Tom to ground control: How lipoproteins communicate extracytoplasmic stress to the decision center of the cell. *J Bacteriol* **199**: 1–13.

- Lamarche, M.G., Wanner, B.L., Crépin, S., and Harel, J. (2008) The phosphate regulon and bacterial virulence: A regulatory network connecting phosphate homeostasis and pathogenesis. *FEMS Microbiol Rev* **32**: 461–473.
- Las Penas, A. De, Connolly, L., and Gross, C.A. (1997) Sigma E is an essential sigma factor in *Escherichia coli*. *J Bacteriol* **179**: 6862–6864.
- Lay, N. De, Schu, D.J., and Gottesman, S. (2013) Bacterial small RNA-based negative regulation: Hfq and its accomplices. *J Biol Chem* **288**: 7996–8003.
- Lazar, S.W., and Kolter, R. (1996) SurA assists the folding of *Escherichia coli* outer membrane proteins. *J Bacteriol* **178**: 1770–1773.
- Leblanc, S.K.D., Oates, C.W., and Raivio, T.L. (2011) Characterization of the induction and cellular role of the BaeSR two-component envelope stress response of *Escherichia coli*. *J Bacteriol* **193**: 3367–3375.
- Lederberg, E.M., and Cohen, S.N. (1974) Transformation of *Salmonella typhimurium* by plasmid deoxyribonucleic acid. *J Bacteriol* **119**: 1072–1074.
- Lee, C.R., Cho, S.H., Yoon, M.J., Peterkofsky, A., and Seok, Y.J. (2007) *Escherichia coli* enzyme IIA^{Ntr} regulates the K⁺ transporter TrkA. *Proc Natl Acad Sci U S A* **104**: 4124–4129.
- Lee, C.R., Park, Y.H., Kim, M., Kim, Y.R., Park, S., Peterkofsky, A., and Seok, Y.J. (2013) Reciprocal regulation of the autophosphorylation of enzyme INtr by glutamine and α -ketoglutarate in *Escherichia coli*. *Mol Microbiol* **88**: 473–485.
- Lee, J., Park, Y.H., Kim, Y.R., Seok, Y.J., and Lee, C.R. (2015) Dephosphorylated NPr is involved in an envelope stress response of *Escherichia coli*. *Microbiol (United Kingdom)* **161**: 1113–1123.
- Lehman, K.M., and Grabowicz, M. (2019) Countering gram-negative antibiotic resistance: Recent progress in disrupting the outer membrane with novel therapeutics. *Antibiotics* **8**(4):163.
- Lim, B., Zimmermann, M., Barry, N.A., and Goodman, A.L. (2017) Engineered Regulatory Systems Modulate Gene Expression of Human Commensals in the Gut. *Cell* **169**: 547–558.
- Lima, S., Guo, M.S., Chaba, R., Gross, C.A., and Sauer, R.T. (2013) Dual molecular signals mediate the bacterial response to outer-membrane stress. *Scienc* **340**: 837–841.
- Lippa, A.M., and Goulian, M. (2009) Feedback inhibition in the PhoQ/PhoP signaling system by a membrane peptide. *PLoS Genet* **5**(12):e1000788.

- Little, R., and Dixon, R. (2003) The amino-terminal GAF domain of *Azotobacter vinelandii* NifA binds 2-oxoglutarate to resist inhibition by NifL under nitrogen-limiting conditions. *J Biol Chem* **278**: 28711–28718.
- London, L.Y., Aubee, J.I., Nurse, J., and Thompson, K.M. (2021) Post-Transcriptional Regulation of RseA by Small RNAs RyhB and FnrS in *Escherichia coli*. *Front Mol Biosci* **8**:668613.
- Lüttmann, D., Göpel, Y., and Görke, B. (2012) The phosphotransferase protein EIINtr modulates the phosphate starvation response through interaction with histidine kinase PhoR in *Escherichia coli*. *Mol Microbiol* **86**: 96–110.
- Lüttmann, D., Göpel, Y., and Görke, B. (2015) Cross-Talk between the Canonical and the Nitrogen-Related Phosphotransferase Systems Modulates Synthesis of the KdpFABC Potassium Transporter in *Escherichia coli*. *J Mol Microbiol Biotechnol* **25**: 168–177.
- Lüttmann, D., Heermann, R., Zimmer, B., Hillmann, A., Rampp, I.S., Jung, K., and Görke, B. (2009) Stimulation of the potassium sensor KdpD kinase activity by interaction with the phosphotransferase protein IINtr in *Escherichia coli*. *Mol Microbiol* **72**: 978–994.
- Mackie, G.A. (2013) RNase E: At the interface of bacterial RNA processing and decay. *Nat Rev Microbiol* **11**: 45–57.
- Madhusudan, S., Paukner, A., Klinge, Y., and Schnetz, K. (2005) Independent regulation of H-NS-mediated silencing of the bgl operon at two levels: Upstream by BglJ and LeuO and downstream by DnaKJ. *Microbiology* **151**: 3349–3359.
- Maki, Y., Yoshida, H., and Wada, A. (2000) Two proteins, YfiA and YhbH, associated with resting ribosomes in stationary phase *Escherichia coli*. *Genes to Cells* **5**: 965–974.
- Martorana, A.M., Motta, S., Silvestre, D. Di, Falchi, F., Dehò, G., Mauri, P., *et al.* (2014) Dissecting *Escherichia coli* outer membrane biogenesis using differential proteomics. *PLoS One* **9**:e100941.
- Martorana, A.M., Moura, E.C.C.M., Sperandio, P., Vincenzo, F. Di, Liang, X., Toone, E., *et al.* (2021) Degradation of Components of the Lpt Transenvelope Machinery Reveals LPS-Dependent Lpt Complex Stability in *Escherichia coli*. *Front Mol Biosci* **8**:758228.
- Martorana, A.M., Sperandio, P., Polissi, A., and Dehò, G. (2011) Complex transcriptional organization regulates an *Escherichia coli* locus implicated in lipopolysaccharide biogenesis. *Res Microbiol* **162**: 470–482.
- May, K.L., Lehman, K.M., Mitchell, A.M., and Grabowicz, M. (2019) A stress response

monitoring lipoprotein trafficking to the outer membrane. *MBio* **10**: 1–14.

Melamed, S., Peer, A., Faigenbaum-Romm, R., Gatt, Y.E., Reiss, N., Bar, A., *et al.* (2016) Global Mapping of Small RNA-Target Interactions in Bacteria. *Mol Cell* **63**: 884–897.

Mengin-Lecreulx, D., and Heijenoort, J. Van (1996) Characterization of the essential gene *glmM* encoding phosphoglucosamine mutase in *Escherichia coli*. *J Biol Chem* **271**: 32–39.

Merritt, M.E., and Donaldson, J.R. (2009) Effect of bile salts on the DNA and membrane integrity of enteric bacteria. *J Med Microbiol* **58**: 1533–1541.

Mi, W., Li, Y., Yoon, S.H., Ernst, R.K., Walz, T., and Liao, M. (2017) Structural basis of MsbA-mediated lipopolysaccharide transport. *Nature* **549**: 233–237.

Miczak, A., Kaberdin, V.R., Wei, C.L., and Lin-Chao, S. (1996) Proteins associated with RNase E in a multicomponent ribonucleolytic complex. *Proc Natl Acad Sci U S A* **93**: 3865–3869.

Milewski, S. (2002) Glucosamine-6-phosphate synthase--the multi-facets enzyme. *Biochim Biophys Acta* **40**: 2712–2722.

Milewski, S., Chmara, H., and Borowski, E. (1986) Anticapsin: an active site directed inhibitor of glucosamine-6-phosphate synthetase from *Candida albicans*. *Drugs Exp Clin Res* **12**: 577–583.

Miller, J.H. (1972) Assay of b-galactosidase. *Exp in Mol Genet* 352–355.

Minagawa, S., Ogasawara, H., Kato, A., Yamamoto, K., Eguchi, Y., Oshima, T., *et al.* (2003) Identification and Molecular Characterization of the Mg²⁺ stimulon of *Escherichia coli*. *J Bacteriol* **185**: 3696–3702.

Missiakas, D., Betton, J.M., and Raina, S. (1996) New components of protein folding in extracytoplasmic compartments of *Escherichia coli* SurA, FkpA and Skp/OmpH. *Mol Microbiol* **21**: 871–884.

Missiakas, D., Mayer, M.P., Lemaire, M., Georgopoulos, C., and Raina, S. (1997) Modulation of the *Escherichia coli* σ (E) (RpoE) heat-shock transcription-factor activity by the RseA, RseB and RseC proteins. *Mol Microbiol* **24**: 355–371.

Mitchell, A.M., and Silhavy, T.J. (2019) Envelope stress responses: balancing damage repair and toxicity. *Nat Rev Microbiol* **17**: 417–428.

Mizuno, T., and Mizushima, S. (1990) Signal transduction and gene regulation through the phosphorylation of two regulatory components: the molecular basis for the osmotic regulation

of the porin genes. *Mol Microbiol* **4**: 1077–1082.

Mohler, K., and Ibba, M. (2017) Translational fidelity and mistranslation in the cellular response to stress. *Nature microbiology* **2**: 17117–17117.

Moller, T., Franch, T., Hojrup, P., Keene, D.R., Bächinger, H.P., Brennan, R.G., and Valentin-Hansen, P. (2002) Hfq: A Bacterial Sm-like protein that mediates RNA-RNA interaction. *Mol Cell* **9**: 23–30.

Mörk-Mörkenstein, M., Heermann, R., Göpel, Y., Jung, K., and Görke, B. (2017) Non-canonical activation of histidine kinase KdpD by phosphotransferase protein PtsN through interaction with the transmitter domain. *Mol Microbiol* **106**: 54–73.

Moscoso, J.A., Schramke, H., Zhang, Y., Tosi, T., Dehbi, A., Jung, K., and Gründling, A. (2016) Binding of cyclic di-AMP to the *Staphylococcus aureus* sensor kinase KdpD occurs via the universal stress protein domain and downregulates the expression of the Kdp potassium transporter. *J Bacteriol* **198**: 98–110.

Moura, E.C.C.M., Baeta, T., Romanelli, A., Laguri, C., Martorana, A.M., Erba, E., *et al.* (2020) Thanatin Impairs Lipopolysaccharide Transport Complex Assembly by Targeting LptC–LptA Interaction and Decreasing LptA Stability. *Front Microbiol* **11**:909.

Mutalik, V.K., Nonaka, G., Ades, S.E., Rhodius, V.A., and Gross, C.A. (2009) Promoter strength properties of the complete sigma E regulon of *Escherichia coli* and *Salmonella enterica*. *J Bacteriol* **191**: 7279–7287.

Nagarajavel, V., Madhusudan, S., Dole, S., Rahmouni, A.R., and Schnetz, K. (2007) Repression by binding of H-NS within the transcription unit. *J Biol Chem* **282**: 23622–23630.

Nagasawa, S., Ishige, K., and Mizuno, T. (1993) Novel members of the two-component signal transduction genes in *Escherichia coli*. *J Biochem* **114**: 350–357.

Nakashima, K., Sugiura, A., Momoi, H., and Mizuno, T. (1992) Phosphotransfer signal transduction between two regulatory factors involved in the osmoregulated *kdp* operon in *Escherichia coli*. *Mol Microbiol* **6**: 1777–1784.

Narayanan, A., Kumar, S., Evrard, A.N., Paul, L.N., and Yernool, D.A. (2014) An asymmetric heterodomain interface stabilizes a response regulator-DNA complex. *Nat Commun* **5**:3282.

Nicoloff, H., Gopalkrishnan, S., and Ades, S.E. (2017) Appropriate Regulation of the sE-Dependent Envelope Stress Response Is Necessary To Maintain Cell Envelope Integrity and Stationary-Phase Survival in *Escherichia coli*. *J Bacteriol* 2017 **199**(12):e00089-17.

Nikaido, H. (2003) Molecular Basis of Bacterial Outer Membrane Permeability Revisited.

Microbiol Mol Biol Rev **67**: 593–656.

Nishino, K., Honda, T., and Yamaguchi, A. (2005) Genome-wide analyses of *Escherichia coli* gene expression responsive to the BaeSR two-component regulatory system. *J Bacteriol* **187**: 1763–1772.

Nitta, T., Nagamitsu, H., Murata, M., Izu, H., and Yamada, M. (2000) Function of the E Regulon in Dead-Cell Lysis in Stationary-Phase *Escherichia coli*. *J Bacteriol* **182**(18):5231-7.

Okuda, S., Freinkman, E., and Kahne, D. (2012) Cytoplasmic ATP hydrolysis powers transport of lipopolysaccharide across the periplasm in *E. coli*. *Science* **338**: 1214–1217.

Okuda, S., Sherman, D.J., Silhavy, T.J., Ruiz, N., and Kahne, D. (2017) Lipopolysaccharide transport and assembly at the outer membrane: the PEZ model. *Nat Rev Microbiol* **14**: 337–345.

Otaka, H., Ishikawa, H., Morita, T., and Aiba, H. (2011) PolyU tail of rho-independent terminator of bacterial small RNAs is essential for Hfq action. *Proc Natl Acad Sci U S A* **108**: 13059–13064.

Özcengiz, G., and Ögülür, I. (2015) Biochemistry, genetics and regulation of bacilysin biosynthesis and its significance more than an antibiotic. *N Biotechnol* **32**: 612–619.

Page, J.E., and Walker, S. (2021) Natural products that target the cell envelope. *Curr Opin Microbiol* **61**: 16–24.

Park, J.T., and Uehara, T. (2008) How bacteria consume their own exoskeletons (turnover and recycling of cell wall peptidoglycan). *Microbiol Mol Biol Rev* **72**: 211–227.

Partridge, J.D., Bodenmiller, D.M., Humphrys, M.S., and Spiro, S. (2009) NsrR targets in the *Escherichia coli* genome: New insights into DNA sequence requirements for binding and a role for NsrR in the regulation of motility. *Mol Microbiol* **73**: 680–694.

Pennetier, C., Domínguez-Ramírez, L., and Plumbridge, J. (2008) Different regions of Mlc and NagC, homologous transcriptional repressors controlling expression of the glucose and N-acetylglucosamine phosphotransferase systems in *Escherichia coli*, are required for inducer signal recognition. *Mol Microbiol* **67**: 364–377.

Pflüger-Grau, K., and Görke, B. (2010) Regulatory roles of the bacterial nitrogen-related phosphotransferase system. *Trends Microbiol* **18**: 205–214.

Pflüger-Grau, K., and Lorenzo, V. de (2014) From the phosphoenolpyruvate phosphotransferase system to selfish metabolism: A story retraced in *Pseudomonas putida*. *FEMS Microbiol Lett* **356**: 144–153.

- Pflüger, K., and Lorenzo, V. De (2008) Evidence of *in vivo* cross talk between the nitrogen-related and fructose-related branches of the carbohydrate phosphotransferase system of *Pseudomonas putida*. *J Bacteriol* **190**: 3374–3380.
- Plumbridge, J. (1995) Co-ordinated regulation of amino sugar biosynthesis and degradation: The NagC repressor acts as both an activator and a repressor for the transcription of the *glmUS* operon and requires two separated NagC binding sites. *EMBO J* **14**: 3958–3965.
- Plumbridge, J. (2001) Regulation of PTS Gene Expression by the Homologous Transcriptional Regulators, Mlc and NagC, in *Escherichia coli* (or How Two Similar Repressors Can Behave Differently). *J Mol Microbiol Biotechnol* **3**: 371–380.
- Plumbridge, J., and Pellegrini, O. (2004) Expression of the chitobiose operon of *Escherichia coli* is regulated by three transcription factors: NagC, ChbR and CAP. *Mol Microbiol* **52**: 437–449.
- Plumbridge, J., and Vimr, E. (1999) Convergent Pathways for Utilization of the Amino Sugars. *Microbiology* **181**: 47–54.
- Plumbridge, J.A. (1991) Repression and induction of the *nag* regulon of *Escherichia coli* K-12: the roles of *nagC* and *nagA* in maintenance of the uninduced state. *Mol Microbiol* **5**: 2053–2062.
- Plumbridge, J.A., Cochet, O., Souza, J.M., Altamirano, M.M., Calcagno, M.L., and Badet, B. (1993) Coordinated regulation of amino sugar-synthesizing and -degrading enzymes in *Escherichia coli* K-12. *J Bacteriol* **175**: 4951–4956.
- Pogliano, K.J., and Beckwith, J. (1993) The *Cs sec* mutants of *Escherichia coli* reflect the cold sensitivity of protein export itself. *Genetics* **133**: 763–773.
- Postma, P.W., Lengeler, J.W., and Jacobson, G.R. (1993) Phosphoenolpyruvate:carbohydrate phosphotransferase systems of bacteria. *Microbiol Rev* **57**: 543–594.
- Powell, B.S., Court, D.L., Inada, T., Nakamura, Y., Michotey, V., Cui, X., et al. (1995) Novel proteins of the phosphotransferase system encoded within the *rpoN* operon of *Escherichia coli*: Enzyme I^{ANtr} affects growth on organic nitrogen and the conditional lethality of an *erats* mutant. *J Biol Chem* **270**: 4822–4839.
- Pratt, L.A., Hsing, W., Gibson, K.E., and Silhavy, T.J. (1996) Micro Review From acids to *osmZ*: multiple factors influence synthesis of the OmpF and OmpC porins in *Escherichia coli*. *Mol Microbiol* **20**: 1–7.

- Prossliner, T., Gerdes, K., Sørensen, M.A., and Winther, K.S. (2021) Hibernation factors directly block ribonucleases from entering the ribosome in response to starvation. *Nucleic Acids Res* **49**: 2226–2239.
- Prossliner, T., Skovbo Winther, K., Sørensen, M.A., and Gerdes, K. (2018) Ribosome hibernation. *Annu Rev Genet* **52**: 321–348.
- Qi, L.S., Larson, M.H., Gilbert, L.A., Doudna, J.A., Weissman, J.S., Arkin, A.P., and Lim, W.A. (2013) Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* **152**: 1173–1183.
- Qiao, S., Luo, Q., Zhao, Y., Zhang, X.C., and Huang, Y. (2014) Structural basis for lipopolysaccharide insertion in the bacterial outer membrane. *Nature* **511**: 108–111.
- Rabus, R., Reizer, J., Paulsen, I., and Saier, M.H. (1999) Enzyme I(Ntr) from *Escherichia coli*. A novel enzyme of the phosphoenolpyruvate-dependent phosphotransferase system exhibiting strict specificity for its phosphoryl acceptor, NPr. *J Biol Chem* **274**: 26185–26191.
- Raetz, C.R.H., Reynolds, C.M., Trent, M.S., and Bishop, R.E. (2007) Lipid A modification systems in gram-negative bacteria. *Annu Rev Biochem* **76**: 295–329.
- Raetz, C.R.H., and Whitfield, C. (2002) Lipopolysaccharide endotoxins. *Annu Rev Biochem* **71**: 635–700.
- Raffa, R.G., and Raivio, T.L. (2002) A third envelope stress signal transduction pathway in *Escherichia coli*. *Mol Microbiol* **45**: 1599–1611.
- Raivio, T.L., Leblanc, S.K.D., and Price, N.L. (2013) The *Escherichia coli* Cpx envelope stress response regulates genes of diverse function that impact antibiotic resistance and membrane integrity. *J Bacteriol* **195**: 2755–2767.
- Raivio, T.L., Popkin, D.L., and Silhavy, T.J. (1999) The Cpx envelope stress response is controlled by amplification and feedback inhibition. *J Bacteriol* **181**: 5263–5272.
- Raivio, T.L., and Silhavy, T.J. (1997) Transduction of envelope stress in *Escherichia coli* by the Cpx two- component system. *J Bacteriol* **179**: 7724–7733.
- Ray, B.L., and Raetz, C.R.H. (1987) The biosynthesis of gram-negative endotoxin. A novel kinase in *Escherichia coli* membranes that incorporates the 4'-phosphate of lipid A. *J Biol Chem* **262**: 1122–1128.
- Reaves, M.L., and Rabinowitz, J.D. (2011) Characteristic Phenotypes Associated with *ptsN*-Null Mutants in *Escherichia coli* K-12 Are Absent in Strains with Functional *ilvG*. *J Bacteriol* **193**: 4576–4581.

- Reichenbach, B., Göpel, Y., and Görke, B. (2009) Dual control by perfectly overlapping σ^{54} - and σ^{70} -promoters adjusts small RNA GlmY expression to different environmental signals. *Mol Microbiol* **74**: 1054–1070.
- Reitzer, L., and Schneider, B.L. (2001) Metabolic Context and Possible Physiological Themes of σ^{54} -Dependent Genes in *Escherichia coli*. *Microbiol Mol Biol Rev* **65**: 422–444.
- Reizer, J., Reizer, A., Merrick, M.J., Plunkett, G., Rose, D.J., and Saier, M.H. (1996) Novel phosphotransferase-encoding genes revealed by analysis of the *Escherichia coli* genome: A chimeric gene encoding an Enzyme I homologue that possesses a putative sensory transduction domain. *Gene* **181**: 103–108.
- Rezuchova, B., Miticka, H., Homerova, D., Roberts, M., and Kormanec, J. (2003) New members of the *Escherichia coli* σ^E regulon identified by a two-plasmid system. *FEMS Microbiol Lett* **225**: 1–7.
- Rhodus, V.A., and Mutalik, V.K. (2010) Predicting strength and function for promoters of the *Escherichia coli* alternative sigma factor, σ^E . *Proc Natl Acad Sci U S A* **107**: 2854–2859.
- Rhodus, V.A., Suh, W.C., Nonaka, G., West, J., and Gross, C.A. (2006) Conserved and variable functions of the σ^E stress response in related genomes. *PLoS Biol* **4**: 0043–0059.
- Rigden, D.J. (2008) The histidine phosphatase superfamily: Structure and function. *Biochem J* **409**: 333–348.
- Rigden, D.J. (2020) Protein Phosphohistidine Phosphatases of the HP Superfamily. *Methods Mol Biol* **2077**: 93–107.
- Riordan, J.T., and Mitra, A. (2017) Regulation of *Escherichia coli* Pathogenesis by Alternative Sigma Factor N. *EcoSal Plus* **7**(2).
- Rodionova, I.A., Goodacre, N., Babu, M., Emili, A., Uetz, P., and Saier, M.H. (2018) The Nitrogen Regulatory PII Protein (GlnB) and N-Acetylglucosamine 6-Phosphate Epimerase (NanE) Allosterically Activate Glucosamine 6-Phosphate Deaminase (NagB) in *Escherichia coli*. *J Bacteriol* **200**(5):e00691-17.
- Rouvière, P.E., and Gross, C.A. (1996) SurA, a periplasmic protein with peptidyl-prolyl isomerase activity, participates in the assembly of outer membrane porins. *Genes Dev* **10**: 3170–3182.
- Ruan, X., Loyola, D.E., Marolda, C.L., Perez-Donoso, J.M., and Valvano, M.A. (2012) The WaaL O-antigen lipopolysaccharide ligase has features in common with metal ion-independent inverting glycosyltransferases. *Glycobiology* **22**: 288–299.

- Salazar, M.E., Podgornaia, A.I., and Laub, M.T. (2016) The small membrane protein MgrB regulates PhoQ bifunctionality to control PhoP target gene expression dynamics. *Mol Microbiol* **102**: 430–445.
- Schnetz, K., Stülke, J., Gertz, S., Krüger, S., Krieg, M., Hecker, M., and Rak, B. (1996) LicT, a *Bacillus subtilis* transcriptional antiterminator protein of the BglG family. *J Bacteriol* **178**: 1971–1979.
- Schramke, H., Tostevin, F., Heermann, R., Gerland, U., and Jung, K. (2016) A Dual-Sensing Receptor Confers Robust Cellular Homeostasis. *Cell Rep* **16**: 213–221.
- Schulte, J.E., and Goulian, M. (2018) The phosphohistidine phosphatase SixA targets a phosphotransferase system. *MBio* **9**: 1–13.
- Schulte, J.E., Roggiani, M., Shi, H., Zhu, J., and Goulian, M. (2020) The phosphohistidine phosphatase SixA dephosphorylates the phosphocarrier NPr. *J Biol Chem* **296**:100090.
- Sharma, R., Shimada, T., Mishra, V.K., Upreti, S., and Sardesai, A.A. (2016) Growth inhibition by external potassium of *Escherichia coli* lacking PtsN (EIIANtr) is caused by potassium limitation mediated by YcgO. *J Bacteriol* **198**: 1868–1882.
- Siebers, A., and Altendorf, K. (1989) Characterization of the phosphorylated intermediate of the K⁺-translocating Kdp-ATPase from *Escherichia coli*. *J Biol Chem* **264**: 5831–5838.
- Silberberg, J.M., Corey, R.A., Hielkema, L., Stock, C., Stansfeld, P.J., Paulino, C., and Hänelt, I. (2021) Deciphering ion transport and ATPase coupling in the intersubunit tunnel of KdpFABC. *Nat Commun* **12**: 1–12.
- Silhavy, T.J., Kahne, D., and Walker, S. (2010) The bacterial cell envelope. *Cold Spring Harb Perspect Biol* **2**(5):a000414.
- Silver, S. (1996) Transport of inorganic cations. In *Escherichia coli* and *Salmonella typhimurium: Cellular and Molecular Biology*. Neidhardt, F.C., Curtiss, R., III., Ingraham, J.L., Lin, C.C., Brooks, L.K., and Magasanik, B., eds. Washington, DC: American Society for Microbiology Press.
- Simpson, B.W., and Trent, M.S. (2019) Pushing the envelope: LPS modifications and their consequences. *Nat Rev Microbiol* **17**: 403–416.
- Sklar, J.G., Wu, T., Kahne, D., and Silhavy, T.J. (2007) Defining the roles of the periplasmic chaperones SurA, Skp, and DegP in *Escherichia coli*. *Genes Dev* **21**: 2473–2484.
- Sperandeo, P., Cescutti, R., Villa, R., Benedetto, C. Di, Candia, D., Dehò, G., and Polissi, A. (2007) Characterization of *lptA* and *lptB*, two essential genes implicated in lipopolysaccharide

transport to the outer membrane of *Escherichia coli*. *J Bacteriol* **189**: 244–253.

Sperandeo, P., Lau, F.K., Carpentieri, A., Castro, C. De, Molinaro, A., Dehò, G., *et al.* (2008) Functional analysis of the protein machinery required for transport of lipopolysaccharide to the outer membrane of *Escherichia coli*. *J Bacteriol* **190**: 4460–4469.

Sperandeo, P., Martorana, A.M., and Polissi, A. (2019) Lipopolysaccharide Biosynthesis and Transport to the Outer Membrane of Gram-Negative Bacteria. *Subcell Biochem* **92**: 9–37.

Sperandeo, P., Villa, R., Martorana, A.M., Šamalikova, M., Grandori, R., Dehò, G., and Polissi, A. (2011) New insights into the Lpt machinery for lipopolysaccharide transport to the cell surface: LptA-LptC interaction and LptA stability as sensors of a properly assembled transenvelope complex. *J Bacteriol* **193**: 1042–1053.

Srinivas, N., Jetter, P., Ueberbacher, B.J., Werneburg, M., Zerbe, K., Steinmann, J., *et al.* (2010) Peptidomimetic antibiotics target outer-membrane biogenesis in *Pseudomonas aeruginosa*. *Science* **327**: 1010–1013.

Stevenson, G., Neal, B., Liu, D., Hobbs, M., Packer, N.H., Batley, M., *et al.* (1994) Structure of the O antigen of *Escherichia coli* K-12 and the sequence of its *rfb* gene cluster. *J Bacteriol* **176**: 4144–4156.

Strauch, K.L., and Beckwith, J. (1988) An *Escherichia coli* mutation preventing degradation of abnormal periplasmic proteins. *Proc Natl Acad Sci U S A* **85**: 1576–1580.

Studholme, D.J., and Buck, M. (2000) Novel roles of $\sigma(N)$ in small genomes. *Microbiology* **146**: 4–5.

Suits, M.D.L., Sperandeo, P., Dehò, G., Polissi, A., and Jia, Z. (2008) Novel Structure of the Conserved Gram-Negative Lipopolysaccharide Transport Protein A and Mutagenesis Analysis. *J Mol Biol* **380**: 476–488.

Svetlov, V., and Nudler, E. (2018) Reading of the non-template DNA by transcription elongation factors. *Mol Microbiol* **109**: 417–421.

Takeda, S.I., Fujisawa, Y., Matsubara, M., Aiba, H., and Mizuno, T. (2001) A novel feature of the multistep phosphorelay in *Escherichia coli*: A revised model of the RcsC $\rightarrow$ YojN $\rightarrow$ RcsB signalling pathway implicated in capsular synthesis and swarming behaviour. *Mol Microbiol* **40**: 440–450.

Tam, C., and Missiakas, D. (2005) Changes in lipopolysaccharide structure induce the σE -dependent response of *Escherichia coli*. *Mol Microbiol* **55**: 1403–1412.

Tepljakov, A., Leriche, C., Obmolova, G., Badet, B., and Badet-Denisot, M.A. (2002) From

Lobry de Bruyn to enzyme-catalyzed ammonia channelling: Molecular studies of D-glucosamine-6P synthase. *Nat Prod Rep* **19**: 60–69.

Thompson, K.M., Rhodius, V.A., and Gottesman, S. (2007) σ E regulates and is regulated by a small RNA in *Escherichia coli*. *J Bacteriol* **189**: 4243–4256.

Tran, A.X., Dong, C., and Whitfield, C. (2010) Structure and functional analysis of LptC, a conserved membrane protein involved in the lipopolysaccharide export pathway in *Escherichia coli*. *J Biol Chem* **285**: 33529–33539.

Tucker, N.P., D’Autréaux, B., Spiro, S., and Dixon, R. (2006) Mechanism of transcriptional regulation by the *Escherichia coli* nitric oxide sensor NorR. *Biochem Soc Trans* **34**: 191–194.

Udekwi, K.I., Darfeuille, F., Vogel, J., Reimegård, J., Holmqvist, E., and Wagner, E.G.H. (2005) Hfq-dependent regulation of OmpA synthesis is mediated by an antisense RNA. *Genes Dev* **19**: 2355–2366.

Ueta, M., Ohniwa, R.L., Yoshida, H., Maki, Y., Wada, C., and Wada, A. (2008) Role of HPF (hibernation promoting factor) in translational activity in *Escherichia coli*. *J Biochem* **143**: 425–433.

Urban, J.H., Papenfort, K., Thomsen, J., Schmitz, R.A., and Vogel, J. (2007) A conserved small RNA promotes discoordinate expression of the *glmUS* operon mRNA to activate GlmS synthesis. *J Mol Biol* **373**: 521–528.

Urban, J.H., and Vogel, J. (2008) Two seemingly homologous noncoding RNAs act hierarchically to activate *glmS* mRNA translation. *PLoS Biol* **6**: 0631–0642.

Valvano, M.A. (2011) Common themes in glycoconjugate assembly using the biogenesis of O-antigen lipopolysaccharide as a model system. *Biochem* **76**: 729–735.

VanBogelen, R.A., Olson, E.R., Wanner, B.L., and Neidhardt, F.C. (1996) Global analysis of proteins synthesized during phosphorus restriction in *Escherichia coli*. *J Bacteriol* **178**: 4344–4366.

Venkatesh, G.R., Koungni, F.C.K., Paukner, A., Stratmann, T., Blissenbach, B., and Schnetz, K. (2010) BglJ-RcsB heterodimers relieve repression of the *Escherichia coli* *bgl* operon by H-NS. *J Bacteriol* **192**: 6456–6464.

Vertommen, D., Ruiz, N., Leverrier, P., Silhavy, T.J., and Collet, J.F. (2009) Characterization of the role of the *Escherichia coli* periplasmic chaperone SurA using differential proteomics. *Proteomics* **9**: 2432–2443.

Vetterli, S.U., Zerbe, K., Müller, M., Urfer, M., Mondal, M., Wang, S.Y., et al. (2018) Thanatin

targets the intermembrane protein complex required for lipopolysaccharide transport in *Escherichia coli*. *Sci Adv* **4**: 1–8.

Villa, R., Martorana, A.M., Okuda, S., Gourlay, L.J., Nardini, M., Sperandio, P., *et al.* (2013) The *Escherichia coli* Lpt transenvelope protein complex for lipopolysaccharide export is assembled via conserved structurally homologous domains. *J Bacteriol* **195**: 1100–1108.

Vogel, J., and Luisi, B.F. (2011) Hfq and its constellation of RNA. *Nat Rev Microbiol* **9**: 578–589.

Vytvytska, O., Jakobsen, J.S., Balcunaite, G., Andersen, J.S., Baccarini, M., and Gabain, A. Von (1998) Host factor I, Hfq, binds to *Escherichia coli* *ompA* mRNA in a growth rate-dependent fashion and regulates its stability. *Proc Natl Acad Sci U S A* **95**: 14118–14123.

Vytvytska, O., Moll, I., Kaberdin, V.R., Gabain, A. Von, and Bläsi, U. (2000) Hfq (HF1) stimulates *ompA* mRNA decay by interfering with ribosome binding. *Genes Dev* **14**: 1109–1118.

Wade, J.T., Roa, D.C., Grainger, D.C., Hurd, D., Busby, S.J.W., Struhl, K., and Nudler, E. (2006) Extensive functional overlap between σ factors in *Escherichia coli*. *Nat Struct Mol Biol* **13**: 806–814.

Walderhaug, M.O., Polarek, J.W., Voelkner, P., Daniel, J.M., Hesse, J.E., Altendorf, K., and Epstein, W. (1992) KdpD and KdpE, proteins that control expression of the *kdpABC* operon, are members of the two-component sensor-effector class of regulators. *J Bacteriol* **174**: 2152–2159.

Wall, E., Majdalani, N., and Gottesman, S. (2018) The Complex Rcs Regulatory Cascade. *Annu Rev Microbiol* **72**: 111–139.

Walsh, N.P., Alba, B.M., Bose, B., Gross, C.A., and Sauer, R.T. (2003) OMP peptide signals initiate the envelope-stress response by activating DegS protease via relief of inhibition mediated by its PDZ domain. *Cell* **113**: 61–71.

Wang, B., Mittermeier, M., and Artsimovitch, I. (2022) RfaH May Oppose Silencing by H-NS and YmoA Proteins during Transcription Elongation. *J Bacteriol* **204**: 1–13.

Wassarman, K.M., Repoila, F., Rosenow, C., Storz, G., and Gottesman, S. (2001) Identification of novel small RNAs using comparative genomics and microarrays. *Genes Dev* **15**: 1637–1651.

White, R.J. (1968) Control of amino sugar metabolism in *Escherichia coli* and isolation of mutants unable to degrade amino sugars. *Biochem J* **106**: 847–858.

- Whitfield, C., Kaniuk, N., and Firdich, E. (2003) Molecular insights into the assembly and diversity of the outer core oligosaccharide in lipopolysaccharides from *Escherichia coli* and *Salmonella*. *J Endotoxin Res* **9**: 244–249.
- Wiktor, J., Lesterlin, C., Sherratt, D.J., and Dekker, C. (2016) CRISPR-mediated control of the bacterial initiation of replication. *Nucleic Acids Res* **44**: 3801–3810.
- Wilson, G.G., Young, K.K.Y., Edlin, G.J., and Konigsberg, W. (1979) High-frequency generalised transduction by bacteriophage T4. *Nature* **280**: 80–82
- Wood, J.M. (2015) Bacterial responses to osmotic challenges. *J Gen Physiol* **145**: 381–388.
- Wösten, M.M.S.M., Kox, L.F.F., Chamnongpol, S., Soncini, F.C., and Groisman, E.A. (2000) A signal transduction system that responds to extracellular iron. *Cell* **103**: 113–125.
- Wu, H.C., and Wu, T.C. (1971) Isolation and characterization of a glucosamine-requiring mutant of *Escherichia coli* K-12 defective in glucosamine-6-phosphate synthetase. *J Bacteriol* **105**: 455–466.
- Wu, T., McCandlish, A.C., Gronenberg, L.S., Chng, S.S., Silhavy, T.J., and Kahne, D. (2006) Identification of a protein complex that assembles lipopolysaccharide in the outer membrane of *Escherichia coli*. *Proc Natl Acad Sci U S A* **103**: 11754–11759.
- Yamamoto, K., and Ishihama, A. (2005) Transcriptional response of *Escherichia coli* to external zinc. *J Bacteriol* **187**: 6333–6340.
- Yamanaka, K., Fang, L., and Inouye, M. (1998) The CspA family in *Escherichia coli*: Multiple gene duplication for stress adaptation. *Mol Microbiol* **27**: 247–255.
- Yethon, J.A., Heinrichs, D.E., Monteiro, M.A., Perry, M.B., and Whitfield, C. (1998) Involvement of *waaY*, *waaQ*, and *waaP* in the modification of *Escherichia coli* lipopolysaccharide and their role in the formation of a stable outer membrane. *J Biol Chem* **273**: 26310–26316.
- Yethon, J.A., Vinogradov, E., Perry, M.B., and Whitfield, C. (2000) Mutation of the lipopolysaccharide core glycosyltransferase encoded by *waaG* destabilizes the outer membrane of *Escherichia coli* by interfering with core phosphorylation. *J Bacteriol* **182**: 5620–5623.
- Yoo, W., Yoon, H., Seok, Y.J., Lee, C.R., Lee, H.H., and Ryu, S. (2016) Fine-tuning of amino sugar homeostasis by EIIA Ntr in *Salmonella Typhimurium*. *Sci Rep* **6**: 1–13.
- Yu, L., Li, W., Xue, M., Li, J., Chen, X., Ni, J., *et al.* (2020) Regulatory Role of the Two-Component System BasSR in the Expression of the EmrD Multidrug Efflux in *Escherichia*

coli. Microb Drug Resist **26**: 1163–1173.

Zhang, X., Li, Y., Wang, W., Zhang, J., Lin, Y., Hong, B., *et al.* (2019) Identification of an anti-Gram-negative bacteria agent disrupting the interaction between lipopolysaccharide transporters LptA and LptC. *Int J Antimicrob Agents* **53**: 442–448.

Zimmer, B., Hillmann, A., and Görke, B. (2008) Requirements for the phosphorylation of the *Escherichia coli* EliANtr protein *in vivo*. *FEMS Microbiol Lett* **286**: 96–102.

Zwir, I., Shin, D., Kato, A., Nishino, K., Latifi, T., Solomon, F., *et al.* (2005) Dissecting the PhoP regulatory network of *Escherichia coli* and *Salmonella enterica*. *Proc Natl Acad Sci U S A* **102**: 2862–2867.