Regulation of glucosamine-6-phosphate synthase synthesis by a hierarchical acting cascade composed of two small regulatory RNAs in *Escherichia coli.*

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List of abbreviations

5’UTR	5’ untranslated region
ABC	ATP binding cassette
ABS	activator binding site
ATP	adenosine triphosphate
bp	base pairs
DIG	digoxygenin
DNA	deoxyribonucleic acid
DTT	Dithiothreitol
EMSA	electro mobility sihft analysis
Fig.	Figure
FMDP	N3-(4-methoxyfumaroyl)-L-2,3-diaminopropanoic acid
Glc6P	glucose-6-phosphate
GlcN	glucosamine
GlcNAc	*N*-Acetylglucosamine
GlcN1P	glucosamine-1-phosphate
GlcN6P	glucosamine-6-phosphate
GlcNAc6P	*N*-Acetylglucosamine-6-phosphate
GlmS	glucosamine-6-phosphate synthase
GTP	guanosine triphosphate
IDA	iodacetamide
IHF	integration host factor
IPTG	isopropyl-β-D-1-thiogalactopyranoside
LB	Luria Bertani
LPS	lipopolysaccharide
mRNA	messanger RNA
NEM	*N*-ethylmaleimide
Neu5Ac	*N*-acetylneuraminic acid
nt	nucleotide
OD	optical density
PAP I	poly(A) polymerase
PCR	polymerase chain reaction
PNPase	polynucleotide phosphorylase

PTS	phosphotransferase system
PVDF	polyvinylidene difluoride membrane
RACE	rapid amplification of cDNA ends
RBS	ribosomal binding site
RNA	ribonucleic acid
rpm	rounds per minute
rRNA	ribosomal RNA
SD	Shine-Dalgarno sequence
SDS	sodium dodecyl sulfate
sRNA	small RNA
TAP	tobacco acid pyrophosphatase
TCS	two component system
UDP	uridin diphosphate
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Summary

Amino sugars are essential for the biosynthesis of the bacterial cell wall and of lipopolysaccharides. The enzyme glucosamine-6-phosphate synthase (GlmS) catalyzes the formation of glucosamine-6-phosohate (GlcN6P) from fructose-6-phosphate and glutamine, which is the first committed step in the biosynthesis of amino sugars. In *Escherichia coli*, GlmS is encoded together with enzyme GlmU in the bi-cistronic *glmUS* operon. While the enzymatic activity of GlmU is needed all the time, GlmS is only required, when no external amino sugars are available. Due to the fact that both genes are co-transcribed, it always was a mystery how differential expression of *glmU* and *glmS* could be achieved. This work solves this mystery. Following transcription, the *glmUS* co-transcript is cleaved within the stop-codon of *glmU* by RNase E, yielding a monocistronic *glmS* transcript. The amount of the *glmS*-transcript is regulated by a feedback mechanism in response to an intracellular limitation of GlcN6P. This regulation relies on the two small RNAs GlmY and GlmZ. GlmY and GlmZ are homologous sRNAs, both in sequence and in secondary structure, and act in a cascade to activate *glmS* expression. When the GlcN6P level decreases, GlmY accumulates and subsequently counteracts processing of GlmZ by RNase E. Only the unprocessed form of GlmZ is able to basepair with the *glmS* transcript. GlmZ basepairs with the left half-site of a stem-loop structure assisted by Hfq. This prevents access of ribosomes to the Shine-Dalgarno sequence. This interaction destroys the inhibitory stem-loop, thereby allowing efficient translation of *glmS*. In addition, the data indicate that the protein YhbJ might might play a role in this process. In a Δ*yhbJ* mutant, processing of GlmZ is abrogated and *glmS* expression is strongly induced. Therefore, it appears feasible that YhbJ is an intermediary factor in the GlmY/GlmZ cascade, which acts between GlmY and GlmZ and regulates GlmZ processing in a GlmY-dependent manner.

Both expression and abundance of GlmY, which acts at the top of this regulatory cascade, is extensively regulated. GlmY is continuously targeted for polynucleotide phosphorylase dependent degradation by poly(A) polymarase dependent polyadenylation. This removes active GlmY species from the cell and keeps the cascade sensitive to the GlcN6P signal. Gene

glmY is expressed from two overlapping σ^{70}- and σ^{54}-dependent promoters. Both promoters initiate transcription at the same nucleotide. Therefore, identical GlmY species are generated from both promoters. The σ^{70}-dependent promoter is active mainly during the exponential growth phase, while activity of the σ^{54}-dependent promoter increased during transition to stationary phase. The σ^{70}-dependent promoter appears to be constitutively active. In contrast, the σ^{54}-dependent promoter subject to regulation by the GlrR/GlrK two component system and YhbJ. GlrR is a σ^{54}-dependent activator protein and is shown to bind to three specific binding sites of the consensus sequence $TGTCN_{10}GACA$, which are present upstream of the *glmY* promoter region. The activity of the σ^{54}-dependent promoter depends on the presence of GlrR. The activity of this promoter is also reduced in a Δ*yhbJ* mutant. This effect is independent from GlmY and GlmZ, but the mechanism of YhbJ-dependent modulation of *glmY* σ^{54}-promoter activity is unknown. Activity of the *glmY*-promoters is not altered in response to GlN6P limitation. Therefore, the GlcN6P signal must be sensed post transcription of *glmY*.

In summary, this work shows how differential expression of the bi-cistronic *glmUS* operon is achieved by post-transcriptional regulation of *glmS* expression by two homologous sRNAs, GlmY and GlmZ. These sRNAs act in a cascade and are the first example for hierarchically acting sRNAs. In addition, this work presents the first example of overlapping σ^{70}- and σ^{54}-dependent promoters that initiate transcription at the same nucleotide.

1. Introduction

Most bacteria live in environments, which may be subject to rapid changes. These changes may be beneficial such as influx of preferred substrates, but may also be potentially life threatening such as heat shock, cold shock, changed osmolarity or attack by antibiotic substances. In order to survive the organism must be able to rapidly adapt to these changes. It can be necessary to turn on the expression of one set of genes, while at the same time the expression of another set of genes must be turned off and the expression levels of other genes is only modulated. Some stress signals require a broad adaptive response involving many different genes, while others might require only the altered expression of very few genes. Bacteria have developed a variety of regulatory systems to deal with these different requirements for adaptation. Regulation can be achieved at the level of transcription through altering transcription rates, post-transcriptionally by alteration of transcript stability, translation efficiency or premature transcription termination or post translation by modulation of enzyme activity or stability. Regulation at the level of transcription initiation can be achieved by DNA-binding regulators of promoter activity such as metabolite binding activators or repressors and two component systems (TCS). TCS consist of a signal sensing histidine kinase which activates a DNA-binding response regulator by phosphorylation in response to the signal. Another option for regulation at the level of transcription are alternative sigma factors. Post-transcriptional regulation may be mediated by metabolite sensing or protein binding riboswitches or by regulatory small RNAs (sRNAs), which bind to their target mRNA by base-pairing and are able to alter transcript stability or may activate or repress translation of the encoded protein.

1.1 Posttranscriptional regulation

Posttranscriptional regulation encompasses all regulatory processes which act after transcription has been completed. There are several options for the cell to perform such a regulation: I) translation rates of proteins may be adjusted, II) transcript stability may be altered. Many different mechanisms have developed, which achieve these regulations by very different mechanisms,

which depend on RNA molecules as regulators. Riboswitches form one class of such regulators. These are mRNA leader sequences, which act in *cis* and are able to fold into alternative secondary structures that change upon an alteration in temperature or binding of metabolites or proteins (Henkin, 2008; Henkin and Yanofsky, 2002; Stülke, 2002; Klinkert and Narberhaus, 2009). Transcripts can also be regulated by small RNA molecules, which can be encoded either in *cis* (=at the same location as the target, e.g. on the non-coding strand of the DNA) or in *trans* (=at a different location on the chromosome) of the regulated mRNA. These sRNAs have in common that they usually act by base-pairing with the target RNA (Brantl, 2009).

1.1.1 Riboswitches and ribozymes

Riboswitches are *cis*-encoded regulatory RNA-elements that reside within the 5' untranslated region (5'UTR) of the regulated mRNA target. A riboswitch may take up two alternative secondary structures. Riboswitches regulate translation or transcription of their targets by alternating between these secondary structures in response to e.g. binding of a metabolite or a protein. Riboswitches attenuate translation by hiding the ribosomal binding sequence in one of the secondary structures. Transcription is controlled by alternation between a transcriptional terminator and an antiterminator structure that allows read-through of RNA-polymerase (Henkin, 2008; Henkin and Yanofsky, 2002; Stülke, 2002).

Essentially, Ribozymes are RNA species that catalyze a chemical reaction. In most cases they catalyze the cleavage of RNA phosphodiester bonds (Scott, 2007), but some ribozymes possess other activities, e.g. the peptidyl transferase activity of the 23S rRNA (Nissen *et al.*, 2000; Steitz and Moore, 2003). Usually RNA-cleaving ribozymes are constitutively active and do not perform regulatory tasks such as hammerhead ribozymes and RNase P (Scott, 2007). So far, only one exception is known: the *glmS* ribozyme of *Bacillus subtilis*. The *glmS* ribozyme is located at the 5'UTR of the *glmS* transcript and regulates *glmS* expression in response to the intracellular availability of glucosamine-6-phosphate (GlcN6P) (Winkler *et al.*, 2004), the product of the reaction catalyzed by the glucosamine-6-phosphate synthase (GlmS). When GlcN6P levels are high in the cell, GlcN6P binds to the *glmS*

ribozyme leading to autocatalyzed cleavage of the *glmS* transcript. Subsequently the *glmS* mRNA is degraded by RNase J1 (Collins *et al.*, 2007). As opposed to riboswitches, the secondary structure of the *glmS* ribozyme does not change upon binding of GlcN6P. Instead, the bound GlcN6P participates in the ribozyme reaction (Winkler *et al.*, 2004). Therefore, *glmS* transcript, which does not contain bound GlcN6P, is stable because the *glmS* ribozyme is inactive in this case.

1.1.2 Small regulatory RNAs

Small noncoding RNAs (sRNAs) are a widespread principle for gene regulation and they occur in all three domains of life. For the first time, sRNAs with regulatory functions were discovered in *E. coli*. In 1981, it was reported that the replication of the plasmid ColE1 is regulated by the ColE1 encoded sRNA RNA I (Tomizawa *et al.*, 1981). The first examples for sRNAs, which controls gene expression, were also found in *E. coli*: the sRNA MicF reduces the amount of *ompF* transcript by direct binding to the *ompF* mRNA (Mizuno *et al.*, 1984) and Spot42 was found to have a regulatory function (Ikemura and Dahlberg, 1973; Rice *et al.*, 1987; Rice and Dahlberg, 1982). After these initial discoveries, only very few bacterial sRNAs were identified for a long time. Instead, regulatory RNAs emerged as a prominent tool for genetic modification of eukaryotic cells (Scherer and Rossi, 2003) and also as a widespread principle for regulation in eukaryotes (Mattick, 2003). Only in recent years, many sRNAs have been identified in prokaryotes, first in *E. coli* (Wassarman *et al.*, 2001; Argaman *et al.*, 2001; Vogel *et al.*, 2003) and subsequently also in diverse prokaryotes (some examples: *Vibrio* (Lenz *et al.*, 2004) *Listeria monocytogenes* (Christiansen *et al.*, 2006), *Salmonella typhimurium* (Padalon-Brauch *et al.*, 2008), *Caulobacter crescentus* (Landt *et al.*, 2008)). It has thus become obvious that regulatory sRNAs are also very important for regulation of gene expression in prokaryotes. For *E. coli*, about 100 sRNAs are known so far. But while the number of known sRNAs increases, the number of sRNAs with an assigned function is still quite small. In this regard, most is known about the sRNAs of *E. coli*.

1.1.3 Types of sRNAs and their preferred modes of action

1.1.3.1 *Cis*-encoded antisense RNAs

Cis-encoded antisense RNAs are encoded on the opposite DNA strand of their respective target genes. This has the consequence that *cis*-encoded RNAs are perfectly complementary to their targets. Therefore, *cis*-encoded RNAs act on their targets by direct base-pairing. Most of them inhibit translation, e.g. in *E. coli* SymR inhibits translation of the *symE* mRNA (Kawano *et al.*, 2007), or facilitate degradation of target mRNA, e.g. RatA of *B. subtilis* facilitates degradation of the *txpA* mRNA (Silvaggi *et al.*, 2005). In some cases, it has been observed that the target RNA is stabilized by the sRNA, e.g. in *E. coli* GadY stabilizes the *gadXW* transcript (Opdyke *et al.*, 2004).

1.1.3.2 *Trans*-encoded sRNAs

Trans-encoded sRNAs are encoded at sites on the chromosome, which are distinct from their target genes. Usually sRNAs do not share perfect sequence complementarities with their targets. They use diverse mechanisms to achieve their regulatory functions. While most sRNAs also act by base-pairing with their target mRNAs, a few act by binding and sequestration of regulatory proteins. The base-pairing with the target RNA is usually achieved by using only small stretches of imperfect complementarities. Therefore, many sRNAs require the Sm-like RNA binding chaperone Hfq for binding (Sittka *et al.*, 2008; Valentin-Hansen *et al.*, 2004). Hfq forms a hexameric ring structure (Brennan and Link, 2007), which possesses binding sites both for the target mRNAs and the regulatory sRNAs. Binding of both the target and the sRNA to Hfq leads to formation of a mRNA:sRNA complex (Brennan and Link, 2007). Base-pairing sRNAs typically act by activation or inhibition of translation of their target mRNAs. The most frequently encountered mechanism for regulation of mRNA targets by sRNAs is inhibition of translation by base-pairing to the Shine-Dalgarno region, making it inaccessible for the ribosomes. Some examples for this mechanism are inhibition of *ptsG* translation by SgrS (Kawamoto *et al.*, 2005), down-regulation of *galK* translation by Spot42 (Møller *et al.*, 2002), inhibition of *ompA* translation by MicA (Udekwu *et al.*, 2005; Rasmussen *et al.*, 2005) and inhibition of *ompC* translation by MicC

(Chen *et al.*, 2004). Activation of mRNA translation is less frequent. In this case the Shine-Dalgarno sequence usually is inaccessible for the ribosome due to a secondary structure in the mRNA. The sRNA is able to open up this structure by base-pairing to the region, which is complementary to the Shine-Dalgarno sequence. Examples for sRNAs acting by this mechanism are DsrA, which activates *rpoS* translation (Majdalani *et al.*, 1998; Lease *et al.*, 1998), and activation of *shiA* translation by RybB (Prevost *et al.*, 2007). Apart from these frequently occurring base-pairing mechanisms, examples for other mechanisms are known, some of which are discussed in more detail below.

Some sRNAs do not act by base-pairing to a target mRNA. Instead, their targets are proteins, which are inhibited by binding of the sRNA. One example for protein binding sRNAs are the *E. coli* CsrB and CsrC sRNAs which regulate the global carbon storage protein CsrA by sequestration. CsrA is an RNA binding protein, which regulates translation of the *glg* mRNA by binding to the Shine-Dalgarno sequence. Both sRNAs possess the sequence motive that is recognized by CsrA and are able to titrate CsrA away from the *glg* mRNA (Weilbacher *et al.*, 2003; Liu *et al.*, 1997; Baker *et al.*, 2002). Another case of regulation at the protein level by a sRNA is 6S RNA, which has the ability to form a complex with σ^{70} RNA polymerase holoenzyme and thereby downregulates transcription from σ^{70}-dependent promoters at the onset of stationary phase (Wassarman, 2007).

1.1.4 Physiological functions and targets of sRNAs in *E. coli*

1.1.4.1 Toxin/antitoxin systems

One major class of regulatory RNAs in *E. coli* comprises the RNA moiety of type I toxin/antitoxin systems (Fozo *et al.*, 2008a). Toxin/antitoxin systems are frequently found in bacteria. They consist of two compounds: a toxin moiety and an antitoxin moiety. The toxin moiety is always a toxic protein. The antitoxin can either be a sRNA (type I system) or a small protein (type II system). The small proteins of the type II systems suppress the toxicity of the toxin by binding the toxic protein. In contrast, type I sRNA antitoxins function by repressing the toxin encoding mRNA (Fozo *et al.*, 2008a). The Hok-Sok system was the first type I system that was discovered (Gerdes *et al.*, 1985;

Gerdes *et al.*, 1986). It is a plasmid encoded system, which ensures that the plasmid is not lost from cells, because loss of the plasmid results in rapid removal of the repressing RNA, but not of the toxic proteins from the cell. Early on, other plasmid encoded type I systems were also discovered, such as the F plasmid encoded *flm*- and SrnB-SrnC-systems (Onishi, 1975; Loh *et al.*, 1988). In addition to plasmid encoded systems, *E. coli* also possesses several chromosomally encoded type I toxin/antitoxin systems and some of these have been characterized in recent years. Examples are the Ibs-Sib-, TisB-IstR-1-, Sho-OhsC-, SymE-SymR- and Ldr-Rdl-systems (Fozo *et al.*, 2008b; Vogel *et al.*, 2004; Kawano *et al.*, 2007; Kawano *et al.*, 2002). The physiological function of chromosomally encoded toxin/antitoxin systems is not yet clear. While some systems might give the cells a competitive advantage under some conditions, it has also been speculated that these systems comprise “selfish DNA” like e.g. transposons (Fozo *et al.*, 2008a). Usually, cells do not benefit from such DNA elements, instead the DNA elements solely aim for their own proliferation.

1.1.4.2 Regulation of outer membrane proteins

A large number of sRNAs, for which targets have been identified so far, regulate outer membrane proteins. The outer membrane represents the outermost barrier against the environment in Gram-negative bacteria. It represents the first permeability border of the cell and provides the first defense against toxins and antibiotics. In pathogenic organisms, proteins of the outer membrane are responsible for interaction with the eukaryotic host. Outer membrane composition is extensively regulated at the transcriptional, as well as the post-transcriptional level involving sRNAs (Vogel and Papenfort, 2006). MicF, which represses translation of the mRNA encoding the outer membrane protein OmpF, was one of the first *trans*-encoded regulatory sRNAs to be discovered. (Mizuno *et al.*, 1984; Schmidt *et al.*, 1995). Various sRNAs are known that regulate sometimes multiple targets, e.g. RybB inhibits expression of *ompC*, *ompW* and *rpoE* (Johansen *et al.*, 2006; Thompson *et al.*, 2007), sometimes just one target, e.g. MicA inhibits translation of *ompA* (Udekwu *et al.*, 2005), which are located at the outer membrane. The expression of some, but not all of these sRNAs is subject to

activation by the σ^E-factor, which regulates the response to extracytoplasmic stress, e.g. MicA and RybB (Johansen *et al.*, 2006; Thompson *et al.*, 2007). As a result, the σ^E-dependent sRNAs are activated and rapidly downregulate their targets in response to this stress. Outer membrane proteins rapidly misfold, when the cell encounters extracytoplasmic stress. Therefore it makes sense for the cell to stop synthesis of the outer membrane proteins, when it encounters this type of stress.

1.1.4.3 Regulation of sugar metabolism

In *E. coli*, altogether five sRNAs are known that regulate sugar metabolism: Spot42, SgrS, ChiX (Görke and Vogel, 2008; Figueroa-Bossi *et al.*, 2009; Overgaard *et al.*, 2009), and GlmY and GlmZ (this work). Spot42 was one of the first sRNAs that was characterized. It is the regulator of *galK*, which encodes galactokinase (GalK)(Møller *et al.*, 2002). GalK catalyzes the ATP-dependent phosphorylation of galactose to galactose-1-phosphate. This is the first step for the utilization of galactose as a carbon source. Gene *galK* is encoded within the *galETKM* operon. While GalK is only needed, when galactose is present as single carbon source, GalE and GalT are needed at all times, because they catalyze the biosynthesis of UDP-sugars, which are needed for biosynthesis of lipopolysaccharides (LPS) of the outer membrane. Therefore, transcription of the *gal*-operon is relatively high, even in the absence of the inducer galactose. Under such conditions, *galK* translation is downregulated by Spot42 in response to carbon source availability, allowing ongoing biosynthesis of UDP-sugars.

The function of sRNA SgrS is the response to phosphosugar stress. In *E. coli* glucose is taken up by the phosphotransferase system (PTS). The key step of the uptake process is catalyzed by the *ptsG* encoded protein $IICB^{Glc}$, which transports glucose across the membrane and phosphorylates it during the transport process (Deutscher *et al.*, 2006). While glucose is the preferred carbon source for *E. coli*, high intracellular levels of glucose-6-phosphate (Glc6P) are toxic for the cell. Therefore, intracellular phosphosugar levels must to be tightly controlled. Upon accumulation of Glc6P, SgrR activates transcription of SgrS (Vanderpool and Gottesman, 2004). SgrS then

counteracts phosphosugar stress by downregulating *ptsG* mRNA through Hfq-assisted base-pairing with the Shine-Dalgarno region (Vanderpool and Gottesman, 2004; Kawamoto *et al.*, 2006; Kawamoto *et al.*, 2005; Morita *et al.*, 2004). While SgrS obviously acts via base-pairing in down-regulation of *ptsG* mRNA, it has been shown that SgrS also encodes the small peptide SgrT. SgrT is a repressor of $IICB^{Glc}$ transporter activity (Wadler and Vanderpool, 2007).

The sRNA ChiX regulates the expression of the gene *chiP*, which encodes an outer membrane porin that is responsible for transport of the amino sugars chitobiose and chitotriose from the environment into the periplasm ((Figueroa-Bossi *et al.*, 2009); for more information on chitobiose metabolism see section 1.3). The mechanism by which ChiX regulates its target mRNA *chiP* is quite unusual. In the absence of the substrate chitobiose, *chiP*-expression is repressed by ChiX through inhibition of ChiP translation (Rasmussen *et al.*, 2009). When chitobiose becomes available as a substrate, the *chb*-operon is induced. This operon encodes functions for uptake and metabolism of chitobiose (Plumbridge and Pellegrini, 2004). The intergenic region between two genes, *chbB* and *chbC*, on the corresponding mRNA is able to bind ChiX sRNA. ChiX preferentially binds to the *chbB-chbC*-intergenic region and therefore the *chb*-mRNA traps ChiX upon induction of *chb*-expression. Under these conditions, ChiX is titrated away from its target *chiP*, which subsequently is no longer repressed and can be translated (Overgaard *et al.*, 2009; Figueroa-Bossi *et al.*, 2009). In light of the discovery of this novel mechanism, it appears possible that many sRNA binding sites, which have been identified at unusual positions within mRNAs, e.g. far removed from the Shine-Dalgarno sequence, may represent indirect targets that are used analogously to the *chbB-chbC* intergenic region.

The two sRNAs GlmY and GlmZ regulate the expression of *glmS*, which encodes GlmS. GlmS catalyzes the first dedicated step of amino sugar biosynthesis (see section 1.3). The regulation of *glmS* expression by GlmY and GlmZ is the focus of this work.

1.1.4.4 Regulation of iron metabolism

Iron is a very important and rarely available nutrient for all organisms: iron is an essential co-factor for enzymes of the tricarboxylic cycle, respiration, DNA synthesis and biosynthesis of metabolites (Andrews *et al.*, 2003). At the same time, under oxygen-rich conditions iron is a source of radicals, which are harmful to the cell. Therefore uptake and use of iron is extensively regulated, depending on the availability of iron. When the intracellular iron level is high in *E. coli*, iron uptake is repressed by the Fur protein (Hantke, 1981), and at the same time expression of many iron using proteins is upregulated by Fur (Hantke, 2001) in a RyhB-dependent manner (Massé and Gottesman, 2002). RyhB is a sRNA, which is repressed by Fur (Massé and Gottesman, 2002; Vassinova and Kozyrev, 2000). As a consequence, limited availability of iron leads to activation of RyhB expression, which subsequently downregulates the expression of non-essential iron using protein (Massé *et al.*, 2005). In these cases RyhB functions by inhibiting translation of its mRNA targets (Morita *et al.*, 2006), which are subsequently degraded by the degradosome and RNase III (Masse *et al.*, 2003; Afonyushkin *et al.*, 2005). One special case of regulation by RyhB is the *iscRSUA* transcript. This transcript encodes the IscR regulator of genes, which depend on Fe-S for their activity, while *iscSUA* encodes a machinery that is necessary for biosynthesis of Fe-S clusters. RyhB regulates the ´differential degradation of the *iscRSUA* transcript under iron-limiting conditions. While the *iscR* part of the transcript remains stable upon regulation by RyhB, the *iscSUA* part of the transcript is rapidly degraded in a RyhB-dependent manner (Desnoyers *et al.*, 2009). Regulation of iron metabolism by a sRNA has also been discovered in other bacteria (Masse *et al.*, 2007).

1.1.4.5 Regulation of *rpoS*

Regulation of the σ^S encoding gene *rpoS* is an interesting case for sRNA-dependent gene regulation. The expression of *rpoS* is not only extensively regulated on the level of transcription initiation, but also post transcription by at least two different sRNAs: DsrA and RprA (Brantl, 2009). DsrA activates translation of *rpoS* during exponential growth at low temperatures (Lease *et*

al., 1998; Sledjeski *et al.*, 1996). The sRNA RprA activates translation of *rpoS* in response to osmotic shock (Majdalani *et al.*, 2002; Majdalani *et al.*, 2001). Although transcription of *rpoS* is initiated at altogether seven different promoters (Gama-Castro *et al.*, 2008; Lange and Hengge-Aronis, 1994; Lange *et al.*, 1995; Takayanagi *et al.*, 1994), these promoters apparently are not sufficient to effectively respond to all environmental signals that demand an altered expression of *rpoS*. The case of *rpoS* shows how gene regulation can occur at multiple levels by diverse mechanisms.

1.2 Control of transcription initiation

Many regulatory processes are conducted at the level of transcription initiation. Control on the level of transcription initiation is usually achieved by repressor or activator proteins that bind to the DNA in the vicinity of the promoter sequence. As a result, the frequency of recognition of the promoter sequence by the RNA polymerase holoenzyme is either decreased or increased and hence the rate of transcription initiation is altered. Another option to achieve a regulation of transcription initiation is the use of alternative sigma factors that usually recognize different promoter sequences.

1.2.1 Two-component systems

Two-component systems (TCS) are signal response systems, which can be found in all domains of life (West and Stock, 2001). They are the predominant form for signal perception and transduction in bacteria (Mitrophanov and Groisman, 2008). A TCS usually consists of a sensor kinase and a response regulator. The sensor kinase contains a conserved histidine residue, which autophosphorylates with ATP. The phosophoryl group is then transferred to a conserved aspartate residue in the response regulator (Mitrophanov and Groisman, 2008). The *E. coli* chromosome encodes for altogether 29 sensor kinases and 32 response regulators (Mizuno, 1997). Often, a sensor kinase is encoded in one operon together with its cognate response regulator. TCS regulate gene expression in response to various signals. The sensor kinase senses the signal. This can either be an external or a cytoplasmic signal. In response to this signal, the sensor kinase changes its rate of autophosphorylation and thereby the subsequent phosphoryl group transfer to

the cognate response regulator is affected accordingly. In addition most histidine kinases also possess a phosphatase activity (Szurmant *et al.*, 2007). Therefore, the response regulator can be dephosphorylated, when the signal is absent from the cell or environment. The response regulator usually regulates the amounts of target proteins by modulation of transcription initiation (Gao *et al.*, 2007). It has different DNA-binding abilities depending on its phosphorylation status. Therefore, an altered phosphorylation will alter its DNA-binding ability and this leads to altered expression of genes that are regulated by the TCS (Mitrophanov and Groisman, 2008; Szurmant *et al.*, 2007). The response regulator regulates the activity of specific promoters by binding to specific sites within the promoter region. Most response regulators act as activators of transcription.

1.2.2 Alternative sigma factors

Sigma factors are proteins, which enable the RNA polymerase to recognize promoters and to initiate transcription. The RNA polymerase core enzyme is unable to recognize promoters. Often, different sigma factors recognize different promoter consensus sequences. *E. coli* possesses altogether seven different sigma factors. σ^{70} is the housekeeping sigma factor of *E. coli*, which is responsible for transcription of housekeeping genes and many non-essential inducible genes (Helmann and Chamberlin, 1988). Most genes possess σ^{70}-dependent promoters. σ^{S} (also called σ^{38}) is associated with general stress responses of the cell, which impair cell growth such as lack of nutrients, oxidative stress, elevated temperatures and others. σ^{S} recognizes similar sequences as σ^{70} and promoter selectivity by σ^{70} and σ^{S} is believed to be achieved by small deviations between both consensus sequences (Gaal *et al.*, 2001). σ^{32} is the general regulator of heat shock response and recognizes a specific consensus promoter sequence (Tobe *et al.*, 1984; Cowing *et al.*, 1985). σ^{E} (also called σ^{24}) is another stress response sigma factor. It is associated with the response to extracytoplasmic or extreme heat stress and also recognizes a distinct promoter consensus sequence (Rhodius *et al.*, 2006). The sigma factors σ^{FecI} and σ^{28} have very specific functions and regulate the ferric acid transporter and flagella synthesis, respectively

(Angerer *et al.*, 1995; Mytelka and Chamberlin, 1996). The seventh sigma factor of *E. coli* is σ^{54}, which is discussed in more detail in section 1.2.3.2.

1.2.3 The *rpoN* operon

σ^{54} is encoded by gene *rpoN* within the *rpoN*-operon. The organization of this operon is fully conserved in *Enterobacteriaceae* and close relatives and well conserved in many other *Proteobacteria* (Comas *et al.*, 2008). It consists of the genes *rpoN*, *hpf*, *ptsN*, *yhbJ* and *npr* (see Fig. 1).

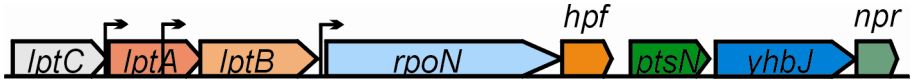


Figure 1: Genetic organization of the *E. coli rpoN* operon. The operon includes genes *lptA*, *lptB*, *rpoN*, *hpf*, *ptsN*, *yhbJ* and *npr*. It is transcribed from three promoters, one is located upstream of *lptA*, one is located upstream of *lptB* within *lptA* and one is located within the *lptB-rpoN* intergenic region.

The *rpoN*-operon is transcribed from a σ^{70}-dependent constitutively active promoter that is located in the *lptB-rpoN* intergenic region (Powell *et al.*, 1995; Castaño and Bastarrachea, 1984; Jones *et al.*, 1994). One σ^{E}-dependent promoter is located upstream of *lptA* (Sperandeo *et al.*, 2007), another σ^{E}-dependent promoter has been predicted to exist within *lptA*, but its existence was not confirmed experimentally (Rhodius *et al.*, 2006). Since no terminator is present between *lptB* and *rpoN*, the *rpoN*-operon might be co-transcribed with *lptA* and *lptB*. Although the proteins, which are encoded in this operon, do not share a common function the majority appear to be important general regulators.

1.2.3.1 *lptAB*

The gene products of *lptBA* and *lptC*, which is encoded directly upstream of *lptA*, but cannot be not considered part of the *rpoN* operon, form an ABC-transporter, which is part of a large complex involved in transport of LPS to the outer membrane (Sperandeo *et al.*, 2008; Sperandeo *et al.*, 2007; Sperandeo *et al.*, 2006). All three genes are essential in *E. coli* (Sperandeo *et al.*, 2006). LptB is the cytoplasmic ATP-binding component, LptA is the periplasmic binding component (Sperandeo *et al.*, 2007) and LptC is the integral membrane component of the ABC-transporter (Sperandeo *et al.*, 2008).

1.2.3.2 *rpoN*

Gene *rpoN* encodes for σ^{54}. This alternative sigma factor greatly differs from all other sigma factors of *E. coli*. In contrast to all other *E. coli* sigma factors, it shares no significant amino acid sequence homology with σ^{70}, except for a short stretch at its C-terminus (Merrick, 1993; Merrick and Gibbins, 1985). In addition, σ^{54} RNA polymerase holoenzyme does not bind to -10 and -35 elements. Instead, highly conserved -12 and -24 motifs are recognized by the polymerase (Reitzer and Schneider, 2001). Upon binding to the promoter sequence, the polymerase forms a closed complex. In this complex, the DNA is still present in its double stranded form and transcription cannot be initiated. For open complex formation, the additional binding of a σ^{54}-specific activator protein to upstream DNA sequences is required. Transcription from σ^{54}-dependent promoters is only initiated after activator protein dependent ATP hydrolysis, which results in melting of the DNA and formation of the open complex (Burrows *et al.*, 2009; Weiss *et al.*, 1991). These σ^{54}-promoter specific activator proteins are unusual for bacterial activators, because binding may occur at large distances from the promoter. For this reason, σ^{54}-activators are often referred to as enhancer binding proteins, since they function partly analogous to eukaryotic enhancer binding proteins. In *E. coli*, 11 such activator proteins are known, namely AtoC, FhlA, HyfR, NtrC, PrpR, PspF, RtcR, YfhA, NorR, YgeV and ZraR (Reitzer and Schneider, 2001; Gardner *et al.*, 2003). Previously, it was believed that also *dhaR* codes for a σ^{54}-activator protein (Reitzer and Schneider, 2001), but it was shown that DhaR activates transcription of a σ^{70}-dependent promoter independently of σ^{54} (Bächler *et al.*, 2005). The targets are known for most of these activator proteins, with the exception of YfhA for which no targets are known and YgeV, which might be the activator of the *xdhA* promoter (Reitzer and Schneider, 2001; Gardner *et al.*, 2003). A target for YfhA is identified in this work. For a long time it was assumed that σ^{54} is a nitrogen specific sigma factor, since the only known targets of σ^{54} were nitrogen related (Thöny and Hennecke, 1989). The activator for these genes is NtrC, which has a large regulon as compared to the other activator proteins (Gyaneshwar *et al.*, 2005). While NtrC regulates the expression of many genes, most other σ^{54}-activators regulate only one

promoter (Reitzer and Schneider, 2001). The NtrC-regulon is activated in response to nitrogen limitation and all other activator proteins activate their targets in response to other distinct and specific stresses. Some, but not all of these stresses are related to nitrogen limitation, making the response to nitrogen limitation the major function of σ^{54} (Reitzer and Schneider, 2001).

1.2.3.3 *hpf*

Gene *hpf* encodes the ribosome hibernation promoting factor Hpf. This factor together with ribosome modulation factor (RMF) is required for the formation of 100S ribosome particles in stationary phase. These particles are thought to preserve ribosomes for rapid reactivation, when cells encounter better growth conditions (Ueta *et al.*, 2005; Maki *et al.*, 2000). Hpf opposes the function of YfiA, which shares some homology with Hpf and is associated with 70S ribosomes during transition to stationary phase (Ueta *et al.*, 2005). Interestingly, most bacteria possess at least one homolog of *hpf* (Ueta *et al.*, 2008). This shows that the function encoded by *hpf* should be important for the cell even though *hpf* is not essential.

1.2.3.4 *ptsN* and *npr*

Enzymes IIA^{Ntr} and Npr, encoded by *ptsN* and *npr*, respectively, form a PTS together with EI^{Ntr}, which is encoded elsewhere on the chromosome by gene *ptsP* (Reizer *et al.*, 1992; Reizer *et al.*, 1996; Powell *et al.*, 1995; Rabus *et al.*, 1999). EI^{Ntr} autophosphorylates with phosphoryl groups, which are donated by PEP, and subsequently transfers the phosphoryl groups to IIA^{Ntr} via NPr (Rabus *et al.*, 1999; Zimmer *et al.*, 2008). This system is conserved in many *Proteobacteria* (Deutscher *et al.*, 2006). For a long time, no clear function for this Ntr-PTS could be defined. Since no phosphoryl group acceptor for IIA^{Ntr}~P was identified, it was speculated that the Ntr-PTS –in contrast to most other PTS- should have a regulatory function instead of being a transport system (Powell *et al.*, 1995). Early works suggested a nitrogen metabolism related function of the system. It was observed that inactivation of *ptsN* results in increased expression of the σ^{54}-dependent promoters $glnA_{p2}$, P_{nifL} and P_{nifH} in *Klebsiella pneumoniae*, although the inactivation of *ptsN* could not be

complemented by ectopically expressed *ptsN* (Merrick and Coppard, 1989). Inactivation of *E. coli ptsN* results in growth defects on some organic nitrogen sources, when a carbon source is additionally present. Activities of the nitrogen source dependent regulated promoters $glnA_{p1}$ and $glnA_{p2}$ are not affected by the *ptsN* mutation (Powell *et al.*, 1995). Unfortunately, no mechanism that would explain these effects was discovered, so far. Another work showed that the dephosphorylated form of IIA^{Ntr} is required for the derepression of the *ilvBN* operon encoding acetohydroxy acid synthase I, which catalyzes the first step of branched-chain amino acid biosynthesis (Lee *et al.*, 2005). In contrast to these reports, which link the Ntr-PTS to nitrogen metabolism, two recent studies show that IIA^{Ntr} is involved in regulation of two different potassium uptake systems. The dephosphorylated form of IIA^{Ntr} inhibits the TrkA subunit of the low affinity K^+ transporter Trk by direct binding (Lee *et al.*, 2007). This form of IIA^{Ntr} also interacts with KdpD, which is the sensor kinase of the KdpD-KdpE TCS, and stimulates phosphorylation of KdpD and KdpE. Phosphorylated KdpE then enhances expression of the *kdpFABC* operon, which encodes the high affinity K^+ transporter KdpFABC (Lüttmann *et al.*, 2009).

1.2.3.5 *yhbJ*

Gene *yhbJ* encodes a protein of unknown function. With some exceptions, homologs of *yhbJ* are present in most bacterial species. It is interesting to note that *yhbJ* often co-localizes with components of the PTS. In Gram-negative bacteria it is found in the *rpoN*-operon, eventually together with genes encoding HprK, EIIA and HPr homologs. In Gram-positive bacteria such as *B. subtilis* the *yhbJ* homolog *yvcJ* localizes to the *yvcIJK-crh-yvcN* operon. In this operon, *crh* encodes a homolog of HPr (Galinier *et al.*, 1997). The protein YhbJ and its homolog YvcJ both possess a P-loop containing ATPase domain, which is important for the function of YhbJ. It was shown that both YvcJ and YhbJ hydrolyze ATP and GTP (Luciano *et al.*, 2009). For *B. subtilis* YvcJ it was shown that inactivation of *yvcJ* results in reduced natural competence and that this defect can be compensated by overexpression of *comK* or *comS* (Luciano *et al.*, 2009). In *E. coli*, deletion of *yhbJ* results in strong overproduction of GlmS, which is involved in amino sugar biosynthesis

((Kalamorz *et al.*, 2007); this work). In addition, YhbJ also regulates the two sRNAs GlmY and GlmZ, which are also involved in regulation of *glmS* expression (this work).

1.3 Amino sugar metabolism in *E. coli*

Amino sugars are essential building blocks of the peptidoglycan component of the bacterial cell wall and they are required for the biosynthesis of the LPS components of the outer membrane. GlmS catalyzes the first and rate-limiting step of *de novo* amino sugar biosynthesis: the formation of GlcN6P and glutamate from fructose-6-phosphate and glutamine (Milewski, 2002; Durand *et al.*, 2008). GlcN6P is subsequently converted to glucosamine-1-phosphate by phosphoglucosamine mutase (GlmM) and is then converted to 5'-diphospho-*N*-acetyl-glucosamine (UDP-GlcNAc) via *N*-acetylglucosamine-1-phosphate by *N*-acetylglucosamine-1-phosphate uridyltransferase/glucosamine-1-phosphate acetyltransferase (GlmU) (Mengin-Lecreulx and van Heijenoort, 1993; Mengin-Lecreulx and van Heijenoort, 1994; Mengin-Lecreulx and van Heijenoort, 1996).

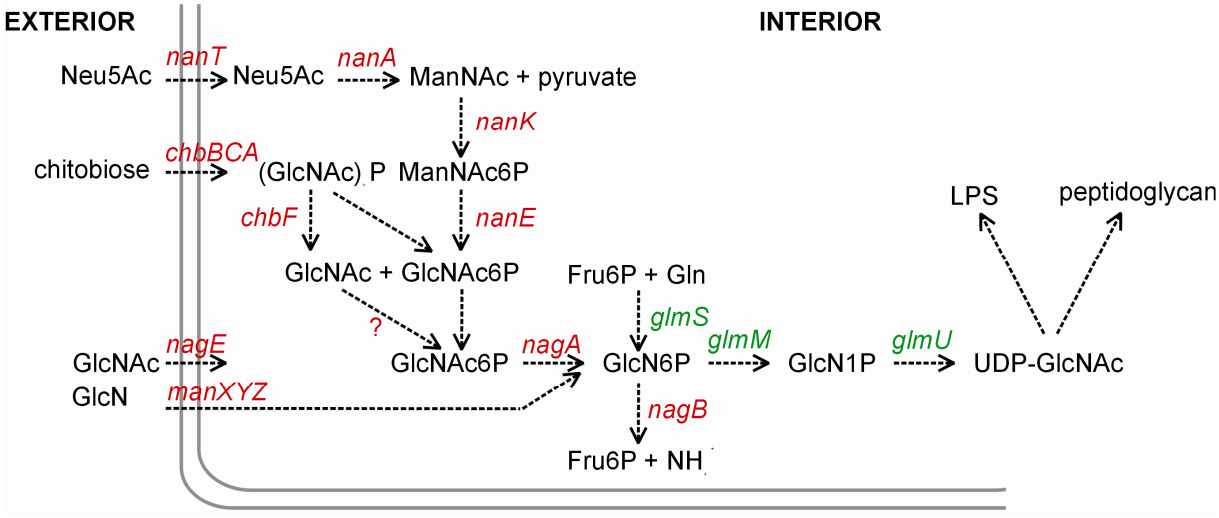


Figure 2: Catabolic pathways for GlcN, GlcNAc, Neu5Ac and chitobiose degradation, and the anabolic pathway for UDP-GlcNAc biosynthesis (modified from (Plumbridge, 1995; Plumbridge and Vimr, 1999)). Genes encoding functions of the anabolic pathway are displayed in green, while genes encoding functions of the catabolic pathway are displayed in red. The substrates chitobiose, GlcNAc and GlcN are taken up and phosphorylated by their respective PTS transporters and subsequently converted to GlcN6P by ChbF and NagA. Neu5Ac is taken up by NanT and subsequently converted to GlcN6P by NanA, NanE and NagA. GlcN6P is then either used as an energy and nitrogen source following the conversion to Fru6P and ammonium by NagB or used for biosynthesis of UDP-GlcNAc by GlmM and GlmU.

While amino sugars are important building blocks for the cell, they are also good carbon and nitrogen substrates. The cell is able to take up and utilize several different amino sugars such as glucosamine (GlcN), *N*-acetylglucosamine (GlcNAc), chitobiose and *N*-acetylneuraminic acid (Neu5Ac) (White, 1968; Keyhani and Roseman, 1997; Vimr and Troy, 1985). For an overview on amino sugar utilization and biosynthesis see Fig. 2. Both GlcN and GlcNAc are PTS-substrates. They are taken up by systems, which are encoded by *manXYZ* and *nagE*, respectively (Postma *et al.*, 1993). Upon transport across the membrane both substrates are phosphorylated, yielding GlcN6P and *N*-actetylglucosamine-6-phosphate (GlcNAc6P), respectively. GlcNAc6P is converted to GlcN6P by GlcNAc6P deacetylase (NagA), which is then further degraded to fructose-6-phosphate and ammonium by GlcN6P deaminase (NagB) (White, 1968). Chitobiose is transported into the periplasm by the chitobiose and chitotriose specific porin ChiP (Figueroa-Bossi *et al.*, 2009) and is then taken up into the cytoplasm by a chitobiose specific PTS, which is encoded by *chbBCA* (Keyhani *et al.*, 2000a; Keyhani *et al.*, 2000c; Keyhani *et al.*, 2000b). Upon transport across the membrane, chitobiose is phosphorylated yielding $(GlcNAc)_2P$ (Keyhani *et al.*, 2000a). $(GlcNAc)_2P$ is subsequently hydrolyzed, probably by the *chbF* encoded hydrolase ChbF, presumably yielding GlcNAc and GlcNAc6P, which can be further utilized. Neu5Ac is taken up from the environment by NanT. Subsequently it is converted into GlcN6P in several enzymatic steps, which are catalyzed by NanA, NanK and NanE (Plumbridge and Vimr, 1999; Vimr and Troy, 1985).

The biosynthetic and the catabolic pathways are connected by the intermediate GlcN6P, which therefore represents the central intermediate of amino sugar metabolism. GlcN6P has to be synthesized by GlmS, when no external amino sugars are available. But when external sources for amino sugars are available, biosynthesis of GlcN6P would be a waste of energy, since all amino sugars are degraded via this intermediate. In contrast, the other biosynthetic enzymes GlmM and GlmU are needed at all times. Genes *glmU* and *glmS* are encoded in a bi-cistronic operon. Expression of *glmUS* is weakly regulated in response to amino sugar availability by the master regulator of amino sugar metabolism, NagC. NagC belongs to the ROK-family of DNA-binding regulators (Titgemeyer *et al.*, 1994). When no amino sugars

are available in the medium NagC binds to its binding sites. In the case of the *glmUS* operon this results in a 3- to 4-fold activation of gene expression, while at the same time NagC functions as a repressor of the expression of the catabolic *nag*- and *chb*-operons (Plumbridge, 1995; Plumbridge *et al.*, 1993), but not of the *nan*-operon, which has its own regulator NanR (Kalivoda *et al.*, 2003). When amino sugars are available, NagC binds the intermediate GlcNAc6P and looses its DNA-binding ability. Thereby, the expression profile for catabolic and anabolic genes is inversed (Plumbridge *et al.*, 1993; Plumbridge, 1995). Although *glmU* and *glmS* are encoded within the same operon, they must be differentially expressed, since *glmU* is always needed and *glmS* is only needed under certain conditions. It always was a mystery, how such a differential expression could be achieved, since neither an additional promoter nor a terminator is present in the *glmUS* intergenic region (Walker *et al.*, 1984). As the following work will show this differential regulation is achieved by the sRNAs GlmY and GlmZ.

1.4 Aims of this study

The subject of this work is the regulation of *glmS* expression by the small RNAs GlmY and GlmZ. At the beginning of this study it was known that deletion of *yhbJ* results in strong overproduction of GlmS without effecting expression of *glmU*. A transposon mutagenesis screen showed that this overproduction is absolutely dependent on the presence of the sRNA GlmZ (formerly SraJ) (Kalamorz, 2009).

Small regulatory RNAs are currently emerging as a previously neglected and over-looked, but very important mechanism for regulation of gene expression in bacteria. Therefore, the focus of this work was on unraveling the underlying mechanisms, which are responsible for the GlmZ- and YhbJ-dependent differential expression of *glmU* and *glmS.* In this respect, it was also the intention of this work to find out the function of YhbJ.

In a first step, it was planned that factors, which are frequently involved in sRNA-mediated gene regulation, such as Hfq and RNase E should be analyzed for a putative function in GlmZ mediated regulation of *glmS*-expression. In parallel, it was planned to analyze the GlmZ sRNA in Δ*yhbJ* mutant strains and in mutant strains of other factors which affect *glmS*-expression.

Subsequently, more factors, which might govern regulation of GlmZ or *glmS* or which might be regulated by YhbJ were to be identified and analyzed further. In this context, other factors (e.g. other sRNAs) that might be regulated by YhbJ were to be screened, and then it was planned to see, whether such factors are also involved in regulation of *glmS*-expression.

1.5 List of publications

Reichenbach,B., Breustedt,D.A., Stülke,J., Rak,B. and Görke,B. (2007) Genetic dissection of specificity determinants in the interaction of HPr with enzymes II of the bacterial phosphoenolpyruvate:sugar phosphotransferase system in *Escherichia coli*. *J Bacteriol*. 189(13):4603-13.

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(6):1518-33.
*contributed equally

Reichenbach,B., Maes,A., Kalamorz,F., Hajnsdorf,E. and Görke,B. (2008) The small RNA GlmY acts upstream of the sRNA GlmZ in the activation of *glmS* expression and is subject to regulation by polyadenylation in *Escherichia coli*. *Nucleic Acids Res*. 36(8):2570-80.

Reichenbach,B., Göpel,Y. and Görke,B. (2009) Dual control by perfectly overlapping σ^{54}- and σ^{70}-promoters adjust**s** small RNA GlmY expression to different environmental signals. *Mol Microbiol*., in revision.

Görke,B. and Reichenbach,B. (2010) Intracellular phosphotransfer and signaling. In: Bacterial signaling, edited by K. Jung and R. Krämer, Wiley-VCH, Weinheim, in press.

2. Feedback control of glucosamine-6-phosphate synthase GlmS expression depends on the small RNA GlmZ and involves the novel protein YhbJ in *Escherichia coli*

The results described in this chapter were published in:

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(6):1518-33.

Author contributions:

This study was designed by F.K., B.Re., B.Ra. and B.G.. B.Re. performed all northern blotting experiments. F.K. performed all western blotting experiments and the transposon mutagenesis. B.Re., W.M. and F.K. constructed strains. Plasmids were constructed by F.K., W.M. and internship students under the supervision of B.Re. and B.G.. W.M. performed the primer extension analysis. β-galactosidase measurements were performed by F.K. except for those in Figure 1, which were performed by B.Re.. B.G. performed the complementation analysis in Figure 1. The paper was written by all authors.

Summary

Amino sugars are essential precursor molecules for the biosynthesis of bacterial cell walls. Their synthesis pathway is initiated by glucosamine-6-phosphate synthase (GlmS) which catalyzes the rate limiting reaction. We report here that expression of the *Escherichia coli glmS* gene is negatively feedback regulated by its product GlcN-6-P at the post-transcriptional level. Initially, we observed that mutants defective for *yhbJ*, a gene of the *rpoN* operon, overproduce GlmS. Concomitantly, a *glmS* mRNA accumulates that is derived from processing of the primary *glmUS* transcript at the *glmU* stop codon by RNase E. A transposon mutagenesis screen in the *yhbJ* mutant identified the small RNA GlmZ (formerly RyiA or SraJ) to be required for *glmS* mRNA accumulation. GlmZ, which is normally processed, accumulates in its full-length form in the *yhbJ* mutant. In the wild type, a decrease of the intracellular GlcN-6-P concentration induces accumulation of the *glmS* transcript in a GlmZ dependent manner. Concomitantly, GlmZ accumulates in its unprocessed form. Hence, we conclude that the biological function of GlmZ is to positively control the *glmS* mRNA in response to GlcN-6-P concentrations and that YhbJ negatively regulates GlmZ. Since in *yhbJ* mutants GlcN-6-P has no effect, YhbJ is essential for sensing this metabolite.

Introduction

Amino sugars are essential building blocks in all living organisms. In bacteria, they are required for synthesis of cell wall peptidoglycan and of lipopolysaccharides (LPS). The first and rate-limiting step in hexosamine synthesis is catalyzed by the enzyme glucosamine-6-phosphate synthase (GlmS), which converts fructose-6-phosphate and glutamine to glucosamine-6-phosphate (GlcN-6-P) and glutamate (Milewski, 2002; Teplyakov *et al.*, 2002). In *Escherichia coli* GlcN-6-P is subsequently isomerized to D-glucosamine-1-phosphate (GlcN-1-P) by enzyme GlmM and further converted to uridine 5'-diphospho-N-acetyl-D-glucosamine (UDP-GlcNAc) by enzyme GlmU (Fig. 1 A; (Mengin-Lecreulx and van Heijenoort, 1993; Mengin-Lecreulx and van Heijenoort, 1994; Mengin-Lecreulx and van Heijenoort, 1996)). UDP-GlcNAc is the major intermediate in the biosynthesis of all amino sugar macromolecules in the cell. All three enzymes in this pathway are essential in

E. coli. As an exception, GlmS is dispensable when exogenous amino sugars like N-acetylglucosamine (GlcNAc) or glucosamine (GlcN) are available. These sugars are taken up and converted to GlcN-6-P which can be utilized as carbon source and at the same time bypasses the reaction catalyzed by GlmS (Fig. 1 A, (Plumbridge and Vimr, 1999)).

Due to its central role in amino sugar synthesis, it is evident that the activity of GlmS must be tightly controlled. Eukaryotic GlmS enzymes are sensitive to strong feedback inhibition by UDP-GlcNAc, i. e. by the final product of the pathway initiated by GlmS (Milewski, 2002). Moreover, in Gram-positive bacteria the 5'-untranslated leader of the *glmS* gene contains a catalytic riboswitch which is activated by GlcN-6-P leading to site-specific self-cleavage of the *glmS* transcript and subsequent repression of *glmS* (Winkler *et al.*, 2004). Hence, in eukaryotes as well as in Gram-positive bacteria the cellular GlmS activity is feedback regulated by the flow of metabolites going through the GlcN-6-P/UDP-GlcNAc pathway. A comparable mechanism has so far been unknown in Gram-negative bacteria, though it has been predicted to exist (van Heijenoort, 1996; Milewski, 2002).

In *E. coli*, GlmU and GlmS are encoded in the bicistronic *glmUS* operon (Fig. 1 B). The operon is transcribed from two promoters present in front of *glmU*, which are subject to regulation by the DNA-binding protein NagC. Binding of NagC to operator sites present in front of *glmU* enhances *glmUS* transcription fourfold (Plumbridge *et al.*, 1993; Plumbridge, 1995). In addition, NagC acts as a repressor of the *nag* and *chb* operons that encode proteins involved in uptake and degradation of exogenous amino sugars like GlcNAc which are sequentially converted to N-acetyl-glucosamine-6-phosphate (GlcNAc-6-P) and GlcN-6-P (Plumbridge and Pellegrini, 2004; Plumbridge, 1991). GlcNAc-6-P is the inducer for NagC, and releases it from its operator sites. Hence, the availability of amino sugars in the medium antagonistically regulates the anabolic (*glm*) and catabolic (*nag, chb*) genes of amino sugar metabolism.

The starting point for the present work was an investigation of the *rpoN* operon of *E. coli*. This operon consists of the genes *rpoN*, *yhbH*, *ptsN, yhbJ* and *ptsO*. Gene *rpoN* encodes the sigma 54 factor that is required for the expression of primarily nitrogen- and stress-related genes (Reitzer and Schneider, 2001). YhbH may have a role in ribosome storage during

stationary phase (Ueta *et al.*, 2005). Genes *ptsN* and *ptsO* encode IIA^{Ntr} and NPr which are homologous to the IIA domain of the mannitol transporter and to HPr, respectively, and belong to the phosphoenolpyruvate:sugar phosphotransferase system (PTS). It was shown *in vitro* that the EI-paralog EI^{Ntr}, NPr and IIA^{Ntr} constitute a protein phosphorylation chain that works in parallel to the canonical PTS-phosphotransferases EI and HPr (Rabus *et al.*, 1999). EI^{Ntr} is encoded by *ptsP* elsewhere on the chromosome. The function of YhbJ has so far been unknown.

In this work we found that mutants defective in *yhbJ* strongly overproduce the GlmS protein. This overproduction results from the accumulation of a monocistronic *glmS* transcript that originates from processing of the *glmUS* transcript by RNase E at the *glmU* stop codon. We found that this mechanism requires the Sm-like protein Hfq and the small RNA of unknown function GlmZ (formerly RyiA or SraJ) that was previously shown to be subject to processing (Argaman *et al.*, 2001; Wassarman *et al.*, 2001). Our data also reveal the physiological meaning of this novel regulatory pathway. We show that a decrease of the intracellular concentration of GlcN-6-P leads to accumulation of the full-length form of GlmZ, which is in turn required for the activation of *glmS* expression. YhbJ appears to control processing and stability of GlmZ. Hence, the current work reveals a novel negative feedback mechanism in *E. coli* that adjusts expression of *glmS* to the intracellular concentration of its product, GlcN-6-P. This mechanism may keep the intracellular hexosamine concentration constant when changing growth conditions require a change in peptidoglycan and LPS synthesis rates. Due to its important role in control of *glmS* expression, we propose to rename RyiA as GlmZ.

Materials and methods

Growth conditions, strains and plasmids

Cells were routinely grown in LB at 37℃ under agit ation (200 rpm). Where necessary, media were supplemented with antibiotics (ampicillin: 100 µg/ml, chloramphenicol: 15 µg/ml, kanamycin: 30 µg/ml, spectinomycin: 50 µg/ml, tetracycline: 12.5 µg/ml). Arabinose and GlcNAc were used at concentrations of 100 mM and 1% (w/v), respectively. For experiments in which inhibitors of the hexosamine pathway and of cell wall biosynthesis were used, cultures

were split at an optical density of 600 nm (OD_{600}) of ~0.3, and growth was continued in the absence and presence of the various inhibitors. Subsequently, aliquots were removed for extraction of total RNA after 30 min or 1 h, for total protein isolation after 3 h and for β-galactosidase assays in one-hour time intervals. All strains and plasmids used and their genotypes and characteristics are listed in Table 1. Gene deletions (designated with a Δ in Table I) constructed *de novo* in this work were made following standard procedures (Hamilton *et al.*, 1989; Datsenko and Wanner, 2000). They were either marker-less clean deletions or the gene of interest was replaced by a chloramphenicol resistance cassette as indicated in Table I (column 2). In all cases, the complete gene was exactly removed. Integration of *lacZ*-fusions into the λ attachment site (*attB*) of the chromosome was achieved according to the method described by (Diederich *et al.*, 1992). Established mutations tagged with an antibiotic resistance marker were moved into other strains by bacteriophage T4GT7 transduction (Wilson *et al.*, 1979) as indicated in Table 1. Strain constructions were verified by PCR using appropriate primers. Details on the strain as well as plasmid constructions are described in the "supplementary material" section. See supplementary Table S1 for a list of the oligonucleotides used in this study.

Table 1: Strains and plasmids used in this study.

Strain/ plasmid	Genotype or relevant structures[1]	Reference, source or construction
A. *strains*		
AM111	*hfq1*::*omega*	(Tsui *et al.*, 1994)
AM112	*hfq2*::*omega*	(Tsui *et al.*, 1994)
GPM83	as JM83, but Δ*glmM*::*neo, rep*^{TS}-plasmid pGMM carrying *glmM, cat*	(Mengin-Lecreulx and van Heijenoort, 1996)
IBPC5321	*thi1 argG6 argE3 his4 mtl1 xyl5 rpsL* Δ*lacX74 mlc1*	(Plumbridge, 1991)
IBPC750	as IBPC5321 but Δ*glmS*::*tc*	(Plumbridge and Vimr, 1999)
JM83	*ara* Δ[*lac-pro*] *rpsL thi* ϕ80 *lacZ*ΔM15	(Yanisch-Perron *et al.*, 1985)
N3431	*lacZ43 (Fs) rne3071(ts) relA1 spoT1 thi1*	(Goldblum and Apririon, 1981)
N3433	as N3431 but wild type *rne*	(Goldblum and Apririon, 1981)
R1279	CSH50 Δ(*pho-bgl*)201 Δ(*lac-pro*) *ara thi*	(Görke and Rak, 1999)
R1653	as R1279, but Δ[*ptsH ptsI crr*]::*neo*	(Görke and Rak, 1999)
R2109	as R1279, but *pcnB80 zad*::Tn*10*	(Görke and Rak, 2001)
R2404	as R1279, but Δ*ptsP*	R1279 × pFDX4255; this work
R2409	as R1279, but Δ*ptsP*, Δ[*ptsH ptsI crr*]::*neo*	T4GT7 (R1653) → R2404; this work
R2413	as R1279, but Δ[*ptsN,* Δ*yhbJ,* Δ*ptsO*]	R1279 × pFDX4259; this work
R2415	as R1279, but Δ[*ptsN,* Δ*yhbJ,* Δ*ptsO*], Δ[*ptsH ptsI crr*]::*neo*	T4GT7 (R1653) → R2413; this work
Z2	as R1279, but Δ[*ptsN,* Δ*yhbJ,* Δ*ptsO*] *strp*^{R} *F'*(*pro*$^{+}$)	R2413→ *strp*^{R} → conjug. × BMH71-18
Z3	as R1279, but *strp*^{R} *F'*(*pro*$^{+}$)	R1279→ *strp*^{R} → conjug. × BMH71-18
Z4	as R1279, but Δ[*ptsN,* Δ*yhbJ,* Δ*ptsO*] *attB*::[*aadA glmU*-5'::*lacZ*] *strp*^{R} *F'*(*pro*$^{+}$)	pBGG15/BamHI → Z2; this work
Z5	as R1279, but Δ[*ptsN,* Δ*yhbJ,* Δ*ptsO*] *attB*::[*aadA glmS*-5'::*lacZ*] *strp*^{R} *F'*(*pro*$^{+}$)	pBGG16/BamHI → Z2; this work

Z6	as R1279, but Δ[*ptsN,* Δ*yhbJ,* Δ*ptsO*] *attB*::[*aadA glmUS'*-5'::*lacZ*] *strp*^{R} *F'*(*pro*$^+$)	pBGG17/BamHI → Z2; this work
Z7	as R1279, but *attB*::[*aadA glmU*-5'::*lacZ*] *strp*^{R} *F'*(*pro*$^+$)	pBGG15/BamHI → Z3; this work
Z8	as R1279, but *attB*::[*aadA glmS*-5'::*lacZ*] *strp*^{R} *F'*(*pro*$^+$)	pBGG16/BamHI → Z3; this work
Z9	as R1279, *attB*::[*aadA glmUS*-5'::*lacZ*] *strp*^{R} *F'*(*pro*$^+$)	pBGG17/BamHI → Z3; this work
Z24	as R1279, but Δ*yhbJ*::*cat*	PCR BG157/BG158→ R1279; this work
Z27	as R1279, but Δ*yhbJ*::*cat attB*::[*aadA glmS*-5'::*lacZ*] *strp*^{R} *F'*(*pro*$^+$)	PCR BG157/BG158→ Z8; this work
Z28	as R1279, but Δ*yhbJ attB*::[*aadA glmS*-5'::*lacZ*] *strp*^{R} *F'*(*pro*$^+$)	Z27 cured from *cat*; this work
Z37	as R1279, but Δ*yhbJ*	Z24 cured from *cat*; this work
Z40	as R1279, but Δ*glmZ*::*cat*, *attB*::[*aadA glmUS*-5'::*lacZ*] *strp*^{R} *F'*(*pro*$^+$)	PCR BG184/BG185→ Z9; this work
Z42	as R1279, but *ΔglmZ*::*cat* Δ[*ptsN,* Δ*yhbJ,* Δ*ptsO*] *attB*::[*aadA glmS*-5'::*lacZ*] *strp*^{R} *F'*(*pro*$^+$)	PCR BG184/BG185→ Z5; this work
Z43	as R1279, but Δ*glmZ* Δ[*ptsN,* Δ*yhbJ,* Δ*ptsO*] *attB*::[*aadA glmS*-5'::*lacZ*] *strp*^{R} *F'*(*pro*$^+$)	Z42 cured from *cat*; this work
Z44	as R1279, but Δ*glmZ*::*cat*	PCR BG184/BG185→ R1279; this work
Z45	as R1279, but Δ*glmZ*	Z44 cured from *cat*; this work
Z46	as R1279, but Δ[*ptsN,* Δ*yhbJ,* Δ*ptsO*], Δ*glmZ*::*cat*	PCR BG184/BG185→ R2413; this work
Z52	as N3431, but Δ*yhbJ*::*cat*	T4GT7 (Z24) → N3431; this work
Z53	as N3433, but Δ*yhbJ*::*cat*	T4GT7 (Z24) → N3433; this work
Z60	as R1279, but *hfq1*	T4GT7 (AM111) → R1279; this work
Z61	as R1279, but Δ*yhbJ*::*cat*, *hfq1*	T4GT7 (AM111) → Z24; this work
Z62	as R1279, but *hfq2*	T4GT7 (AM112) → R1279; this work
Z63	as R1279, but Δ*yhbJ*::*cat*, *hfq2*	T4GT7 (AM112) → Z24; this work
B.		
Plasmids		
pBGG15	transcriptional fusion of *glmU*-5' (*glmU* -392 to +240) to *lacZ*	this work
pBGG16	transcriptional fusion of *glmS*-5' (*glmS* -311 to +129) to *lacZ*	this work
pBGG17	transcriptional fusion of *glmUS*' (*glmU* -392 to *glmS* +129) to *lacZ*	this work
pBGG56	*glmS* under P_{tac}-control, ori p15A, *tet*	this work
pBGG59	transcriptional fusion of *glmZ*-5' (*glmZ* -424 to +31) to *lacZ*	this work
pBGG64	transcriptional fusion of *glmS*-5' (*glmS* -183 to +129) to *lacZ*	this work
pBGG65	transcriptional fusion of *glmS*-5' (*glmS* -115 to +129) to *lacZ*	this work
pBGG66	as pBGG16 but mutation: *glmS* (-155 to -152): CCGG → ATTA	this work
pBGG67	as pBGG16 but mutation: *glmS* (-129 to -126): GGAG → TCCT	this work
pBGG84	*glmZ* (-100 to +254) under P_{Ara}-control in pBAD30	this work
pFDX3400	pSC101-rep^{TS}-derivative, *cat*, XbaI-site	this work
pFDX4255	flanking regions of *ptsP* in XbaI-site of pFDX3400	this work
pFDX4259	regions 5' of *ptsN* and 3' of *ptsO* in XbaI-site of pFDX3400	this work
pFDX4291	pSC101-ori, *cat*, operator-less P_{tac}, BglII, *sacB*-RBS, NdeI, XbaI, HincII	this work
pFDX4292	*ptsO* under P_{tac}-control in pFDX4291	this work
pFDX4294	*ptsN* under P_{tac}-control in pFDX4291	this work
pFDX4296	*ptsN*, *yhbJ*, *ptsO* under P_{tac}-control in pFDX4291	this work
pFDX4320	*yhbJ*, *ptsO* under P_{tac}-control in pFDX4291	this work
pFDX4322	*ptsO* and *ptsN* under P_{tac}-control in pFDX4291	this work
pFDX4324	*yhbJ* under P_{tac}-control in pFDX4291	this work
pKEM4	ori p15A, *neo*, *attP*, *aadA*, *T4gene32*-terminator, promoterless *lacZ*	(Nagarajavel, 2007)
pKES99	as pKEM04 but P_{lacUV5} in front of *lacZ*	(Nagarajavel *et al.*, 2007)
pKESK18	Tn*10*-transposase under λP_R-control, λcI_{857}, mTn*10*::*cat*, pSC101-rep^{TS}, *neo*	(Madhusudan *et al.*, 2005)

[1]RBS = ribosomal binding site; ori = origin of replication.

Transposon mutagenesis

Transposon mutagenesis was performed as described previously (Madhusudan *et al.*, 2005). Briefly, transformants of strains Z8 and Z28 carrying the temperature-sensitive *mTn*10 delivery plasmid pKESK18 were grown at 30℃ in LB medium supplemented with kanamycin and chloramphenicol. Appropriate dilutions were plated on M9 minimal medium plates supplemented with 1% glucose, thiamine (1 µg/ml), X-gal (40 µg/ml)

and chloramphenicol and incubated at 42℃ to select for transposon mutants. Mutants that exhibited a change in the phenotype were re-streaked on M9 plates as above to re-evaluate their phenotype. The *mTn*10 insertion sites were determined by semi-random, two-step PCR using the primers and PCR conditions as described previously (Madhusudan *et al.*, 2005). PCR-products were purified and sequenced. The screens yielded the mutants carrying mTn10 insertions at positions as follows. Derivatives of strain Z8: *glmS*(-7)::*mTn*10 (= Z8-TM1); *glmS*(+5)::*mTn*10 (= Z8-TM2); *glmS*(-63)::*mTn*10 (= Z8-TM3). Derivatives of strain Z28: *glmZ*(+10)::*mTn*10 (= Z28-TM1), *glmZ*(+6)::*mTn*10 (= Z28-TM2), *glmZ*(+12)::*mTn*10 (= Z28-TM4), *glmZ*(+84)::*mTn*10 (= Z28-TM5), *glmZ*(+16)::*mTn*10 (= Z28-TM6), *glmZ*(+74)::*mTn*10 (= Z28-TM7), *glmZ*(-59)::*mTn*10 (= Z28-TM9), *glmZ*(+30)::*mTn*10 (= Z28-TM10), *glmZ*(-88)::*mTn*10 (= Z28-TM12), *glmZ*(+83)::*mTn*10 (= Z28-TM13), *glmZ*(-1)::*mTn*10 (= Z28-TM15).

LC-MS/MS protein identification

The identification of GlmS by mass spectrometry is described under “supplementary material”.

β-galactosidase assays

Overnight cultures grown in LB containing the appropriate antibiotics were inoculated into the same medium to an OD_{600} of 0.1. When necessary, 1 % (w/v) GlcNAc, 100 mM L-arabinose and 1 mM IPTG were added. If not otherwise indicated, the cultures were grown at 37°C to an OD_{600} of 0.5 - 0.7, and subsequently harvested. Determination of β-galactosidase activities was performed as described previously (Miller, 1972). Enzyme activities are expressed in Miller units. Enzyme assays were performed in triplicate from at least two independent cultures.

Primer extension analysis

The total RNA of cultures grown to exponential phase in LB was isolated using the hot phenol method of RNA extraction as described (Khodursky *et al.*, 2003). For primer extension reactions, 10 µg RNA were incubated with 1.2

pMol primer in a final volume of 5 µl. The mixture was incubated at 70℃ for 3 min followed by a 3 min incubation at room temperature and was then placed on ice. Two µl of this annealing mix were mixed with 0.37 mM dNTPs and 3 µCi [α-^{32}P]CTP, 1 unit AMV reverse transcriptase (Promega, USA) and the appropriate buffer in a final volume of 6 µl and incubated for 15 min at 48 ℃. The reaction products were analyzed by electrophoresis on a 7M urea/TBE/8% polyacrylamide gel alongside a sequencing ladder obtained with the same primer and plasmid pBGG17 as template. Sequencing reactions were performed using the CycleReader™ DNA Sequencing Kit (Fermentas, Canada) and 3 µCi [α-^{32}P]CTP for labeling according to the manufacturer's protocol.

RNA extraction and Northern blot analysis

Preparation of total RNA from cultures grown to exponential phase was performed using the RNeasy Mini kit (Qiagen, Germany) according to the manufacturer's instructions. Digoxygenin-labeled RNA probes specific for *glmU, glmS* and GlmZ RNAs were obtained by *in vitro* transcription using the DIG Labeling kit (Roche Diagnostics, Germany) and PCR-generated fragments as templates, respectively. The primers used for PCR were BG147 and BG148 for *glmU*, BG149 and BG150 for *glmS,* and BG230 and BG231 for *glmZ*. The reverse primers contained a T7 RNA polymerase recognition sequence, respectively. For Northern blot analyses of *glmU* and *glmS* mRNAs, 5 µg total RNA were separated by formaldehyde agarose gel electrophoresis and subsequently transferred to a positively charged nylon membrane (Roche Diagnostics, Germany) using the VacuGene XL Vacuum blotting System (Amersham Biosciences, USA) following the supplier's protocol. For the detection of GlmZ, 5 µg total RNA were separated on 7M urea/TBE/8% polyacrylamide gels and thereafter transferred to the nylon membrane by electroblotting in 0.5× TBE at 15 V for 1 h. Probe hybridization and signal detection were carried out according to the manufacturer's instructions (DIG RNA labeling kit and detection chemicals; Roche Diagnostics).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

If not otherwise indicated, *E. coli* cells were grown to an OD_{600} of ~ 0.5 and harvested. The cells were resuspended in SDS sample buffer and 3.25 µg total protein of each sample was loaded on 12.5 % SDS/polyacrylamide gels. Subsequently the gels were stained with Coomassie brilliant blue R-250 or electro-blotted to a PVDF membrane (Bio-Rad laboratories, USA) by a semi-dry transfer (90 min at 0.8 mA/cm^2) as described by the supplier. GlmS was detected with polyclonal anti-GlmS rabbit antibodies diluted 1:10000, and the antibodies were visualized using rabbit IgG-AP secondary antibodies (Promega, USA) and the CDP* detection system (Roche Diagnostics, Germany).

Results

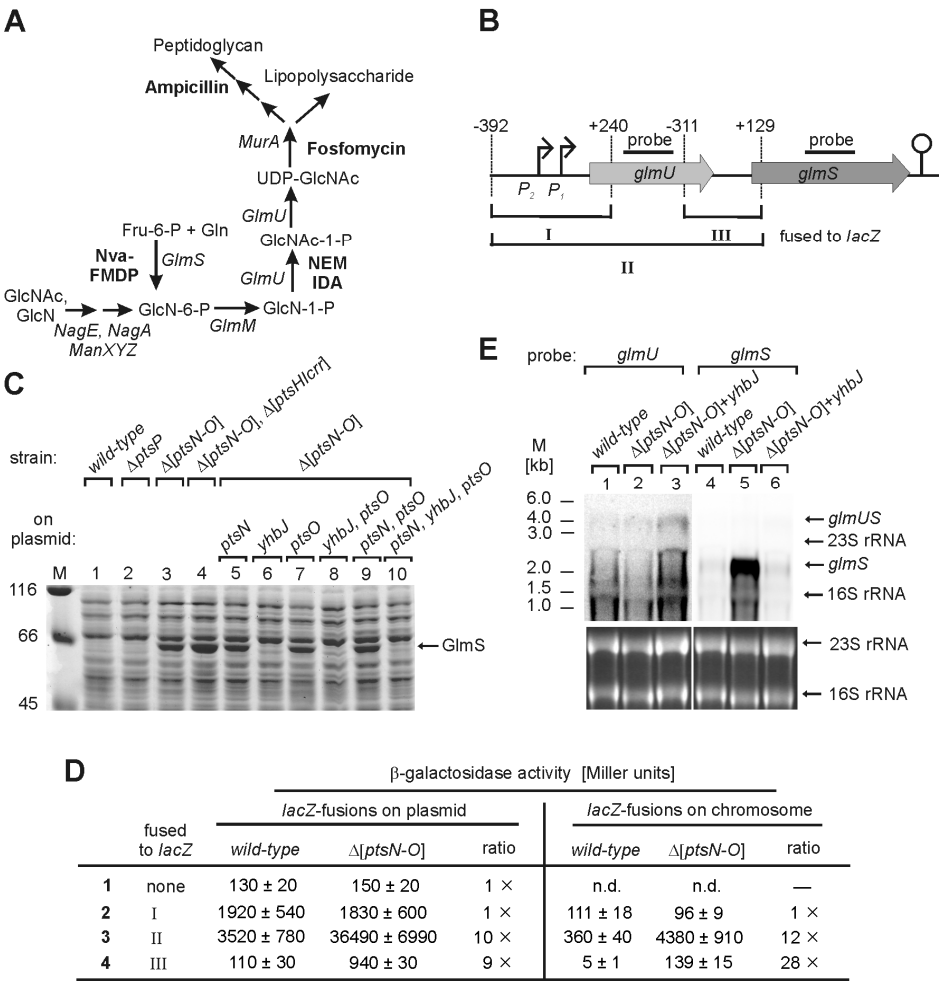
Deletion of *yhbJ*, a gene of the *rpoN* operon, leads to accumulation of GlmS protein.

When we analyzed the total cell protein of strains lacking gene *ptsP* or the three distal genes *ptsN, yhbJ, ptsO* of the *rpoN* operon by SDS/polyacrylamide gel electrophoresis and subsequent Coomassie-staining, we observed that in the Δ[*ptsN-O*] mutant a protein of ~65 kDa was strongly overproduced (Fig. 3 C, lanes 1 and 3). Mass spectrometry identified this protein as glucosamine 6-phosphate synthase (GlmS). No overproduction of GlmS was detectable in mutants carrying deletions of *ptsP* and/or of the *ptsHIcrr* operon encoding the canonical phosphotransferases of the PTS (Fig. 3 C, lane 2 and data not shown). Moreover, the additional deletion of the *ptsHIcrr* operon in the Δ[*ptsN-O*] mutant had no effect on GlmS overexpression (Fig. 3 C, lanes 3 and 4). Complementation studies with plasmids constitutively expressing *ptsN*, *yhbJ* or *ptsO,* either alone or in various combinations, revealed that overproduction of GlmS in the *ptsN-ptsO* deletion strain was abolished by the presence of *yhbJ*, while the absence or presence of *ptsN* and/or *ptsO* had no effect (Fig. 3 C, lanes 5-10). Finally, a *yhbJ* deletion appeared to be sufficient for overproduction of GlmS (Fig. S2, lanes 1-3). Hence, YhbJ is necessary and sufficient to prevent overproduction

of GlmS. The PTS-paralogs EI^{Ntr}, NPr and IIA^{Ntr} as well as the canonical PTS-enzymes appear not to be involved in the control of GlmS expression by YhbJ.

A new *glmS*-specific transcript emerges in the *yhbJ* mutant

To investigate the molecular level responsible for overproduction of GlmS, we employed plasmid-born transcriptional fusions of the *lacZ* reporter gene to *glmU* and *glmS*. Both, the wild type and the Δ[*ptsN-O*] mutant strain produced similar levels of β-galactosidase activity of ~1850 units when they contained a plasmid carrying a *glmU'-lacZ* fusion (Fig. 3 B, fusion I and Fig. 3 D, line 2). These activities were 12-15 times higher in comparison to transformants carrying the vector that lacked an insert in front of *lacZ* (Fig. 3 D, lines 1 and 2) and reflect the activities of the known two promoters in front of *glmU*. Next, we tested a construct that carries a *glmUS'-lacZ* operon fusion, i.e. *lacZ* was transcriptionally fused to *glmS*' in the context of the *glmUS* upstream sequences (Fig. 3 B, fusion II). This fusion generated 3520 units of β-galactosidase activity in the wild type strain, and activities increased 10-fold in the Δ[*ptsN-O*] mutant (Fig. 3 D, line 3). Similar ratios of expression were obtained when the *lacZ* fusions were present in single copy on the chromosome (Fig. 3 D right panel, lines 2 and 3). Translational fusions of *lacZ* to *glmU'* and *glmS'* yielded comparable activities (data not shown). Taken together, these data suggest that overproduction of GlmS as the result of the *yhbJ* deletion is caused by increased *glmS* transcript levels and that this transcriptional up-regulation occurs downstream of the known promoters of *glmUS*.



D

β-galactosidase activity [Miller units]

	fused to *lacZ*	*lacZ*-fusions on plasmid: *wild-type*	*lacZ*-fusions on plasmid: Δ[*ptsN-O*]	ratio	*lacZ*-fusions on chromosome: *wild-type*	*lacZ*-fusions on chromosome: Δ[*ptsN-O*]	ratio
1	none	130 ± 20	150 ± 20	1 ×	n.d.	n.d.	—
2	I	1920 ± 540	1830 ± 600	1 ×	111 ± 18	96 ± 9	1 ×
3	II	3520 ± 780	36490 ± 6990	10 ×	360 ± 40	4380 ± 910	12 ×
4	III	110 ± 30	940 ± 30	9 ×	5 ± 1	139 ± 15	28 ×

Figure 3: Knockout of *yhbJ* induces overproduction of GlmS which results from the appearance of a monocistronic *glmS* transcript. **A.** The hexosamine pathway in which GlmS holds a key role by catalyzing synthesis of GlcN-6-P from Fru-6-P and Gln. This reaction is essential in the absence of external amino sugars. The metabolic reactions leading to the synthesis of UDP-GlcNAc, the precursor of peptidoglycan, are depicted. Enzymes are in italics and their inhibitors are shown in bold. **B.** Organization of the *glmUS* operon. Promoters are indicated by arrows. Horizontal lines indicate the location of the RNA-probes used for Northern analyses. For reporter gene studies the regions I, II and III were fused to *lacZ* as indicated by square brackets. **C.** Analysis of total protein extracts by 12.5% SDS-PAGE and subsequent Coomassie staining. The following strains were employed: lane 1, R1279; lane 2, R2404; lane 3 and lanes 5-10, R2413; lane 4, R2415. In lanes 5-10 strain R2413 carried plasmids with the genes as indicated: lane 5, pFDX4294; lane 6, pFDX4324; lane 7, pFDX4292; lane 8, pFDX4320; lane 9, pFDX4322; lane 10, pFDX4296. **D.** β-galactosidase activities of strains carrying *glmU'*- (line 2), *glmUS'*- (line 3) and *glmS'-lacZ* (line 4) reporter gene fusions. Left panel: The wild type strain R1279 and the Δ[*ptsN-O*] mutant R2413 were transformed with plasmids pBGG15 (line 2), pBGG17 (line 3) and pBGG16 (line 4) which carry sections of the *glmUS* operon fused to *lacZ* as indicated in B. In lane 1 the empty *lacZ* fusion plasmid pKEM4 was employed. Right panel: The *lacZ* fusions were present in the chromosomal *attB* site of the *wild type* strain and the [*ptsN-O*] mutant, respectively. The corresponding strains were Z7 and Z4 (line 3), Z8 and Z5 (line 4), and Z9 and Z6 (line 5), respectively. **E.** Northern blot analysis of the transcripts of the *glmUS* operon. The total RNAs of strains R1279 (lanes 1 and 4), R2413 (lanes 2 and 5) and R2413/pFDX4324 (lanes 3 and 6) were hybridized to probes specific for *glmU* (lanes 1-3) or *glmS* (lanes 4-6). The ethidium-bromide stained gel is shown as loading control at the bottom.

To investigate this possibility, we performed Northern blot analyses with total RNAs of the wild type strain, of the Δ[*ptsN-O*] mutant and of the mutant

complemented with plasmid-encoded YhbJ. Using a *glmU*-specific probe, a weak band at ~3.5 kb corresponding to the full-length transcripts could be detected and no prominent differences between the strains were detectable (Fig. 3 E, lanes 1-3). The low amount of the *glmUS* co-transcript might indicate its instability as suggested by a recent study (Joanny *et al.*, 2007) and the smear visible below could represent the degradation products. In contrast, with the *glmS*-specific probe a prominent transcript of ~2 kb appeared in the Δ[*ptsN-O*] mutant that was almost undetectable in the wild type as well as in the Δ[*ptsN-O*] mutant carrying *yhbJ* on a plasmid (Fig. 3 E, lanes 4-6). Note that in comparison to lanes 1-3, a ten times lower concentration of the probe was used in these Northern experiments in order to detect the *glmS* transcript as a distinct band. Using a higher probe concentration, the *glmUS* co-transcript and the smear below became also detectable with the *glmS* probe (data not shown). Taken together, YhbJ negatively controls the amount of a *glmS* transcript, which according to its size could have its 5' end in the *glmUS* intergenic region or within the 3'-end of *glmU*.

The monocistronic *glmS* mRNA results from RNase E dependent processing of the *glmUS* full length transcript at the *glmU* stop codon

Next, we performed primer extensions to determine the exact 5' end of the *glmS* transcript appearing in the *yhbJ* mutant. These experiments revealed two strong signals on the gel with RNA isolated from the Δ[*ptsN-O*] strain whereas no signal could be detected with the wild type RNA (Fig. S1; see legend to Fig. S1 for further information). These signals correspond to G and A, both located within the stop codon of *glmU*, respectively. No other signals were detectable. Inspection of the nucleotide sequence upstream of the primer extension signals did not reveal any putative promoter.

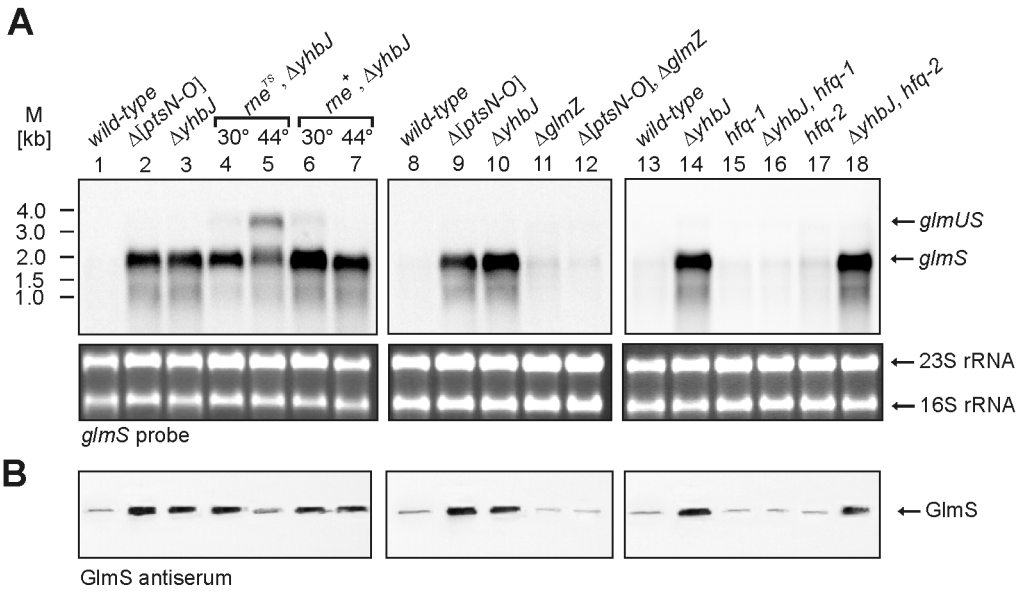


Figure 4: Accumulation of the *glmS* transcript in the *yhbJ* mutant depends on GlmZ and Hfq and results from processing of the *glmUS* full-length transcript by RNase E. A. Northern blot analysis of the *glmS* transcripts using a *glmS*-specific probe. Total RNAs of the following strains were employed: R1279 (lanes 1, 8, 13), R2413 (lanes 2, 9), Z24 (lanes 3, 10, 14), Z52 (lanes 4, 5), Z53 (lanes 6, 7), Z44 (lane 11), Z46 (lane 12), Z60 (lane 15), Z61 (lane 16), Z62 (lane 17), Z63 (lane 18). Genotypes were as indicated on top of the figure. The ethidium-bromide stained gel is shown at the bottom as loading control. **B.** Determination of GlmS amounts in cell extracts separated by SDS/12.5% polyacrylamide gel electrophoresis and visualized by Western blotting. The strains used were the same as in A.

Quite recently it has been reported that a *glmS*-specific transcript with its 5'-end mapping to the *glmU* stop codon appears in mutants lacking *pcnB* encoding poly (A) polymerase I (PAP I). It was shown that this transcript results from RNase E mediated processing of the *glmUS* mRNA at the 3'-end of *glmU* (Joanny *et al.*, 2007). To determine whether our findings are related, we tested whether accumulation of the *glmS* transcript in the *yhbJ* mutant also depends on RNase E and employed the temperature-sensitive *rne*3071^{TS} mutation. To this end, we introduced the *yhbJ* deletion into the *rne*^{TS} mutant N3431 and its isogenic wild type strain N3433. As expected, the resulting *yhbJ* deletion strains produced the *glmS*-specific transcript at 30℃ also in the N3433 background (Fig. 4 A, lanes 1-4 and 6). In the parental *yhbJ*$^{+}$ wild type strains N3431 and N3433 the *glmS* mRNA was only weakly detectable (data not shown;). A temperature shift to 44℃ had no effect on the *glmS* mRNA level in the *rne*$^{+}$ Δ*yhbJ* strain (Fig. 4 A, lanes 6 and 7). In contrast, in the *rne*3071^{TS} Δ*yhbJ* mutant the amount of *glmS* transcript decreased and the full-length *glmUS* transcript became detectable (Fig. 4 A, lane 5).

Consequently, we studied the impact of RNase E on the GlmS protein level. Whole cell extracts were analyzed by Coomassie staining (data not shown) and Western blotting using antiserum directed against *E. coli* GlmS (Fig. 4 B). The data show that the cellular amount of the GlmS protein indeed decreases in the *rne*3071^{TS} Δ*yhbJ* mutant at 44℃ in comparison to 30℃ (Fig. 4 B, la nes 4 and 5). In the *rne*$^{+}$ Δ*yhbJ* strain the temperature-shift did not affect overproduction of GlmS (Fig. 4 B, lanes 6 and 7). In the isogenic *yhbJ*$^{+}$ strains no GlmS overproduction occurred (data not shown). Hence, the results suggest that the *glmS* transcript that accumulates in the Δ*yhbJ* mutant results from processing of the *glmUS* mRNA at the *glmU* stop codon in an RNase E dependent manner.

Overproduction of *glmS* in the *yhbJ* mutant requires the small RNA GlmZ

Our results suggested that *glmS* expression is controlled by an RNase E mediated processing event and YhbJ. To further elucidate this pathway and to identify additional factors that might be involved, we performed a transposon mutagenesis screen for insertions that abolish the high *glmS* expression in the *yhbJ* mutant. For this purpose, the sequence upstream of *glmS* (*glmS*: -311 to +129) encompassing the RNase E processing site was inserted upstream of *lacZ* (Fig. 3, fusion III). In this fusion, expression of *glmS'-lacZ* is driven by a constitutive promoter located upstream of the *glmS'* fragment within the *aadA* resistance cassette, which directs weak read through transcription into *lacZ*. Therefore, expression of this fusion is independent of the *glmUS* promoters. This fusion was expressed nine-fold higher in the Δ[*ptsN-O*] or the Δ*yhbJ* mutant in comparison to the wild type strain when it was present on a plasmid and more than 20-fold higher when it resided on the chromosomes of these strains (Fig. 3 D, lines 4 and Fig. 5 A, columns 1-3). Control experiments verified the absence of a promoter within the *glmS'* fragment and showed that the *aadA-glmS'-lacZ* fusion mRNA is processed by RNase E like the *glmUS* transcript (Fig. S3 B, see legend for details).

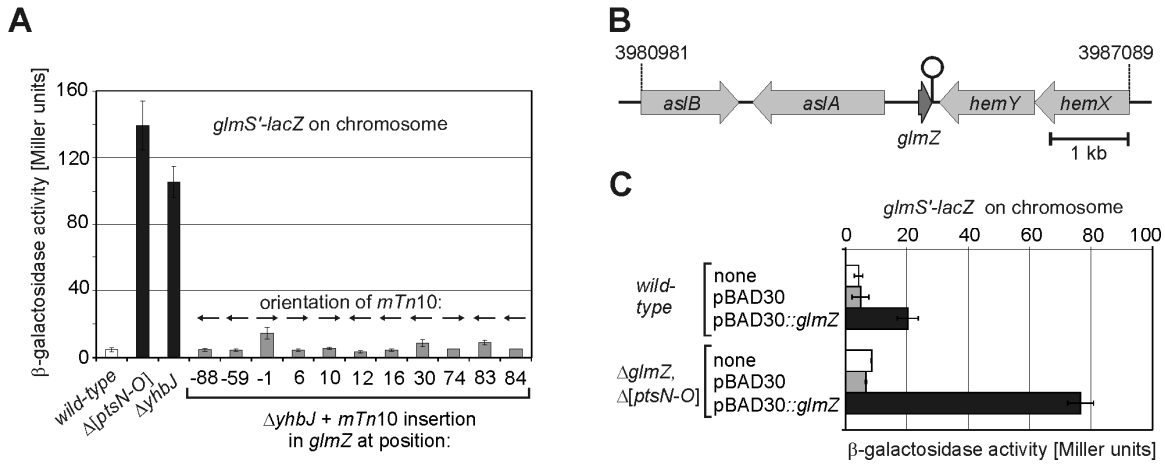


Figure 5: GlmS overexpression caused by deletion of *yhbJ* is eliminated by mutations in the small RNA gene *glmZ*. A. β-galactosidase activities of Δ*yhbJ glmZ::mTn*10 double mutants carrying the *glmS'-lacZ* reporter fusion on the chromosome. The various *mTn*10 mutants (columns 4-14) were obtained in a transposon mutagenesis screen of strain Z28 (Δ*yhbJ*) for abolishment of *glmS'-lacZ* expression. The positions of the *mTn*10 insertions relative to *glmZ* are depicted and arrows indicate the orientation of the transposons. For comparison, the β-galactosidase activities of strains Z8 (column 1), Z5 (column 2) and of the parental strain Z28 (column 3) are shown. **B.** Genomic context of the *glmZ* gene. Genes are depicted as arrows in the direction of their transcription. The *rho* independent terminator present at the 3' end of *glmZ* is symbolized by a lollipop. Positions are according to http://www.biocyc.org/. **C.** Complementation analysis demonstrating that plasmid-driven expression of *glmZ* restores the high expression of the *glmS'-lacZ* fusion in the Δ*glmZ* Δ[*ptsN-O*] strain. Strains Z8 (wild type) and Z43 *(*Δ*glmZ* Δ[*ptsN-O*]) were transformed with plasmid pBAD30 (vector control, grey bars) and pBGG84 (*glmZ*, black bars), respectively, and the β-galactosidase activities were determined. Arabinose was added for induction of *glmZ* expression. The untransformed strains (white bars) served as controls.

Most important for the screen, the *glmS'*(-311 to +129)-*lacZ* fusion when present in single copy caused the formation of blue colonies on X-gal plates in the Δ*yhbJ* mutant but not in the wild type. Hence, the Δ*yhbJ* mutant was subjected to transposon mutagenesis and screened for colorless colonies. Twenty-four positive clones reproducibly exhibiting lower levels of *lacZ* expression were isolated, of which eight carried an insertion in *lacZ*. The remaining 16 insertions all mapped in gene *glmZ* (formerly *ryiA* or *sraJ)* or its immediate upstream region at altogether 11 different positions (Fig. 5 A). This gene codes for a small RNA (sRNA) of hitherto unknown function (Argaman *et al.*, 2001; Wassarman *et al.*, 2001). The *glmZ* gene is present in the intergenic region between gene *aslA* encoding an arylsulfatase and the *hemCDXY* gene cluster important for tetrapyrrole synthesis, and it is transcribed in opposite direction to these adjacent genes (Fig. 5 B). In these Δ*yhbJ glmZ*::*mTn*10 mutants the *glmS'-lacZ* fusion was only weakly expressed, and simultaneously overproduction of the GlmS protein was abolished (Fig. 5 A

and Fig. S2), suggesting that in addition to the absence of YhbJ a functional *glmZ* gene may be required for GlmS overproduction. Therefore, Δ*glmZ* and Δ*glmZ* Δ[*ptsN-O*] mutants were constructed and investigated in Northern and Western experiments. Indeed, accumulation of *glmS* mRNA and of GlmS protein could no longer be observed in the Δ*glmZ* Δ[*ptsN-O*] double mutant (Fig. 4 A and 4 B, lanes 8-12, respectively). To confirm the importance of the small RNA GlmZ in *glmS* regulation, a complementation analysis was performed. A plasmid carrying *glmZ* downstream of the P_{ara}-promoter was introduced into the Δ*glmZ* Δ[*ptsN-O*] mutant that in addition carried the *glmS'-lacZ* fusion on the chromosome, and the β-galactosidase activities were determined. Presence of this plasmid resulted in expression of the *glmS'-lacZ*-fusion whereas only low expression levels were obtained in the presence of the empty expression plasmid or in the absence of a plasmid (Fig. 5 C). In addition, we tested the effect of plasmid-driven GlmZ expression in the wild type strain. Interestingly, presence of the plasmid slightly enhanced expression of the *glmS'-lacZ* fusion indicating that overexpression of GlmZ can partially overcome the negative effect exerted by YhbJ on *glmS* expression.

The accumulation of the *glmS* transcript in the *yhbJ* mutant requires Hfq

It has been reported that GlmZ binds the Hfq protein (Wassarman *et al.*, 2001) that is often required for sRNAs to act. To analyze its possible contribution to accumulation of the processed *glmS* transcript, we tested the *glmS* transcript levels and GlmS amounts in double mutants carrying insertions in the *hfq* gene in addition to a deletion of *yhbJ* (Fig. 4 A and 4 B, lanes 13-18, respectively). No overproduction of *glmS* transcript and GlmS protein occurred in the Δ*yhbJ hfq*1::Ω strain, which carries an insertion disrupting *hfq*. In contrast, *glmS* mRNA levels and GlmS overproduction were unaltered in the Δ*yhbJ hfq*2::Ω mutant which carries the identical insertion cassette but inserted closer to the 3'-end of *hfq* so that, although truncated, the Hfq protein is still functional (Tsui *et al.*, 1994). Hence, the results show that accumulation of the *glmS* transcript and of GlmS protein in the *yhbJ* mutant requires the small RNA GlmZ and the Sm-like protein Hfq.

Mutation of *yhbJ* affects processing of GlmZ

The next important step was to clarify the relationship between YhbJ and GlmZ. Our data indicated that GlmZ may act downstream of YhbJ in the pathway controlling *glmS* expression, suggesting that YhbJ controls either the amount or the activity of the sRNA GlmZ. To determine whether YhbJ affects transcription of *glmZ* we tested a transcriptional *glmZ'-lacZ* fusion. This fusion yielded ~40% of the expression level produced by a constitutively expressed P_{lacUV5}-*lacZ* fusion indicating that the *glmZ*-5' fragment contains promoter activity. Expression of the *glmZ-lacZ* fusion was not affected by the Δ*yhbJ* and the Δ*glmZ* mutations, making it unlikely that *glmZ* transcription is controlled by YhbJ or auto-regulated (Fig. 6 A).

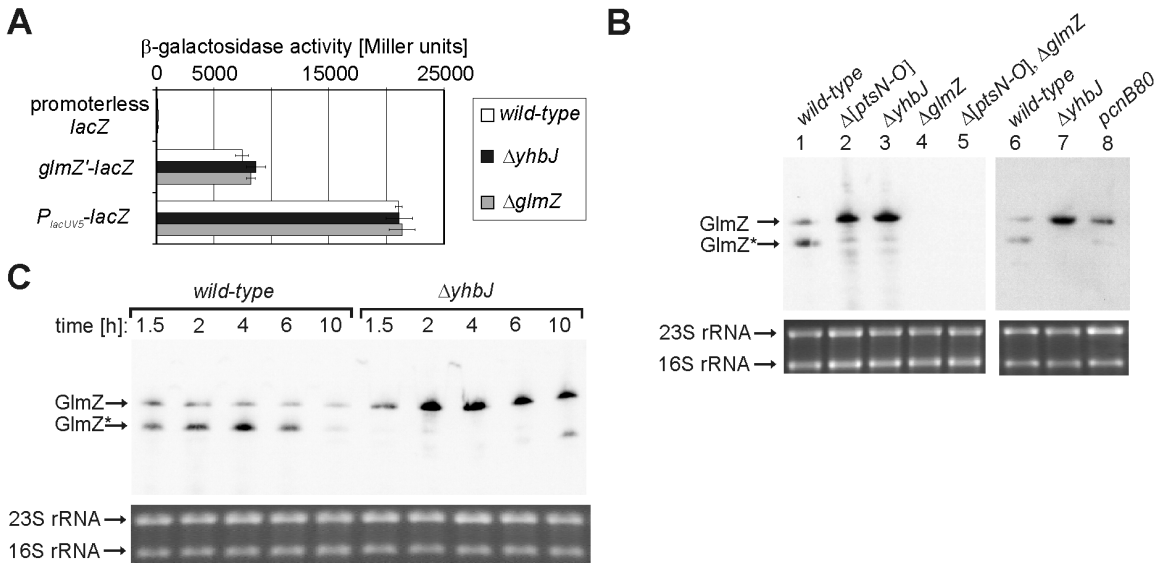


Figure 6: Post-transcriptional control of GlmZ by YhbJ. A. Expression of a *glmZ'-lacZ* fusion and therefore activity of the *glmZ* promoter is not affected by YhbJ. Plasmid pBGG59 carrying a *glmZ'-lacZ* fusion was introduced into strains R1279, Z37 and Z45 and the β-galactosidase activities were determined. Plasmids pKEM4 and pKES99 carrying no insert and the constitutive P_{lacUV5} promoter in front of *lacZ*, respectively, were included as controls. **B.** Northern blot analysis of GlmZ. Total RNAs of strains R1279 (lanes 1, 6), R2413 (lane 2), Z24 (lanes 3, 7), Z44 (lane 4), Z46 (lane 5), and R2109 were hybridized to a probe specific for GlmZ. **C.** Detection of GlmZ at various times during growth. Strains R1279 and Z24 were inoculated to an OD_{600} of 0.075 and total RNAs of aliquots withdrawn at the indicated times were analyzed by Northern blotting using a GlmZ probe.

To test whether YhbJ controls GlmZ at a post-transcriptional level we performed Northern analyses using a probe specific for GlmZ. It has previously been shown that two forms of GlmZ are present in wild type cells and that the shorter variant derives from processing. The full length form of GlmZ ends with a transcriptional terminator and has a size of ~210 nt whereas

the processed form was estimated to be 155 nt long (Argaman *et al.*, 2001; Wassarman *et al.*, 2001). Two bands corresponding to full-length GlmZ and its processed derivative GlmZ* were indeed detectable in the wild type strain, and they were absent in strains lacking the *glmZ* gene (Fig. 6 B, lanes 1, 4 and 5). In contrast, in the Δ[*ptsN-O*] and the Δ*yhbJ* mutants the amount of full-length GlmZ strongly increased while the processed form was almost undetectable (Fig. 6 B, lanes 1, 2 and 3). Analysis of GlmZ at different time points during growth revealed that in the wild type strain the processed form of GlmZ was more prevalent than its unprocessed form during the exponential growth phase. In contrast, in the Δ*yhbJ* mutant GlmZ was always almost exclusively present in its full-length form and in higher amounts in comparison to the wild type (Fig. 6 C). Taken together, it appears that YhbJ controls GlmZ post-transcriptionally by modulating its processing and/or stability. Interestingly, the amount of non-processed GlmZ was also increased in an isogenic *pcnB80* mutant coding for a less active PAP I protein (Fig. 6 B, lanes 6 and 8). Hence, it appears possible that PAP-I affects a factor acting upstream in the signal cascade rather than to regulate the *glmS* transcript directly by adding a poly(A) tail to its 3' end.

A decrease in cellular GlmS activity induces expression of a *glmS'-lacZ* fusion

Our data demonstrated that expression of GlmS can be modulated in a process that requires the sRNA GlmZ, Hfq and processing of the *glmUS* transcript by RNase E. However, so far high GlmS expression was exclusively detected in the artificial situation when either *yhbJ* or *pcnB* are defective. To identify the signal that may induce high expression in the wild type strain, we again performed a transposon mutagenesis screen. In this case we used the wild type strain carrying the *glmS'-lacZ* fusion and screened for insertions that resulted in blue colonies on X-gal-plates and therefore in high expression of *lacZ*.

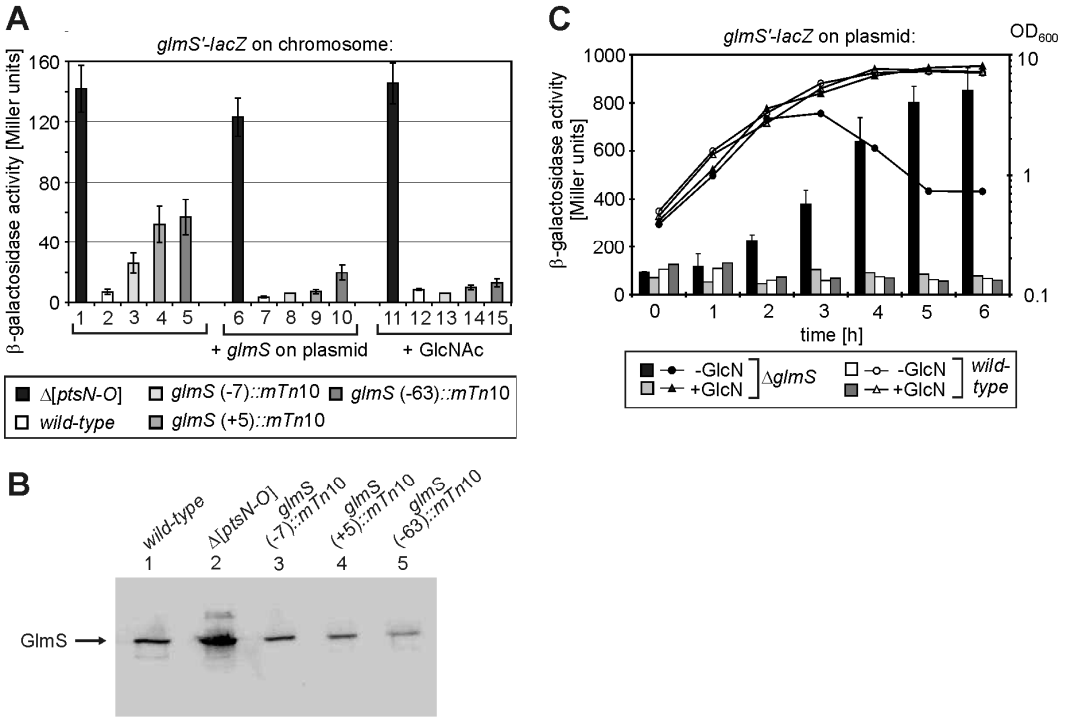


Figure 7: Reduction of GlmS synthesis up-regulates expression of the *glmS'-lacZ* reporter fusion in the wild type strain. A. β-galactosidase activities of *glmS*::*mTn*10 mutants obtained in the transposon mutagenesis screen for elevated expression of the *glmS'-lacZ* fusion in the wild type strain Z8. For comparison strain Z8 and the Δ[*ptsN-O*] mutant derivative Z5 are shown. Cells were grown in LB (bars 1-5) or in LB supplemented with GlcNAc (bars 11-15). In the experiments shown in bars 6-10 the strains were transformed with plasmid pBGG56 which encodes *glmS* under control of the *tac* promoter. IPTG (1 mM) was added for induction of expression. **B.** Western blotting experiment demonstrating that the *glmS::mTn*10 insertions reduce the GlmS amount below the level present in the wild type strain. **C.** The *glmS'-lacZ* reporter fusion is highly expressed in a Δ*glmS* mutant and can be repressed by the addition of GlcN. Strains IBPC750 (Δ*glmS*) and its isogenic wild type IBPC5321 were transformed with plasmid pBGG16 carrying a *glmS'-lacZ* fusion and grown to an OD_{600} of ~0.3 in the presence of GlcN. The culture was split and growth was continued in the absence and presence of GlcN and the β-galactosidase activities were determined in one-hour intervals. Also given are the growth curves.

Twenty-four positive clones were isolated of which 15 carried an insertion in front of the *lacZ* reporter gene. Six mutants carried the *mTn*10 inserted in *yhbJ* at altogether four different positions (pos. -6, +678, +716, +756). In the three remaining mutants, the transposon was inserted upstream of or in the extreme 5'-part of the genuine *glmS* gene (pos. -63, -7, +5). In these three mutants, expression of *glmS'-lacZ* was partially induced yielding 20 to 45 % of the level measured in the Δ[*ptsN-O*] mutant (Fig. 7 A, columns 1-5). Western blotting experiments showed that these mutants still synthesize GlmS, but to amounts lower than the wild type strain (Fig. 7 B, compare lanes 3-5 with lane 1). Introduction of a plasmid expressing the *glmS* gene from a *tac* promoter repressed the *glmS'-lacZ* fusion in the *glmS::mTn*10 mutants (Fig. 7 A, lanes

6-10). These data suggested that a low cellular amount (and thereby low activity) of the GlmS enzyme may induce expression of the *glmS'-lacZ* fusion. To test this idea, we investigated the activity of the *glmS'-lacZ* fusion in a Δ*glmS* mutant (Fig. 7 C). Although *glmS* is normally essential, the mutant is viable in the presence of GlcN or GlcNAc in the medium. The strains IBPC750 (Δ*glmS*) and its isogenic wild type IBPC5321 were transformed with the *glmS'-lacZ* fusion construct. After a period of initial growth, the cultures were split and growth was continued in the absence or presence of GlcN and the β-galactosidase activities of samples collected in periodical time intervals were determined. In the absence of GlcN the Δ*glmS* mutant continued to grow for three hours before the cells started to lyse (Fig. 7 C). Over the time, expression of the *glmS'-lacZ* fusion increased up to 9-fold (Fig. 7 C, black columns). This increase was undetectable in the corresponding wild type strain or in the Δ*glmS* mutant carrying a constitutively expressed control *lacZ* fusion (data not shown). Interestingly, presence of GlcN or GlcNAc in the medium prevented the high expression of the *glmS-lacZ* fusions in the Δ*glmS* strain as well as in the *glmS::mTn*10 mutants (Figs. 7 C, light grey columns and Fig. 7 A, columns 11-15). GlcN and GlcNAc are converted to GlcN-6-P and therefore bypass the reaction catalyzed by GlmS. These results thus suggest that the high expression of the *glmS'-lacZ* fusions in the *glmS* mutants is the result of the cellular depletion of GlcN-6-P or of a metabolite downstream in the pathway leading to UDP-GlcNAc (Fig. 3 A).

Depletion of GlcN-6-P up-regulates *glmS*

To identify the metabolite in the pathway from GlcN-6-P to UDP-GlcNAc and to peptidoglycan, which is responsible for up-regulation of *glmS* expression when its cellular concentration drops, we used different enzyme inhibitors known to be active within this pathway *in vivo* (Fig. 3 A). First, killing curves were performed to determine the lethal concentrations of these inhibitors in liquid cultures (data not shown). Subsequently, the wild type strains that carry the *glmUS'-lacZ* and the *glmS'-lacZ* fusions on the chromosome were grown in the presence of sub-lethal as well as lethal concentrations of the respective inhibitors, and following their addition the β-galactosidase activities were

determined at different time-points (Fig. 8 A and data not shown). Fosfomycin and ampicillin that inhibit cell wall synthesis at a step downstream of GlmU had no effect on expression of *lacZ* irrespective of the concentration used (data not shown). Similarly, iodoacetamide (IDA) (data not shown) and N-ethyl maleimide (NEM) (Fig. 8 A, dark grey bars) which inhibit GlmU activity (Burton *et al.*, 2006) had no effect. In contrast, the addition of Nva-FMDP induced expression of the *lacZ* reporter fusion in a concentration-dependent manner (Figs. 8 A and 8 B). Nva-FMDP is a derivative of N3-(4-methoxyfumaroyl)-L-2,3-diaminopropanoic acid (FMDP) and was shown to selectively inhibit GlmS activity (Marshall *et al.*, 2003). No deleterious effect of Nva-FMDP on the growth could be observed (growth curves in Fig. 8 B). To explore the effect of Nva-FMDP further, we analyzed *glmS* expression by Northern and Western analyses. The addition of Nva-FMDP led to the accumulation of the monocistronic *glmS* mRNA and concomitantly to overexpression of the GlmS protein, whereas the other inhibitors had no significant effects (Fig. 8 C and 8 D, upper panels). When GlcNAc was added to the culture treated with Nva-FMDP, the *glmS* transcript became undetectable, confirming that the intracellular exhaustion of a metabolite in the hexosamine pathway is responsible for up-regulation of *glmS* expression (Fig. 8 D, lanes 5-8). Nva-FMDP had no effect in the *yhbJ* mutant. The high *glmS* and GlmS levels could not be increased further by the inhibition of GlmS activity (Fig. 8 C, lower panel, lanes 1 and 5; Fig. 8 E, lanes 9 and 10). Moreover, in the *yhbJ* mutant the presence of GlcNAc lowered the *glmS* mRNA amount only four-fold and GlmS protein levels two-fold (Fig. 8 C, lower panel, lanes 5 and 6; Fig. 8 E, lanes 10 and 11).

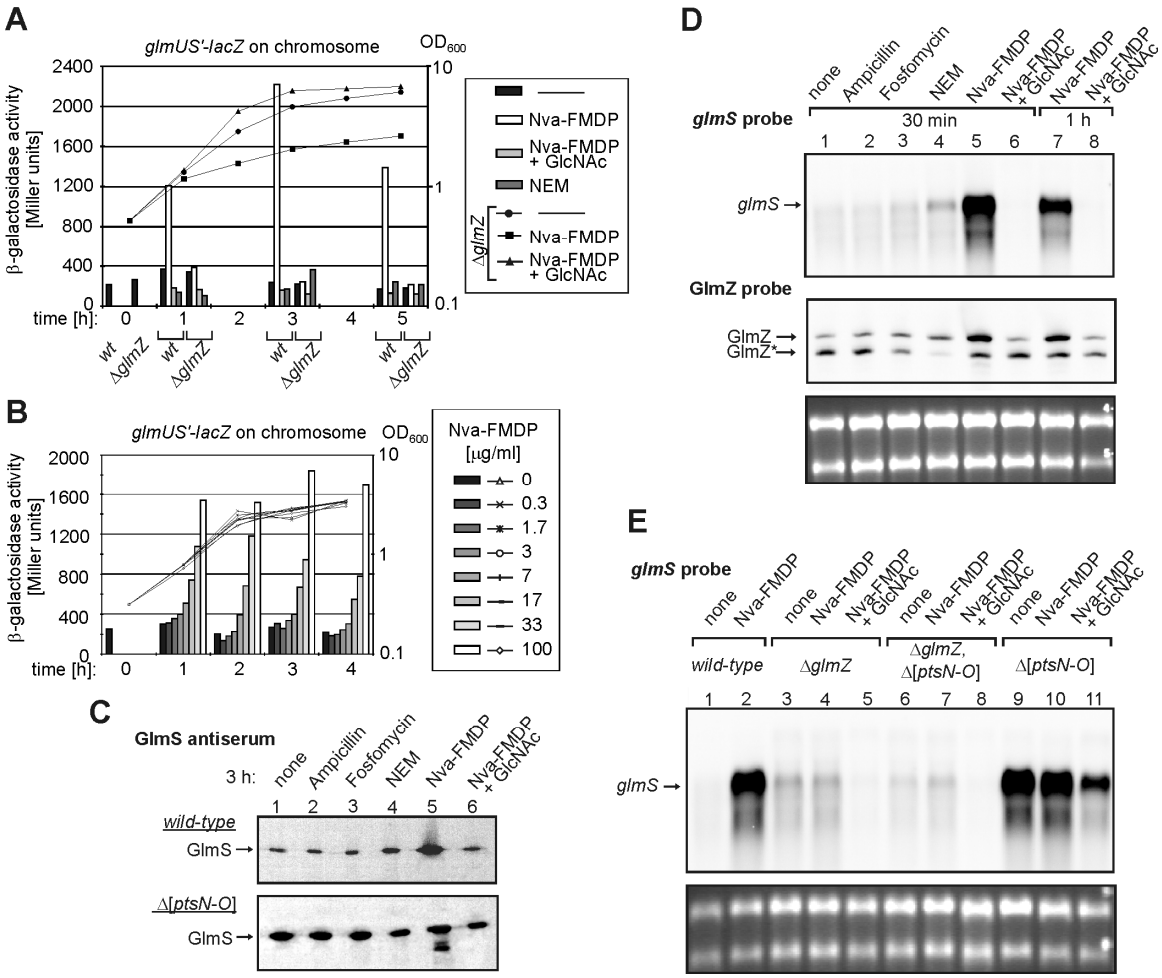


Figure 8: Depletion of intracellular GlcN-6-P induces GlmS expression in a GlmZ-dependent manner. A. Effect of inhibitors of GlmS and GlmU on the expression of the chromosomal *glmUS'-lacZ* fusion in the wild type strain Z9 and the Δ*glmZ* mutant Z40. Cultures of strains Z9 and Z40 were split at time-point 0 and growth was continued in the absence or presence of sub-lethal concentrations of Nva-FMDP (100 µg/ml) or NEM (10 µg/ml). GlcNAc was added where indicated. Aliquots of the cultures were removed at the indicated times and the β-galactosidase activities were determined (depicted by columns). In addition, the growth curves of the cultures of strain Z40 are shown. **B.** Nva-FMDP activates expression of the *glmUS'-lacZ* fusion in a concentration-dependent manner. A culture of strain Z9 was split at time-point 0, and growth was continued in the presence of various concentrations of Nva-FMDP as indicated, and the β-galactosidase activities were determined in one hour time-intervals. In addition, the growth curves of the respective cultures are shown. **C.** Western blotting experiments to determine the effects of inhibitors of the peptidoglycan biosynthesis pathway on the GlmS amount in wild type strain R1279 (top) and in the Δ[*ptsN-O*] mutant R2413 (bottom). The following concentrations were used in Fig. 7 C, D and E: ampicillin (2 µg/ml), fosfomycin (2 µg/ml), NEM (10 µg/ml), Nva-FMDP (100 µg/ml). **D.** Northern blotting experiments to determine the effects of inhibitors of the peptidoglycan biosynthesis pathway on the *glmS* transcript (*glmS* probe; top) and on GlmZ (GlmZ probe; bottom) in wild type strain R1279. Samples were harvested at the time indicated after addition of the inhibitors. The ethidium-bromide stained formaldehyde agarose gel is shown at the bottom. **E.** Northern blotting experiments using a *glmS* probe to study the impact of Nva-FMDP and GlcNAc on the *glmS* transcript in strains R1279 (lanes 1, 2), Z44 (lanes 3-5), Z46 (lanes 6-8) and R2413 (lanes 9-11).

This four-fold repression of *glmS* corresponds well to the known four-fold reduction of *glmUS* promoter activity by GlcNAc that results from the release

of NagC binding (Plumbridge, 1995). In sum, the data suggest that functional YhbJ is required for metabolite dependent down-regulation of *glmS* expression.

Our data indicate that expression of *glmS* is controlled by either the concentration of GlcN-6-P or GlcN-1-P (see Fig. 3 A). To distinguish between the two possibilities, we used the conditional *glmM* knockout strain GPM83 and its isogenic wild type JM83. First, we verified by Northern analysis that this pair of strains properly accumulates *glmS* transcripts upon addition of Nva-FMDP (data not shown). Strain GPM83 carries an inactivated *glmM* gene on the chromosome and a wild type copy of *glmM* on a plasmid with a temperature-sensitive origin of replication. Hence, shift to high temperature leads to cessation of GlmM synthesis in the growing culture and concomitantly to depletion of GlcN-1-P and accumulation of GlcN-6-P (Mengin-Lecreulx and van Heijenoort, 1996). As revealed by Northern- and Western analyses, a temperature-shift to 44℃ did not induce higher *glmS* expression levels (data not shown). Hence, GlcN-6-P is the signaling molecule responsible for modulating expression of *glmS*.

The small RNA GlmZ is essential for the GlcN-6-P-dependent control of *glmS* expression and is in turn controlled by GlcN-6-P

To see whether the sRNA GlmZ is required for the GlcN-6-P dependent modulation of *glmS* expression, we tested the effect of Nva-FMDP on *glmS* expression in the Δ*glmZ* mutant. As revealed by *lacZ*-fusion data and Northern analysis, Nva-FMDP had no inducing effect on *glmS* expression in the Δ*glmZ* or the Δ*glmZ* Δ[*ptsN-O*] mutant (Fig. 8 A and 6 E, lanes 3-8). Intriguingly, in contrast to the wild type strain, the presence of 100 µg/ml Nva-FMDP caused a strong growth defect of the Δ*glmZ* mutant. The growth defect could be suppressed by the addition of GlcNAc (Fig. 8 A). Finally, we tested the effects of the various inhibitors used above on GlmZ in Northern blot analyses. The addition of Nva-FMDP, but not of the other inhibitors, led to the accumulation of the full-length form of GlmZ, and this could be prevented by the additional presence of GlcNAc (Fig. 8 D, lower panel). These data indicate

that GlmZ is modulated in response to the GlcN-6-P concentration and transmits this signal to the *glmUS* transcript.

Discussion

The lack of feedback inhibition of GlmS activity in *E. coli* has been a long-standing mystery that is clarified by the mechanism discovered here. We demonstrate that the synthesis of the *E. coli* GlmS protein is feedback regulated by its product, glucosamine-6-phosphate, at a post-transcriptional level (see model in Fig. 9). We show that the depletion of GlcN-6-P, the product of GlmS enzymatic activity, but not of one of the metabolites further downstream in the pathway, strongly induces GlmS synthesis. Responsible for this induction is the accumulation of a *glmS* specific transcript that is derived from RNase E catalyzed processing of the primary *glmUS* transcript within the *glmU* stop codon (Fig. 9). This feedback control of *glmS* expression could also account for a differential regulation of *glmU* and *glmS* expression under conditions when amino sugars are present in the medium and GlmU but not GlmS is required (Fig. 3 A). Secondly, we show that the accumulation of *glmS* mRNA requires the small RNA GlmZ. In a *glmZ* mutant, up-regulation of *glmS* expression in response to a decrease in GlcN-6-P did not occur anymore. Moreover, processing and turnover of the small RNA GlmZ turned out to be controlled by the concentration of GlcN-6-P: Full-length GlmZ accumulates upon depletion of GlcN-6-P. The degree of *glmS* expression correlates with the amount of the unprocessed form of GlmZ (Fig. 9). Thirdly, we identified an additional factor, YhbJ, that strongly affects *glmS* expression. In a *yhbJ* mutant, the production of the *glmS* transcript is de-repressed and GlmS accumulates. Actually, it was this initial observation that enabled us to perform a transposon mutagenesis screen in which we identified GlmZ as a module in the feedback regulation of *glmS* expression by GlcN-6-P.

In the *yhbJ* mutant the production of *glmS* mRNA from the *glmUS* transcript is no longer sensitive to GlcN-6-P. Therefore, in contrast to the *glmS* riboswitch in *B. subtilis* (Winkler *et al.*, 2004), it appears unlikely that the *glmS* transcript can directly sense GlcN-6-P. Thus, YhbJ or GlmZ are good candidates as being the GlcN-6-P sensory molecule. It is interesting to note, however, that in both, *B. subtilis* and *E. coli*, the regulatory switch that couples *glmS*

expression to GlcN-6-P occurs at the post-transcriptional level and in both cases involves a processing event of the mRNA 5' to the *glmS* coding region. This strategy may allow an immediate adaptation of *glmS* expression when the intracellular GlcN-6-P concentration suddenly shifts in response to altered environmental conditions that dictate a change in peptidoglycan synthesis rates.

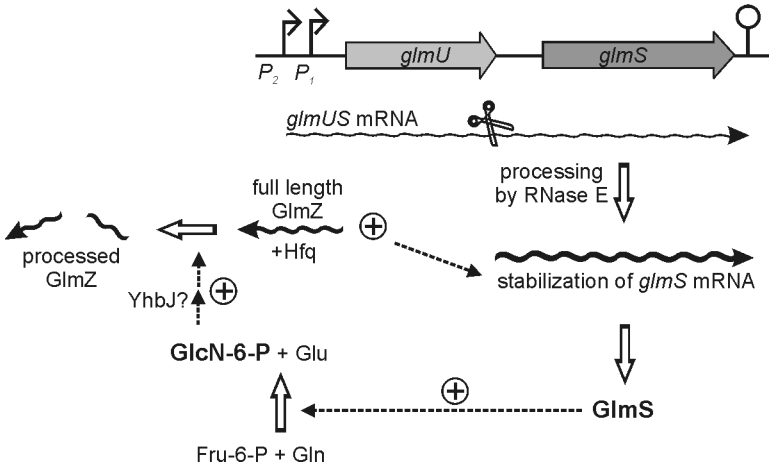


Figure 9: Model for the feedback regulation of *glmS* expression by the small RNA GlmZ in response to GlcN-6-P. When the intracellular GlcN-6-P concentration decreases, the full length form of the small RNA GlmZ accumulates and positively regulates GlmS synthesis in an Hfq dependent manner. This is achieved by the accumulation of a *glmS* specific mRNA that emerges from processing of the *glmUS* co-transcript by RNAse E at the *glmU* stop codon. Software analysis predicts that GlmZ could bind to sequences upstream of *glmS* and prevent formation of an inhibitory stem loop structure overlapping with the RBS (see Fig. S3 A). Thus, active translation by deblocking the RBS could account for the observed stabilization of the *glmS* mRNA. Note that it cannot be completely excluded that GlmZ might also be necessary for recruiting RNase E to its target *glmUS* mRNA leading to production of the stable *glmS* mRNA. Accumulation of the *glmS* mRNA causes high-level synthesis of GlmS, which refills the GlcN-6-P pool. Higher GlcN-6-P concentrations in turn repress *glmS* expression by destabilizing GlmZ. YhbJ, a protein encoded in the *rpoN* operon, governs this process in a yet unknown way.

At first view, the way by which GlmZ controls *glmS* expression appears unorthodox. Where known, the majority of small RNAs down-regulate gene expression by blocking translation and/or stimulating mRNA degradation, whereas GlmZ positively controls gene expression. A few examples are known where sRNAs stimulate translation of target mRNAs (Repoila *et al.*, 2003; Hammer and Bassler, 2007; Prevost *et al.*, 2007), and at least in one case, GadY, the sRNA increases stability of a mRNA (Opdyke *et al.*, 2004). However, GadY interacts with the 3' end of its target mRNA, whereas in the case of GlmZ the target site should be present between positions -183 and +129 relative to *glmS*, since a fusion of only this region to *lacZ* is still

regulated by YhbJ and GlmZ (Fig. S3 B, bars 1 and 2). Software analysis predicts five potential secondary structures that can form within the intergenic region of the *glmUS* mRNA (Fig. S3 A). Interestingly, the RBS of *glmS* is buried in the stem of the most distal secondary structure suggesting that it could be inaccessible to the ribosome. Strikingly, according to software prediction the positions 151-169 of GlmZ can potentially baise-pair with the left half-site of this stem and with nucleotides in front of it (positions -40 to -26 relative to *glmS*; Fig. S3 A). This interaction would disrupt the stem loop structure and make the RBS accessible to the ribosome. Hence, it appears possible that GlmZ regulates *glmS* expression by relieving a translational block and that the observed stabilization of *glmS* mRNA by GlmZ is a secondary effect that results from ongoing translation. Rapid target mRNA degradation as a result of translation inhibition has also been observed for other sRNAs (Morita *et al.*, 2006; Prevost *et al.*, 2007).

We demonstrated that up-regulation of *glmS* expression requires a functional *hfq* gene, at least in the *yhbJ* mutant. In *E. coli*, a large group of sRNAs that pair with their mRNA targets depends on the Hfq protein to function properly. Hfq stabilizes the interaction of the small RNA with its target mRNA and often recruits RNase E that subsequently initiates the concomitant degradation of both RNAs (Gottesman, 2005; Aiba, 2007). The region surrounding the *glmU* stop codon resembles typical RNase E target sequences (Diwa *et al.*, 2000), i.e. it contains an A/U rich sequence upstream and two adjacent stem-loops downstream of the target site (Fig. S3 A). However, disruption of either one of these secondary structures by mutation has no effect on the regulated expression of a *glmS'-lacZ* fusion (Fig. S3 B, compare bars 1, 4 and 5). The complete deletion of the RNase E-site and of the adjacent stem loops globally reduces expression of the *glmS'-lacZ* fusion ~5-fold in all tested strains suggesting that this region is important for *glmS* transcript stability. However, the remaining activity is still dependent on GlmZ and regulated by YhbJ like the activity of the parental construct containing the RNase E site (Fig. S3 B, bars 1 and 3). These observations make it unlikely that GlmZ directly targets the region surrounding the *glmU* stop codon, which is in agreement with the software-predicted GlmZ binding site next to the *glmS* translation start. Therefore, processing of the *glmUS* co-transcript by RNase E and regulation

of *glmS* expression by GlmZ are likely to be independent, and GlmZ may stabilize the *glmS* monocistronic transcript after its generation by RNase E (Fig. 9). However, care has to be taken with premature conclusions about the details of the mechanism, since a second small RNA, SroF (Vogel *et al.*, 2003), is additionally involved in control of *glmS* expression. This sRNA shares some sequence identity with GlmZ, and we found that its processing is also sensitive to GlcN-6-P and controlled by *yhbJ* (our unpublished data). Using a large scale screening approach, GlmZ and SroF and their stimulatory effects on *glmS* expression have also been identified by the Vogel lab, and SroF was accordingly renamed GlmY (Jörg Vogel, personal communication).

How does YhbJ affect processing of GlmZ and thus expression of *glmS*? YhbJ has no similarity to other proteins of known function. It contains a nucleotide binding motif, and software analysis predicts a putative RNA-binding domain at the C-terminus. Hence, YhbJ may bind directly to the *glmS* or GlmZ RNAs, thereby destabilizing them. However, YhbJ may have a more pleiotropic function, e.g. it could control activity of PAP-I or of some other gene/protein involved in control of RNA turnover that in turn controls stability of GlmZ. Interestingly, *yhbJ* is highly conserved in bacteria. It exists in almost all *proteobacteria* where it is present within the *rpoN* operon (Boël *et al.*, 2003). In Gram-positive bacteria, the *yhbJ* ortholog is co-transcribed with another highly conserved gene designated *yvcK* in *B. subtilis*. We previously showed that under certain growth conditions *B. subtilis* mutants defective in *yvcK* are unable to provide precursor molecules required for the synthesis of the cell wall (Görke *et al.*, 2005). An ortholog of *yvcK* is also present in *E. coli*. It is tempting to speculate that in these two unrelated bacteria YhbJ and YvcK may have important and perhaps interconnected roles in the control of synthesis of precursor molecules required for cell wall synthesis.

Due to its central function, bacterial GlmS has been extensively explored as a target for the design of novel antibiotics. Most attention has been focused on utilizing glutamine analogues that selectively inhibit GlmS and of which derivatives of FMDP are the most effective compounds (Milewski, 2002; Teplyakov *et al.*, 2002). While FMDP-peptides like Nva-FMDP effectively inhibit growth of Gram-positive bacteria in micromolar concentrations (Andruszkiewicz *et al.*, 1990; Chmara *et al.*, 1998), no growth inhibitory effect

on wild type *E. coli* in a range of 1-300 µM was observed in this work (Fig. 8 B). In contrast, a *glmZ* mutant is severely growth inhibited by 300 µM Nva-FMDP (Fig. 8 A). Accordingly, the high intrinsic resistance of wild type *E. coli* to Nva-FMDP may be the consequence of the GlmZ mediated overexpression of *glmS* in response to a decrease in intracellular GlcN-6-P concentration. Under this new aspect, compounds that would prevent GlmS overproduction e.g. by inhibition of GlmZ, Hfq or RNase E, can be predicted to increase the antimicrobial potential of GlmS inhibitors against *E. coli* and perhaps related bacteria.

Acknowledgements

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Supplementary material

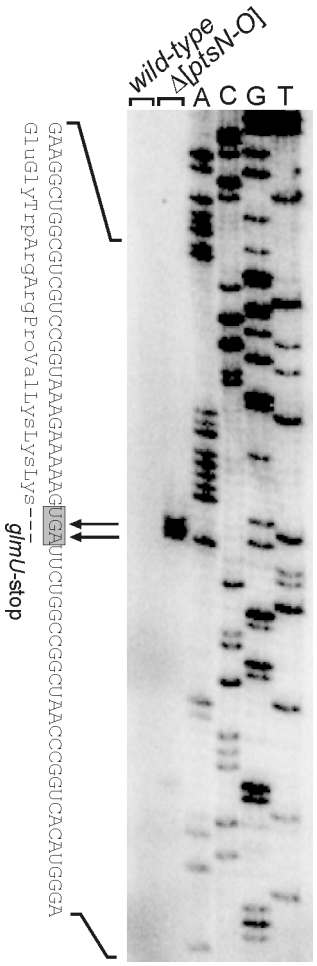

Figure S1: The 5' end of the monocistronic *glmS* mRNA that accumulates in the *yhbJ* mutant maps to the *glmU* stop codon. Primer extension analysis of total RNAs of strains R1279 (wild type) and R2413 (Δ[*ptsN-O*]) using primer 949 which anneals to positions -53 to -71 upstream of *glmS*. The reaction products were separated on urea/polyacrylamide gels alongside a sequencing ladder generated with the same primer. The identical 5' ends were mapped when primer 910 annealing closer to *glmS* (*glmS*: +6 to –21) was used whereas no products were obtained with primer 967 which anneals upstream of the *glmU* stop codon (data not shown).

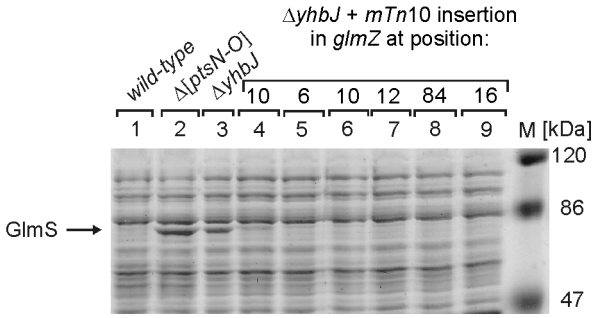

Figure S2: Determination of the amount of GlmS in cell extracts of the Δ*yhbJ glmZ::mTn*10 mutants. Several of the *mTn*10 mutants are shown as examples. The *mTn*10 insertion sites are indicated at the top. Two independent mutants carrying the mTn*10* at position +10 were tested. Proteins were separated by 12.5% SDS/PAGE and analyzed by Coomassie staining. As controls, the strains Z8, Z5 and Z28 were employed (lanes 1-3).

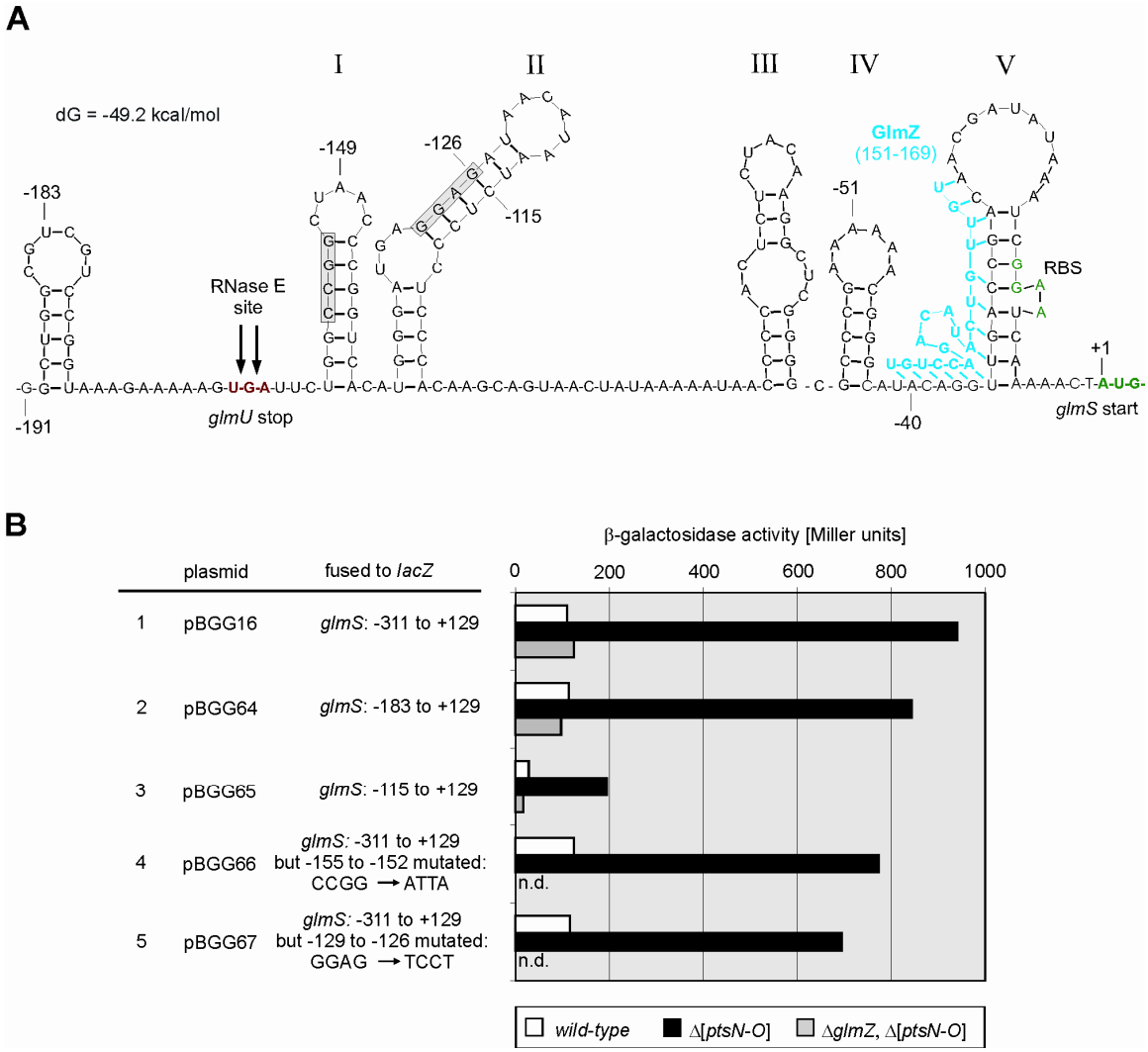

Figure S3: Analysis of the *glmU-glmS* intergenic region. A. Software prediction for the folding of the intergenic region of the *glmUS* mRNA and its basepairing with the sRNA GlmZ (Mathews *et al.*, 2004; Zuker, 2003). Positions are relative to the first nucleotide of the *glmS* gene. The nucleotides subjected to mutational analysis are highlighted in grey. GlmZ is depicted in blue. **B.** β-galactosidase activities of strains R1279 (*wild-type*), R2413 (Δ[*ptsN-O*]) and Z46 (Δ*glmZ*, Δ[*ptsN-O*]) carrying fragments of the *glmUS* intergenic region fused to *lacZ* on plasmids as indicated. n.d. = not determined. Several control experiments confirmed the absence of a promoter within the *glmS* (-311 to +129) fragment: Primer extension analyses using a primer annealing to *lacZ* showed that a single *glmS'-lacZ* mRNA with its 5'-end mapping to the *glmU* stop codon was detectable in the Δ*yhbJ* mutant but not in the wild type (data not shown). This result excludes the possibility that a promoter is present downstream of the RNase E cleavage site. In addition, we confirmed the absence of a promoter upstream of the RNase E cleavage site using a fusion of a shorter *glmS'* (-183 to +129) fragment to *lacZ* (Fig. S3, compare bars 1 and 2). Finally, we constructed a *glmS'-lacZ* fusion in a different vector that carries no promoter directing its expression, and inserted this fusion into the *attB*-site on the chromosome. This fusion was indeed not expressed, neither in the wild-type nor in the Δ[*ptsN-O*] mutant (data not shown). Taken together, the data confirm that expression of the *glmS'-lacZ* fusion (Fig. 3 D, line 4) is driven by read-through from a promoter located in the *aadA* resistance gene cassette, and that the *aadA-glmS'-lacZ* fusion mRNA is processed by RNase E like the *glmUS* transcript.

Supplemental experimental procedures

Construction of strains

Strains R2404 and R2413 carrying deletions of *ptsP* and [*ptsN, yhbJ, ptsO*], respectively, were constructed according to (Hamilton *et al.*, 1989) using plasmid pFDX3400 which contains a temperature-sensitive origin of replication and a *cat*-resistance gene. The flanking regions of *ptsP* were amplified by PCR using the primer pairs 718/719 and 720/721 and the flanking regions of *ptsN-O* were amplified using the primer pairs 724/725 and 747/748 and cloned in tandem into the unique XbaI-site of plasmid pFDX3400, respectively. The resulting plasmids pFDX4255 and pFDX4259 were used to eliminate the genes of interest in strain R1279 by subsequent 42°/28°C temperature shifts as described previously (Görke and Rak, 1999). Deletions of *yhbJ* and *glmZ* in strains Z24, Z27, Z40, Z42, Z44 and Z46 were constructed using the method of (Datsenko and Wanner, 2000). Briefly, the target strains R1279, R2413, Z5 and Z8 were transformed with the temperature-sensitive plasmid pKD46 delivering the λRed recombination genes. Subsequently, the transformants were electroporated with PCR-fragments carrying a *cat* resistance cassette flanked by ends homologous to the target genes. The primer pairs BG157/BG158 and BG184/BG185 were used to generate the PCR-fragments for the *yhbJ* and *glmZ* deletions, respectively. Transformants were selected at 37°C or 30°C for chloramphenicol-resistance and subsequently plated at 37°C to cause loss of plasmid pKD46. Subsequently, the *cat*-cassette was removed in strains Z27, Z24, Z42 and Z44 using helper plasmid pCP20, which resulted in strains Z28, Z37, Z43 and Z45, respectively.

Integration of *lacZ*-fusions into the λ attachment site (*attB*) of the chromosome was performed using the helper plasmid pLDR8 as described by (Diederich *et al.*, 1992). Briefly, plasmids pBGG15, pBGG16 and pBGG17 were digested with BamHI and the fragments encompassing the various *lacZ*-fusions were isolated. These fragments in addition carried an *aadA* resistance gene and the *attP*-sequence but no origin of replication. They were self ligated and used to transform strains Z2 and Z3 harboring the temperature-sensitive λ integrase expression plasmid pLDR8. Selection at 42°C on spectinomycin-containing

plates resulted in strains Z4-Z9, carrying the respective *lacZ* fusions on the chromosome. Z2 and Z3 are isogenic with strains R2413 and R1279, respectively, but in addition carry an F' plasmid that was introduced by conjugation with strain BMH71-18 (Kramer *et al.*, 1984) in order to complement the proline auxotrophy of the parental strains. Proline prototrophy was important in order to test whether the nitrogen source affects *glmS* expression (data not shown).

Construction of plasmids

For DNA cloning strain DH5α was used following standard procedures (Sambrook and Russell, 2001). Plasmids pKEM4, pKES15 and pKES99, which are suitable for integration of *lacZ* reporter fusions into the chromosomal *attB* site are derivatives of plasmid pKESD8 (Dole *et al.*, 2002). Plasmid pKES15 carries a *bgl* promoter *lacZ* fusion, plasmid pKES99 carries a *lacUV5* promoter *lacZ* fusion and pKEM4 carries a promoter-less *lacZ* gene (Nagarajavel, 2007; Nagarajavel *et al.*, 2007). For the construction of the *glmU*-, *glmS*-, and *glmZ-lacZ* fusions in plasmids pBGG15, pBGG16, pBGG17, pBGG59, pBGG64 and pBGG65, respectively, the *bgl*-promoter on plasmid pKES15 was removed by SalI/XbaI digestion. Subsequently, SalI/XbaI-digested PCR fragments were inserted that were generated using the primer pairs BG119/BG122 (*glmU*: -392 to +240), BG121/BG123 (*glmS*: -311 to +129), BG119/BG123 (*glmU* -392 to *glmS* +129), BG199/BG202 (*glmZ*: -424 to +31), BG213/BG123 (*glmS*: -183 to +129) and BG214 + BG123 (*glmS*: -115 to +129), respectively. Plasmids pBGG66 and pBGG67 are isogenic to plasmid pBGG16 but carry point mutations in the *glmS'* fragment. They were constructed following the multiple-mutation reaction protocol as described (Hames *et al.*, 2005). Briefly, the *glmS'* fragments carrying the desired mutations were generated in PCR reactions containing a thermo-stable DNA ligase, the primer pair BG121/BG123 and the 5'-phosphorylated mutagenic primer BG215 (*glmS* -171 to -136) or BG216 (*glmS* -147 to -108), respectively. Subsequently, the SalI/XbaI-digested PCR-fragment was inserted between these sites in plasmid pKES15. Plasmid pBGG56 carrying *glmS* under *tac* promoter control was constructed by a three fragment ligation

combining the XbaI-SacII- and the SacII-BamHI-fragments of plasmid pFDX3453 (Görke and Rak, 1999) with a PCR fragment encompassing *glmS* which was obtained by amplification with primers BG180 and BG181 and digestion with BamHI and XbaI. For the construction of plasmid pBGG84 carrying *glmZ* under control of the *ara*-promoter, *glmZ* was amplified by PCR using the primer combinations BG235/BG237, respectively. The PCR fragments were digested at their extremities with SacI and XbaI and inserted between these sites in plasmid pBAD30 (Guzman *et al.*, 1995). For the complementation analyses shown in Fig. 3 C genes of the *rpoN* operon were cloned into plasmid pFDX4291 to allow for their constitutive expression. This plasmid carries a pSC101 origin of replication, a *cat* resistance gene and an operator-less *tac* promoter followed by the Shine-Dalgarno (SD) sequence of the *Bacillus subtilis sacB* gene. A singular BglII-site is present between P_{tac} and the *sacB*-SD and singular NdeI/XbaI/HincII-sites are present downstream of the *sacB*-sequence, suitable for cloning. Details on the construction of this plasmid are available on request. Plasmid pFDX4291 was opened by NdeI/XbaI digestion and ligated to PCR fragments digested with the same enzymes and amplified with the primer pairs as follows: 826/827 (*ptsO*), 828/829 (*ptsN*), 828/827 (*ptsN, yhbJ, ptsO*), 856/827 (*yhbJ*, *ptsO*). This yielded plasmids pFDX4292, pFDX4294, pFDX4296 and pFDX4320, respectively. To construct plasmid pFDX4322 carrying *ptsO* and *ptsN* in tandem, plasmid pFDX4292 was opened by HincII digestion and ligated to the blunt-made BglII-HincII fragment of plasmid pFDX4294. For the construction of plasmid pFDX4324 carrying *yhbJ*, plasmid pFDX4291 was opened by BglII and ligated to a BglII-digested PCR fragment amplified with primers 723/825 from plasmid pFDX4320 as template.

LC-MS/MS protein identification

Excised polyacrylamide gel pieces of stained protein bands were digested with trypsin according to (Shevchenko *et al.*, 1996). Tryptic peptides extracted from each gel slice were injected onto a reversed-phase liquid chromatographic column (Dionex-NAN75-15-03-C18 PM) by using the *ultimate* HPLC system (Dionex, Netherlands) to further reduce sample complexity prior to mass analyses with an LCQ DecaXP mass spectrometer

(Thermo Electron Corp, San Jose, CA), equipped with a nano-electrospray ion source. Cycles of MS spectra with m/z ratios of peptides and four data-dependent MS2 spectra were recorded by mass spectrometry. The MS2 spectra with a total ion current higher than 10,000 were used to search for matches to peptides from the *NCBI Escherichia coli* Database using the Sequest algorithm of the Bioworks software (Version 3.1, Thermo Electron Corp). The search parameters included are based on the Sequest algorithm: (i) precursor ion mass tolerance less than 1.4 amu, (ii) fragment ion mass tolerance less than 1.0 amu, (iii) up to three missed tryptic cleavages allowed, and (iv) fixed cysteine modification by carboxyamidomethylation (plus 57.05 amu) and variable modification by methionine oxidation (plus 15.99 amu), and phosphorylation of serine, threonine, or thyrosine (plus 79.97 amu). Matched peptide sequences of identified proteins must pass the following criteria: (i) the cross-correlation scores (Xcorr) of matches are greater than 2.0, 2.5, and 3.0 for peptide ions of charge state 1, 2, and 3, respectively, (ii) ΔCn values of the best peptide matches were at least 0.4, and (iii) the primary scores (Sp) were at least 600. Protein identification required at least two different peptides matching these criteria.

Table S1: Oligonucleotides used in this study.

Primer	Sequence[a]	Res. sites	Position[b]
718	GCTCTAGACAGCTCCCATTGG	XbaI	*lgt* +682 to +666
719	CGCGGATCATATACATATCTT		*lgt* -150 to -130
720	TTAACCTCTTTTACGTCGATAA		*nduH* +530 to +508
721	GCTCTAGAAAGCTGGGATAACTGTG	XbaI	*mutH* -202 to -216
723	TCATGGTTTACGTTTTTCCAG		*yrbL* -21 to -2
724	TCTTCATCACTTTGACATACAA		*ptsO* +273 to +295
725	GCTCTAGAGCAAACTTACCCGGTC	XbaI	*yrbL* +802 to +783
747	GCTCTAGACAGCCAGTTTATCCGCAG	XbaI	*rpoN* +943 to +962
748	AAGAACCTGCCCACTCAAAC		*ptsN* -10 to -27
825	CTCGAGACTTGACAATTAATCATCGG	XhoI	
826	AAACGTAACATATGACCGTCAAGC	NdeI	*ptsO* +1 to +13
827	GTGATCTAGATTAATCTTCATCAAA	XbaI	*ptsO* +258 to +275
828	CTTAGGTGAACATATGACAAATAA	NdeI	*ptsN* +1 to +11
829	CATTCTAGAATAACTACGCTTCATC	XbaI	*ptsN* +498 to +481
856	CAGCATATGGTACTGATGATCGTCAG	NdeI	*yhbJ* +1 to +19
910	ACACATATGTTTTGATTCCGATTTATA		*glmS* +6 to -21
949	TCGGGCGCCCCGAGCCTTG		*glmS* -53 to -71
967	TCACTTTTTCTTTACCGGAC		*glmS* -162 to -181
BG119	GCACGCGTCGACCAGCTGCGCGTTATCGAGTTG	SalI	*glmU* -392 to -371
BG121	GCACGCGTCGACCAGCTGGTGGCCCCGGTAAC	SalI	*glmS* -311 to -292
BG122	GCTCTAGACAGCTGCTCTGCCTGAAGCAC	XbaI	*glmU* +240 to +220
BG123	GCTCTAGACATATGACCTTCTGCATCAACAAC	XbaI	*glmS* +129 to +106
BG147	AAAACGGCAAAGTTACCGGC		*glmU* +433 to +453
BG148	CTAATACGACTCACTATAGGGAGATTCGCATCTTCCACAACG		*glmU* +959 to +939
BG149	CTGGCGCGGAAGTAAAACG		*glmS* +676 to +694
BG150	CTAATACGACTCACTATAGGGAGAAGAACCCGGAACGTTA		*glmS* + 1144 to +1125
BG157	GTTATTCGGTAATGTCTCTTTTAGACGTTGTGAGGAGAAACAGTACG		

	TGTAGGCTGGAGCTGCTTCG		
BG158	GGCATGCATGCCCAGCTTGTTTGTGATTTCAACAGTTTGCTTGACG GTCATATGAATATCCTCCTTAGTTCCTATTCC		
BG184	GGGATGTTATTTCCCGATTCTCTGTGGCATAATAAACGAGTGTAGGC TGGAGCTGCTTCG		
BG185	CACCCGGAGGCAAGCACCTCCGGGGCCTTCCTGATACATCATATGA ATATCCTCCTTAGTTCCTATTCC		
BG180	GCGGGATCCTCGAGAAATCGGAATCAAAAACTATGTGTGG	BamHI	*glmS* -18 to +8
BG181	GCGTCTAGATTACTCAACCGTAACCGATTTTGCC	XbaI	*glmS* +1829 to +1805
BG199	GCACGCGTCGACGCAAAATGCTCCGGTTTCATG	SalI	*glmZ* -424 to -404
BG202	GCGTCTAGAGGCGAACATAAGAGATGGAATGAGC	XbaI	*glmZ* +31 to +7
BG213	GCACGCGTCGACTCGTCCGGTAAAGAAAAAGTG	SalI	*glmS* -184 to -163
BG214	GCACGCGTCGACTCCCTCCCACAAGCAGTAAC	SalI	*glmS* -115 to -94
BG215	P-GAAAAAGTGATTCTGG**ATTA**CTAACCCGGTCACATG		*glmS* -171 to -136
BG216	P-CCCGGTCACATGGGATGA**TCCT**ATAACATAATCTCCCTCC		*glmS* -147 to -108
BG230	GTAGATGCTCATTCCATCTC		*glmZ* +1 to +20
BG231	CTAATACGACTCACTATAGGGAGAAAACAGGTCTGTATGACAAC		*glmZ* +172 to 152
BG235	GGCGAGCTCTCAGGAAGTTATTACTCAGGAAGC	SacI	*glmZ* -100 to -83
BG237	GGCTCTAGATTCCTTCTCACCCGGAGGCAAGCACC	XbaI	*glmZ* +254 to +229

[a]Restriction sites are underlined; nucleotide positions that differ from the wild-type sequence are in boldface.
[b]Positions are relative to the first nucleotide of the respective gene. Gene names are as in http://biocyc.org, except for *glmZ* which was formerly named *sraJ*.

3. The small RNA GlmY acts upstream of the sRNA GlmZ in the activation of *glmS* expression and is subject to regulation by polyadenylation in *Escherichia coli*

The results described in this chapter were published in:

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Author contributions:

This study was designed by B.R., E.H. and B.G.. A.M. performed the 3'RACE and the GlmY and GlmZ half-life measurements. B.R. performed all northern blots except for the half-life blots. Strains were constructed by F.K. and B.R.. Plasmids were constructed by an internship student under the supervision of B.R.. F.K. performed β-galactosidase measurements. B.R., E.H. and B.G. wrote the paper.

Abstract

In *Escherichia coli* the *glmS* gene encoding glucosamine 6-phosphate (GlcN-6-P) synthase GlmS is feedback regulated by GlcN-6-P in a pathway that involves the small RNA GlmZ. Expression of *glmS* is activated by the unprocessed form of GlmZ, which accumulates when the intracellular GlcN-6-P concentration decreases. GlmZ stabilizes a *glmS* transcript that derives from processing. Overexpression of a second sRNA, GlmY, also activates *glmS* expression in an unknown way. Furthermore, mutations in two genes, *yhbJ* and *pcnB*, cause accumulation of full-length GlmZ and thereby activate *glmS* expression. The function of *yhbJ* is unknown and *pcnB* encodes poly(A) polymerase PAP-I known to polyadenylate and destabilize RNAs. Here we show that GlmY acts indirectly in a way that depends on GlmZ. When the intracellular GlcN-6-P concentration decreases, GlmY accumulates and causes in turn accumulation of full-length GlmZ, which finally activates *glmS* expression. In *glmZ* mutants, GlmY has no effect on *glmS*, whereas artificially expressed GlmZ can activate *glmS* expression also in the absence of GlmY. Furthermore, we show that PAP-I acts at the top of this regulatory pathway by polyadenylating and destabilizing GlmY. In *pcnB* mutants, GlmY accumulates and induces *glmS* expression by stabilizing full-length GlmZ. Hence, the data reveal a regulatory cascade composed of two sRNAs, which responds to GlcN-6-P and is controlled by polyadenylation.

Introduction

In recent years it became evident that in bacteria many genes are regulated at the post-transcriptional level in addition to control of transcription initiation. In this respect, the *glmS* gene encoding glucosamine-6-phosphate synthase (GlmS) received much attention because in the *Firmicutes* group of Gram-positive bacteria its expression is feedback regulated by a riboswitch mechanism (Winkler *et al.*, 2004; Barrick and Breaker, 2007). In this case, the *glmS* mRNA is capable to bind the product of GlmS enzymatic activity, glucosamine 6-phosphate (GlcN-6-P), leading to activation of an intrinsic ribozyme that catalyzes self-cleavage of the *glmS* mRNA. This self-cleavage initiates the rapid degradation of the *glmS* mRNA by RNase J1 shutting off GlmS synthesis (Collins *et al.*, 2007). Recently, it has been reported that in

the Gram-negative bacterium *Escherichia coli* synthesis of GlmS is likewise feedback regulated by GlcN-6-P, but by a mechanism that involves a small RNA rather than a riboswitch (Kalamorz *et al.*, 2007).

The synthesis of GlcN-6-P by GlmS is the rate-limiting reaction in the hexosamine pathway that delivers precursor molecules for biosynthesis of peptidoglycan and lipopolysaccharides (LPS), which are essential elements of bacterial cell walls and Gram-negative outer membranes. In *E. coli*, *glmS* is encoded in the bi-cistronic *glmUS* operon that is transcribed from two promoters in front of *glmU* (Plumbridge, 1995). The primary *glmUS* transcripts are subject to processing by RNase E at the *glmU* stop codon (Kalamorz *et al.*, 2007; Joanny *et al.*, 2007). Upon a decrease of the intracellular GlcN-6-P concentration, the *glmS* mono-cistronic transcript is stabilized in a process that depends on the sRNA GlmZ encoded in the *hemY-aslA* intergenic region (Kalamorz *et al.*, 2007). This sRNA is synthesized as a 210 nt long precursor and subsequently processed, presumably by RNase III, to yield a form of ~155 nt (Argaman *et al.*, 2001; Wassarman *et al.*, 2001). Upon a decrease in the intracellular GlcN-6-P concentration, the full-length form of GlmZ accumulates and concomitantly stabilizes the *glmS* transcript, giving rise to higher GlmS synthesis levels (Kalamorz *et al.*, 2007). Software analysis predicts that base-pairing of GlmZ with the *glmS* message would disrupt an inhibitory stem loop structure within the *glmS* leader RNA that buries the ribosomal binding site. Therefore, the observed stabilization of the *glmS* mRNA could be the consequence of activation of *glmS* translation (Kalamorz *et al.*, 2007). Interestingly, most of the supposedly base-pairing nucleotides are removed from GlmZ upon processing, which explains that exclusively full-length GlmZ can activate *glmS*.

A second sRNA, GlmY, was identified to cause GlmS overproduction, when overexpressed from a plasmid (Urban *et al.*, 2007). GlmY is encoded in the *purL-yfhK* intergenic region and evidence suggests that it is transcribed from a σ^{54}-dependent promoter (Urban *et al.*, 2007). GlmY has been reported to exist in two different sizes of 184 nt and 148 nt, respectively. The shorter and more abundant form was suggested to result from 3' end processing of the longer variant (Urban *et al.*, 2007; Vogel *et al.*, 2003). However, the mechanism by

which GlmY may activate the *glmS* mRNA and whether there is an interference with GlmZ is currently not known.

In addition, mutations in two genes, *yhbJ* and *pcnB*, have been identified to cause overexpression of GlmS by activation of the GlmZ-mediated pathway: In both mutants full-length GlmZ accumulates and stabilizes the *glmS* mRNA resulting in dramatic overexpression of GlmS (Kalamorz *et al.*, 2007; Joanny *et al.*, 2007). Gene *yhbJ* is present in the *rpoN* operon coding for σ^{54} and homologues of the phosphotransferase system (PTS). YhbJ contains an ATP-binding motif and a putative RNA binding domain, but the mechanism by which it stimulates processing of GlmZ remains elusive. Gene *pcnB* codes for poly(A) polymerase I (PAP I) responsible for adding short poly (A) tails to the 3' ends of transcripts, which may facilitate their subsequent degradation (Hajnsdorf *et al.*, 1995; O'Hara *et al.*, 1995; Xu *et al.*, 1993). The activity of PAP I is in particular required for the degradation of RNA molecules that contain tightly folded secondary structures at their 3' end and lack a terminal single-stranded region (Cheng and Deutscher, 2005; Khemici and Carpousis, 2004). These may be decay intermediates or primary transcripts carrying a *rho*-independent terminator at the 3'-end (Mohanty and Kushner, 2006). Polyadenylation is believed to make these substrates accessible to further degrading RNases like RNase II and polynucleotide phosphorylase (PNPase), the latter being part of the degradosome. In addition, PAP I plays a role in plasmid copy number control by governing the turnover of regulatory RNAs and is involved in the disposal of defective RNA molecules (Kushner, 2007; Condon, 2007).

In the present study, we analyzed how GlmZ, GlmY and PAP I act together in the regulation of *glmS* expression. We show that upon an increase of the cellular amount of sRNA GlmY, the full-length form of the sRNA GlmZ accumulates and in turn activates *glmS* expression. GlmY has no effect on *glmS* expression in the absence of GlmZ, while GlmY is not necessarily required for GlmZ-dependent activation of *glmS* expression. Hence, GlmY controls GlmZ, which then targets the *glmS* mRNA. In addition, we demonstrate that GlcN-6-P controls *glmS* expression by modulating the

amount of GlmY. GlmY subsequently transmits the signal to GlmZ, which finally regulates the *glmS* mRNA. Our further data show that the half-life of GlmY is tightly controlled by polyadenylation. PAP I polyadenylates and destabilizes GlmY and thereby indirectly contributes to the regulation of cellular GlmZ and *glmS* amounts.

Materials and methods

Growth conditions, strains and plasmids

Cells were grown in LB at 37℃ under agitation (200 r.p.m.). When necessary, media were supplemented with antibiotics (ampicillin: 50 µg/ml, chloramphenicol: 15 µg/ml, kanamycin: 30 µg/ml). Nva-FMDP was added at a concentration of 100 µg/ml when the cultures reached an OD_{600} of 0.3. The strains and plasmids used and their relevant genotypes and characteristics are listed in Table 2. See Table 3 for the list of oligonucleotides used in this study.

Table 2: Strains and plasmids used in this study.

Strain/ plasmid	Genotype or relevant structures[1]	Reference, source or construction
A. *strains*		
IBPC903	as N3433, but Δ *pcnB::kan*	(Joanny *et al.*, 2007)
N3433	HfrH, *lacZ*43, *λ, relA*1, *spoT*1, *thi*1	(Goldblum and Apririon, 1981)
R1279	CSH50 Δ(*pho-bgl*)201 Δ(*lac-pro*) *ara thi*	(Görke and Rak, 1999)
R2413	as R1279, but Δ[*ptsN, ΔyhbJ, ΔptsO*]	(Kalamorz *et al.*, 2007)
Z8	as R1279, but *attB::*[*aadA glmS*-5'::*lacZ*] $strp^R$ *F'*(*pro*$^+$)	(Kalamorz *et al.*, 2007)
Z24	as R1279, but Δ*yhbJ*::*cat*	(Kalamorz *et al.*, 2007)
Z28	as R1279, but Δ*yhbJ, attB::*[*aadA glmS*-5'::*lacZ*] $strp^R$ *F'*(*pro*$^+$)	(Kalamorz *et al.*, 2007)
Z37	as R1279, but Δ*yhbJ*	(Kalamorz *et al.*, 2007)
Z38	as R1279, but Δ*glmZ*::*cat, attB::*[*aadA glmS*-5'::*lacZ*] $strp^R$ *F'*(*pro*$^+$)	PCR BG184 / BG185→ Z8; this work
Z44	as R1279, but Δ*glmZ*::*cat*	(Kalamorz *et al.*, 2007)
Z45	as R1279, but Δ*glmZ*	(Kalamorz *et al.*, 2007)
Z46	as R1279, but Δ[*ptsN, ΔyhbJ, ΔptsO*], Δ*glmZ*::*cat*	(Kalamorz *et al.*, 2007)
Z47	as R1279, but Δ[*ptsN, ΔyhbJ, ΔptsO*], Δ*glmZ*	Z46 cured from *cat*; this work
Z95	as R1279, but Δ*glmY::cat*	PCR BG248/BG 249→ R1279; this work
Z96	as R1279, but Δ*glmY*	Z95 cured from *cat*; this work
Z105	as R1279, but Δ*glmZ,* Δ*glmY::cat*	T4GT7 (Z95) → Z45; this work
Z107	as R1279, but Δ[*ptsN, ΔyhbJ, ΔptsO*], Δ*glmZ,* Δ*glmY::cat*	T4GT7 (Z95) → Z47; this work
Z115	as R1279, but Δ*yhbJ*, Δ*glmY::cat*	T4GT7 (Z95) → Z37; this work
Z116	as R1279, but Δ*yhbJ*, Δ*glmZ::cat*	T4GT7 (Z44) → Z37; this work
Z129	as R1279, but Δ*pcnB::kan*	T4GT7 (IBPC903) → R1279; this work
Z152	as R1279, but Δ*pcnB::kan,* Δ*glmY::cat*	T4GT7 (Z95) → Z129; this work
B. *Plasmids*		
pBAD30	ori p15A, P_{ara}, MCS, *bla*	(Guzman *et al.*, 1995)
pBGG84	*glmZ* under P_{Ara}-control in pBAD30	(Kalamorz *et al.*, 2007)
pBGG149	as pBGG179, but *glmY* downstream of λP_L	this work
pBGG179	ori pMB1, λP_L, MCS, *bla*	this work

[1]ori = origin of replication; MCS = multiple cloning site

Table 3: Oligonucleotides used in this study.

Primer	Sequence[a]	Res. sites	Position[b]
BG149	CTGGCGCGGAAGTAAAACG		*glmS* +676 to +694
BG150	CTAATACGACTCACTATAGGGAGAAGAACCCGGAACGTTA		*glmS* +1144 to +1125
BG184	GGGATGTTATTTCCCGATTCTCTGTGGCATAATAAACGAGTGTAGGC TGGAGCTGCTTCG		*glmZ* -39 to -1
BG185	CACCCGGAGGCAAGCACCTCCGGGGCCTTCCTGATACATCATATGA ATATCCTCCTTAGTTCCTATTCC		*glmZ* +248 to +207
BG230	GTAGATGCTCATTCCATCTC		*glmZ* +1 to +20
BG231	CTAATACGACTCACTATAGGGAGAAAACAGGTCTGTATGACAAC		*glmZ* +172 to +152
BG248	CAACAAAGCCGGGAATTACCCGGCTTTGTTATGGAAGTGTAGGCTG GAGCTGCTTCG		*glmY* +185 to +150
BG249	CTATTTTCTTTATTGGCACAGTTACTGCATAATAGTAACCCATATGAA TATCCTCCTTAGTTCCTATTCC		*glmY* -40 to -1
BG260	AGTGGCTCATTCACCGAC		*glmY* +1 to +18
BG261	CTAATACGACTCACTATAGGGAGATAAGGCGGTGCCTAACTC		*glmY* +150 to +131
BG361	GCGAATTCAGTGGCTCATTCACCGAC	EcoRI	*glmY* +1 to +18
BG373	GGCGGATCCAGCGTTTCAAGGTGTTACTC	BamHI	*glmY* +254 to +233
BG418	P-AATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAG GCATGCAAGCTTG	MCS[c] of pBAD33	
BG419	P-GATCCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCG GGTACCGAGCTCG	MCS[c] of pBAD33	
DEOXYLI	GATCCCGGGATCCACCACCA	BamHI	
RIBOLI	P-UGGUGGUGGAUCCCGGGAUC		
Pforw	GATCCTGCAGAGTGGCTCATTCACCGAC	PstI	*glmY* +1 to +18

[a]Restriction sites are underlined. 5'-phosphorylated oligonucleotides are marked with a P. [b]Positions are relative to the first nucleotide of the respective gene. [c]MCS: multiple cloning site

For DNA cloning, strain DH5α was used following standard procedures (Sambrook and Russell, 2001). For construction of plasmid pBGG149, *glmY* was amplified by PCR using primers BG361 and BG373. The obtained DNA fragment was digested with EcoRI and BamHI and subsequently inserted between the sames sites on plasmid pBGG179. Plasmid pBGG179 carries the multiple cloning site of plasmid pBAD33 (Guzman *et al.*, 1995) downstream of the strong λP_L promoter. It was constructed by replacing the EcoRI-BamHI fragment encompassing *bglG* in plasmid pFDX1088 (K. Schnetz, unpublished) with a fragment obtained by hybridizing the 5' phosphorylated oligonucleotides BG418 and BG419, which are complementary to each other. Newly constructed gene deletions were made following standard procedures (Datsenko and Wanner, 2000). They were either marker-less clean deletions obtained with the help of plasmid pCP20 as described or the deleted gene was replaced by a chloramphenicol resistance cassette. T4GT7 transduction was used to move established deletions tagged with antibiotic resistance markers between strains (Wilson *et al.*, 1979). All strains constructed in this work were checked by PCR using appropriate primers.

β-Galactosidase assays

LB cultures were inoculated from overnight cultures in the same medium to an OD_{600} of 0.1. The cultures were grown to an OD_{600} of 0.5-0.7 and harvested. Determination of β-galactosidase activities was performed as described (Miller, 1972). Enzyme activities are presented in Miller units and are mean values of measurements performed with samples from at least three independent cultures.

RNA extraction and northern analysis

RNA extraction was performed from samples harvested from the exponential growth phase or from a set of samples harvested along the growth curve of a single culture using the RNeasy mini kit (Qiagen, Germany) according to the manufacturer's instructions. Digoxigenin-labeled RNA probes against *glmS*, GlmZ and GlmY RNAs were obtained by *in vitro* transcription using the DIG-Labelling kit (Roche Diagnostics, Germany) and specific PCR generated fragments as templates. The primers used for PCR were BG149 and BG150 for *glmS*, BG230 and BG231 for *glmZ* and BG260 and BG261 for *glmY*. T7 RNA polymerase recognition sequences were introduced into the PCR fragment by the reverse primer. For Northern blot analysis of *glmS* mRNA, 5 µg of total RNA was separated by formaldehyde agarose gelelectrophoresis. The RNA was then transferred to a positively charged nylon membrane (Roche Diagnostics, Germany) using the VacuGene XL vacuum blotting system (Amersham Biosciences, USA) following the manufacturer's protocol. For Northern blot analysis of GlmY and GlmZ, 5 µg of total RNA was separated on 7 M urea/TBE/8% polyacrylamide gels and subsequently transferred to the nylon membrane by electroblotting in 0.5x TBE at 15 V for 1 h. Probe hybridization and detection were carried out according to the supplier's instruction (DIG RNA Labelling kit, Roche Diagnostics, Germany).

Determination of GlmY and GlmZ half-lifes

To measure sRNA half-lifes, transcription initiation was inhibited by adding rifampicin to exponentially growing cells to a final concentration of 500 µg/ml (time 0). 10 ml aliquots of the culture were harvested at suited time intervals

and rapidly mixed with an equal volume of ethanol pre-equilibrated at -70℃. Total RNAs were extracted as described previously (Hajnsdorf *et al.*, 1994). Five μg were loaded on a high resolution 6% denaturing polyacrylamide gel, electrotransferred and hybridized with ^{32}P-labeled RNA probes as described previously (Hajnsdorf *et al.*, 1994). To normalize the data, the same membrane was subsequently hybridized with a ^{32}P-labeled 5S rRNA specific probe (5'-ACTACCATCGGCGCTACGGC). The signals were detected and quantified using a PhosphoImager.

3'RACE analysis of GlmY 3' ends

Total RNA was prepared as described (Hajnsdorf *et al.*, 1994) and 2.5 μg were ligated with 100 pmol oligonucleotide RIBOLI using 20 units T4 RNA ligase (Promega) in a reaction buffer containing 12.5 mM ATP, 50 mM HEPES pH 7.5, 20 mM $MgCl_2$, 3.3 mM DTT, 0.01 μg/ul BSA and 10% DMSO (Donis-Keller, 1979). After precipitation with ethanol, the pellet was re-suspended in 20 μl water. Five μl of this solution was annealed to 100 pmol oligonucleotide DEOXYLI in 10 μl 50 mM Tris-HCl (pH 8.5), 8 mM $MgCl_2$, 30 mM KCl, 100 mM DTT. Synthesis of cDNA was performed by incubating the annealing mix with 10 units of AMV reverse transcriptase and 100 mM dNTPs at 42 ℃ for 1 h. After addition of 100 pmol primer Pforw, the entire cDNA reaction was subjected to PCR amplification. After digestion of the PCR fragments at the PstI and BamHI-sites introduced by the primers, the DNA fragments were cloned into the vector pT3T718U (Pharmacia) digested with the same enzymes. After isolation of recombinant clones, the inserts were amplified using vector-specific primers and the PCR fragments were sequenced.

Results

YhbJ affects the amount of the small RNA GlmY

The localization of *yhbJ* in the *rpoN* operon and its high degree of conservation in the genomes of *proteobacteria* (Boël *et al.*, 2003) raised the possibility that YhbJ might have a global function and that it could also be a regulator of sRNAs other than GlmZ. Therefore, we tested in Northern

experiments whether a *yhbJ* mutation would also affect other candidate sRNAs known to be expressed in *E. coli* (Argaman *et al.*, 2001; Wassarman *et al.*, 2001). These experiments revealed that the sRNA GlmY is less prevalent in the *yhbJ* mutant in comparison to the wild-type strain whereas other tested sRNAs were unaffected (our unpublished results). To investigate the fate of GlmY in more detail, we isolated total RNAs of the wild-type strain and the *yhbJ* mutant at different time points during growth and analyzed them in Northern experiments using probes specific for *glmS*, GlmZ and GlmY, respectively. In the wild-type strain, both forms of the sRNA GlmZ were detectable and almost no *glmS* transcript accumulated (Fig. 10, first and second panel, respectively). In contrast, in the *yhbJ* mutant processing of GlmZ was prevented resulting in accumulation of full-length GlmZ and concomitantly in the accumulation of *glmS* mRNA, which is in perfect agreement with previous results (Kalamorz *et al.*, 2007).

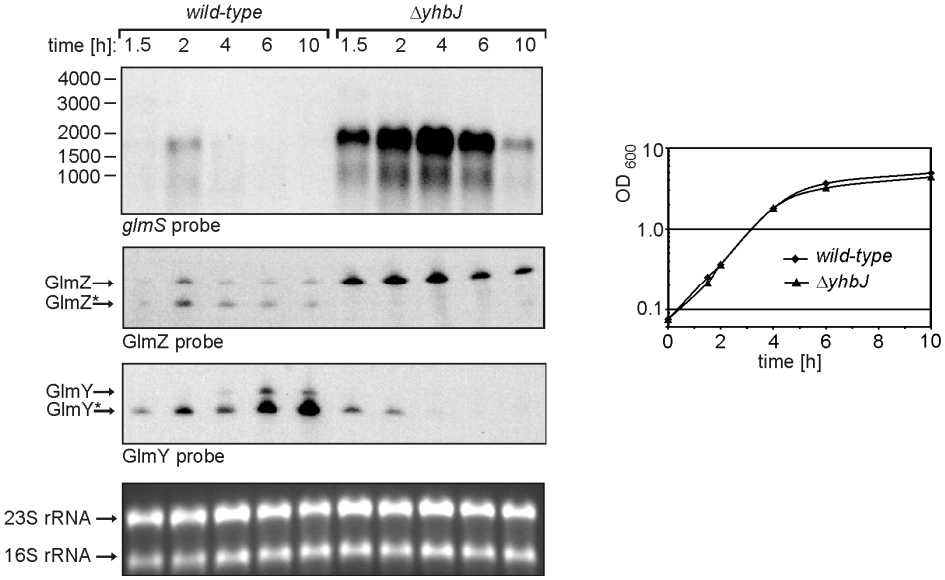

Figure 10: YhbJ has opposite effects on the amounts of GlmZ and GlmY. Northern blot analysis of RNA samples collected at various time points during growth of strains R1279 (wild-type) and Z37 (Δ*yhbJ*). The corresponding growth curves are shown at the top. The RNAs were hybridized with probes specific for *glmS* (first panel), for GlmZ (second panel) and for GlmY (third panel). The ethidium-bromide stained gel is shown as loading control at the bottom. The shorter variants of GlmZ and GlmY are designated with an asterisk (throughout this study). The sizes of the molecular weight marker (in kb) are given at the left (first panel).

Interestingly, GlmY behaved very different from GlmZ: in the wild-type strain the short variant of GlmY (subsequently designated GlmY* in this report) was detectable at all time-points and accumulated when the cells entered the

stationary growth phase as observed previously (Vogel *et al.*, 2003), whereas in the *yhbJ* mutant GlmY* was exclusively detectable in the early exponential growth phase (Fig. 10, third panel). Full-length GlmY was present in much lower amounts in the wild-type, and only detectable in stationary phase, whereas in the *yhbJ* mutant it was not detectable at all. In conclusion, it appears that a *yhbJ* mutation has opposite effects on the two sRNAs: Whereas GlmZ is stabilized in its full-length form, GlmY becomes destabilized.

GlmY is dispensable for the GlmZ-mediated activation of *glmS* expression

Our data suggest that YhbJ controls the cellular amounts of the sRNAs GlmZ and GlmY. Therefore, we asked whether GlmY would also have a role in the GlmZ-mediated control of *glmS* expression. To address this question, we deleted the *glmY* gene and combined this mutation with Δ*glmZ* and/or Δ*yhbJ* mutations.

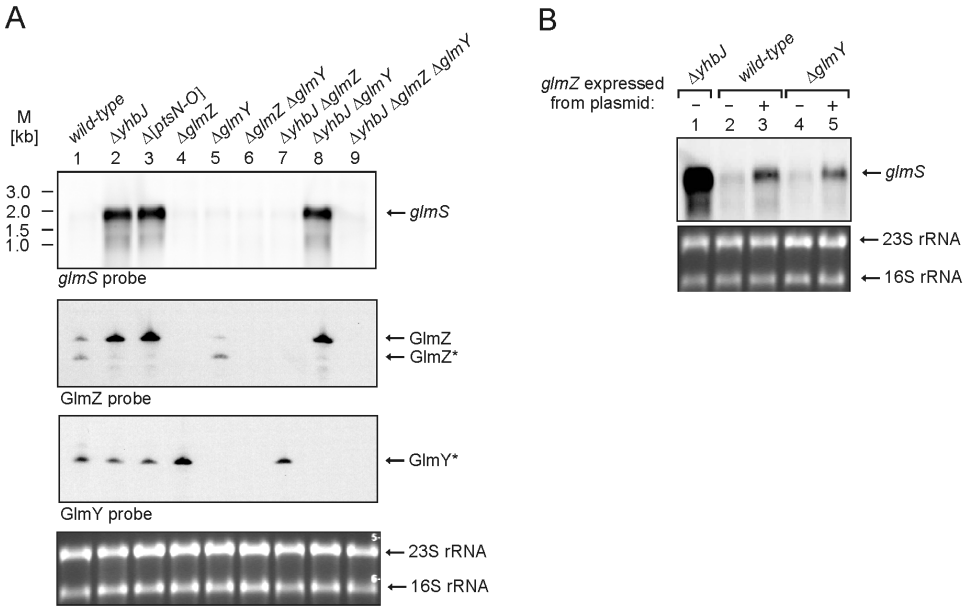

Figure 11: Activation of *glmS* expression by GlmZ is independent of GlmY. A. Northern blot analyses to determine the effects of Δ*yhbJ*, Δ*glmZ* and Δ*glmY* mutations, alone or in various combinations, on the *glmS*, GlmY and GlmZ transcript levels. Total RNAs of strains R1279 (lane 1), Z37 (lane 2), R2413 (lane 3), Z44 (lane 4), Z95 (lane 5), Z105 (lane 6), Z116 (lane 7), Z115 (lane 8) and Z107 (lane 9) were hybridized with a *glmS* specific probe (top panel), a GlmZ specific probe (medium panel) and a GlmY specific probe (bottom panel). The relevant genotypes are given at the top. **B.** Northern blot experiment to determine the effect of GlmZ overexpression on *glmS* transcript levels. Strains R1279 (wild-type) and Z96 (Δ*glmY*) were transformed with pBAD30 (empty vector; lanes 2 and 4) or pBGG84 (*glmZ* on pBAD30, lanes 3 and 5) and total RNA was isolated from arabinose-induced cultures and hybridized with a *glmS* probe. The untransformed Δ*yhbJ* mutant served as control (lane 1).

The resulting strains were grown to exponential phase and total RNAs were prepared and subsequently probed in Northern experiments for the *glmS*, GlmZ and GlmY RNAs, respectively (Fig. 11 A). As already shown before, the *glmS* transcript as well as full-length GlmZ strongly accumulate in *yhbJ* mutants, whereas the amount of GlmY* decreases in comparison to the wild-type (Fig. 11 A, lanes 1-3). As expected from previous data (Kalamorz *et al.*, 2007), the accumulation of the *glmS* transcript was abolished in the Δ*glmZ* Δ*yhbJ* double mutant (Fig. 11 A, top panel, lane 7), demonstrating once again that up-regulation of *glmS* in the *yhbJ* mutant relies on GlmZ. In contrast, the Δ*yhbJ* Δ*glmY* double mutant still overproduced the *glmS* transcript whereas it was undetectable in the Δ*yhbJ* Δ*glmZ* Δ*glmY* triple mutant (Fig. 11 A, top panel, lanes 8 and 9). In addition, in the Δ*yhbJ* Δ*glmY* double mutant, full-length GlmZ accumulated like in the Δ*yhbJ* single mutant (Fig. 11 A, medium panel, lanes 2 and 8). Furthermore, no prominent differences in *glmS* and GlmZ RNA amounts were detectable between the wild-type and the Δ*glmY* mutant (Fig. 11 A, compare lanes 5 and 1). Taken together, a Δ*glmY* mutation appears to have no effect on *glmS*- and GlmZ-levels, neither in the wild type nor in the *yhbJ* mutant. These results suggested that GlmY is dispensable for the GlmZ dependent activation of *glmS* expression, at least in this mutant background. To see, whether this is also the case in *yhbJ*$^+$ strains, we tested the effects of GlmZ overexpression in the wild-type strain and the Δ*glmY* mutant. We have shown before that GlmZ overexpression activates *glmS* expression to some extent even in the wild-type suggesting that GlmZ overproduction is able to partially overcome the negative effect exerted by YhbJ (Kalamorz *et al.*, 2007). To see, whether this is also the case in a Δ*glmY* mutant, we introduced a plasmid carrying *glmZ* downstream of the arabinose-inducible P_{BAD} promoter into the wild-type strain and the Δ*glmY* mutant. Transformants carrying the empty expression vector pBAD30 served as controls. The cells were grown in the presence of arabinose and total RNAs were extracted and subsequently analyzed in a Northern experiment using a probe directed against *glmS*. As expected, in the wild-type strain the presence of the GlmZ overproduction construct caused accumulation of the *glmS* mRNA whereas the empty expression vector had no effect (Fig. 11 B, lanes 2

and 3). The virtually same result was obtained when the Δ*glmY* mutant was tested (Fig. 11 B, lanes 4 and 5). This result clearly demonstrates that GlmZ *per se* does not require the presence of GlmY for the activation of *glmS* expression, which suggests that base-pairing between GlmZ and *glmS* mRNA does not depend on GlmY.

Overexpression of GlmY induces *glmS* expression in a GlmZ-dependent manner

Next, we investigated the effect of GlmY overexpression. For this purpose, the *glmY* gene was cloned on a plasmid under control of the strong constitutively active λP_L promoter. The resulting plasmid was introduced into the wild-type strain that carried a *glmS'-lacZ* reporter fusion expressed from a constitutive promoter on the chromosome (Kalamorz *et al.*, 2007). This fusion is perfectly regulated by GlmZ and YhbJ.

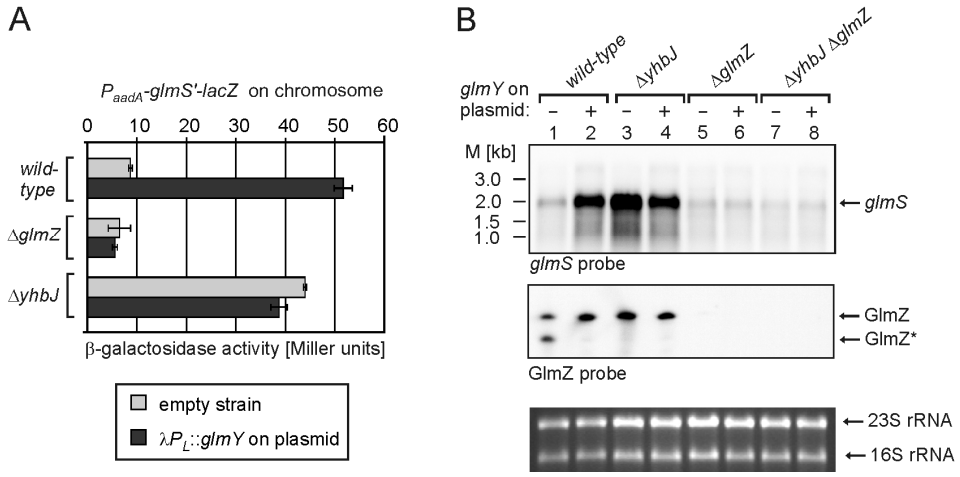

Figure 12: GlmY requires GlmZ for the activation of *glmS* expression. A. Overexpression of *glmY* induces expression of the *glmS'-lacZ* reporter fusion in the wild-type but not in the Δ*glmZ* mutant. Strains Z8 (wild-type), Z38 (Δ*glmZ*) and Z28 (Δ*yhbJ*) were grown in the absence (grey bars) or presence of the *glmY* overproducing plasmid pBGG149 (black bars) and the β-galactosidase activities were determined. **B.** Northern blot analysis of *glmS* and GlmZ RNAs in strains overproducing GlmY. Total RNAs were isolated from strains R1279 (wild-type), Z37 (Δ*yhbJ*), Z45 (Δ*glmZ*) and Z116 (Δ*yhbJ*, Δ*glmZ*), which were either untransformed (lanes 1, 3, 5 and 7) or transformed with plasmid pBGG149 overproducing GlmY (lanes 2, 4, 6 and 8). The RNAs were hybridized with a *glmS* probe (upper panel) and a GlmZ probe (second panel).

The presence of the *glmY* expression plasmid led to induction of *glmS'-lacZ* expression (Fig. 12 A), whereas no increase in β-galactosidase activity was detectable when the empty expression vector was present (data not shown). To confirm these results we performed Northern experiments using probes

specific for *glmS* and GlmZ. In the wild-type strain, overexpression of *glmY* caused the strong accumulation of the *glmS* transcript and concomitantly of full-length GlmZ sRNA (Fig. 12 B, lanes 1 and 2). Hence, it can be concluded that GlmY positively regulates the *glmS* mRNA, which is in agreement with a recent publication demonstrating that GlmY overexpression causes overproduction of GlmS protein (Urban *et al.*, 2007). Our additional observation that GlmY overproduction stabilizes full-length GlmZ, raises the possibility that GlmY acts on *glmS* indirectly via GlmZ. To test this idea, we repeated the experiments described above in Δ*glmZ* and Δ*glmZ* Δ*yhbJ* mutants. In these strains, GlmY overproduction had no stimulatory effect, neither on expression of the *glmS'-lacZ* reporter fusion (Fig. 12 A) nor on the *glmS* transcript level as detected by Northern analysis (Fig. 12 B, lanes 5-8). Next, we tested the effect of GlmY overexpression in the *yhbJ* mutant. In this strain *glmS* strongly accumulates and the *glmS'-lacZ* reporter fusion is highly expressed (Fig. 12 A and Fig 12 B, lane 3). Additional overexpression of the *glmY* construct, however, had no additive effect on the GlmZ and *glmS* RNA levels (Fig. 12 B, lanes 3 and 4) and on the expression of the *glmS'-lacZ* fusion (Fig. 12 A).

So far, our data show that a high cellular amount of GlmY induces *glmS* expression in a process that depends on GlmZ, whereas GlmZ can positively regulate the *glmS* mRNA independently from GlmY. Hence, GlmY acts upstream and may act in concert with YhbJ to regulate GlmZ, which in turn targets the *glmS* mRNA.

GlmY receives and transmits the GlcN-6-P signal to *glmS* via GlmZ

We have recently shown, that the sRNA GlmZ mediates the feedback control of *glmS* expression by GlcN-6-P. When the intracellular GlcN-6-P concentration decreases, full-length GlmZ accumulates and activates *glmS* expression (Kalamorz *et al.*, 2007). Our results above demonstrate that GlmY acts upstream of GlmZ in the activation of *glmS* mRNA. This raised the possibility that GlmY receives the GlcN-6-P signal and relays it to GlmZ, which then stimulates *glmS* expression. To address this question, we used Nva-FMDP, a derivative of N3-(4-methoxyfumaroyl)-L-2,3-diaminopropanoic acid.

This compound selectively inhibits GlmS enzymatic activity *in vivo* (Marshall *et al.*, 2003) and thereby causes a decrease in the intracellular GlcN-6-P concentration, which leads to induction of *glmS* expression via accumulation of full-length GlmZ (Kalamorz *et al.*, 2007). To see whether the intracellular GlcN-6-P concentration also affects the amount of GlmY present in the cell, the wild-type strain was grown to exponential phase and after splitting of the culture, growth was continued in either the absence or presence of Nva-FMDP. Subsequently, cells were harvested at three different time-points (30 min, 1 h and 2h) and total RNAs were isolated and subjected to Northern analyses using probes directed against *glmS*, GlmZ and GlmY.

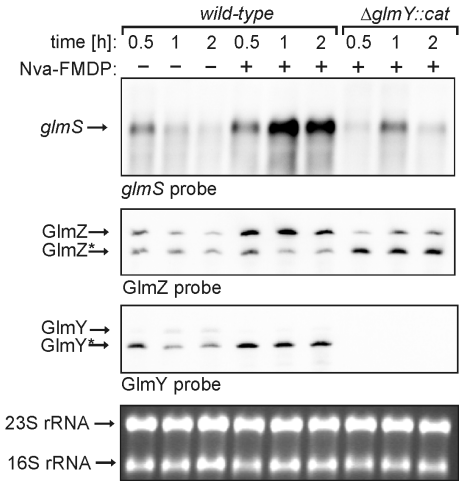

Figure 13: GlmY is essential for transduction of the GlcN-6-P signal to *glmS* and is itself regulated by GlcN-6-P. Northern blotting experiments to determine the effect of the inhibitor of GlmS enzymatic activity, Nva-FMDP, on the *glmS* transcript (*glmS* probe; top), on GlmZ (GlmZ probe; second panel) and on GlmY (GlmY probe; third panel) in strains R1279 (wild-type) and Z95 (Δ*glmY::cat*). Samples were harvested at the time indicated after addition of Nva-FMDP.

As expected from previous data (Kalamorz *et al.*, 2007), Nva-FMDP caused the accumulation of *glmS* transcript and simultaneously of full-length GlmZ (Fig. 13, first and second panel, compare lanes 1-3 with lanes 4-6). Intriguingly, presence of Nva-FMDP also caused the accumulation of GlmY* (Fig. 13, third panel), demonstrating that the GlmY* amount in the cell is controlled by GlcN-6-P. To test whether the accumulation of GlmZ and *glmS* RNAs upon depletion of GlcN-6-P is the direct consequence of accumulation of GlmY, we repeated the experiment using a Δ*glmY* mutant. Indeed, Nva-FMDP had no large effect in this mutant, i.e. accumulation of full-length GlmZ and up-regulation of *glmS* mRNA was abolished (Fig. 13, first and second

panel, lanes 7-9). However, it appears that the processed form of GlmZ was present in a somewhat higher amount in the Nva-FMDP treated Δ*glmY* mutant in comparison to the other conditions (Fig. 13, second panel). The reason for this phenomenon remains to be determined. In sum, the data show that GlcN-6-P controls *glmS* expression by regulating the amount of GlmY, which subsequently transmits the signal via GlmZ to *glmS* mRNA.

Mutation of poly (A) polymerase PAP-I increases the stabilities of both GlmZ and GlmY sRNAs

Mutation of *pcnB* encoding poly (A) polymerase PAP-I leads to strong accumulation of *glmS* mRNA and hence to overproduction of GlmS (Joanny *et al.*, 2007). In addition, full-length GlmZ accumulates in a *pcnB* mutant (Kalamorz *et al.*, 2007) suggesting that PAP-I affects a factor upstream in the signaling cascade controlling *glmS* expression rather than the *glmS* mRNA itself. To find out which of the known factors governing *glmS* mRNA accumulation is controlled by PAP-I, we analyzed the fates of *glmS*, GlmZ and GlmY RNAs. For this purpose, we isolated total RNAs of the *pcnB* mutant and the wild-type at different time points during growth and analyzed them in Northern experiments using probes specific for the various RNAs.

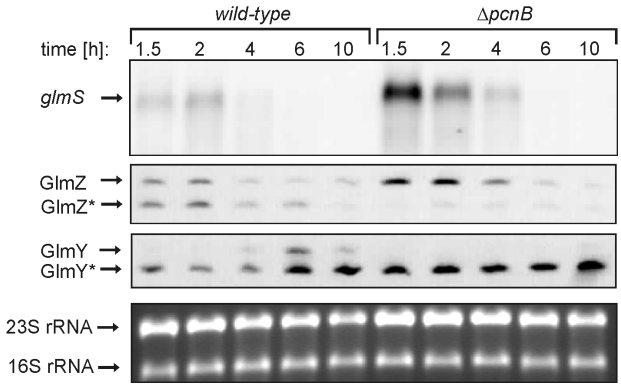

Figure 14: Mutation of *pcnB* results in accumulation of *glmS*, full-length GlmZ and GlmY* RNAs. Northern blot analysis of RNA samples collected at various times during growth of strains R1279 (wild-type) and Z129 (Δ*pcnB*). Specific RNAs were detected using probes directed against *glmS* (upper panel), GlmZ (second panel) and GlmY (third panel).

These experiments revealed that in the Δ*pcnB* mutant *glmS* mRNA and concomitantly full-length GlmZ strongly accumulate in the exponential growth phase (Fig. 14, top and medium panels). The shorter form of GlmZ was hardly

detectable in the Δ*pcnB* mutant, suggesting that GlmZ processing is affected. In parallel, much higher amounts of GlmY* were detectable in the Δ*pcnB* mutant in comparison to the wild-type strain (Fig. 14, third panel). When cells entered stationary phase the amounts of *glmS* mRNA and GlmZ drastically decreased both in the wild-type as well as in the Δ*pcnB* strain. This suggests superimposition of a negative control mechanism down-regulating GlmZ and therefore *glmS* during this growth phase, regardless of the activity of PAP-I.
The higher amounts of GlmY* and full-length GlmZ detectable in the Δ*pcnB* mutant during exponential growth could either mean that these sRNAs are stabilized in the Δ*pcnB* mutant or alternatively that their expression level is altered. To discriminate between these possibilities, we determined the half-lifes of GlmZ and GlmY in the Δ*pcnB* mutant and the wild-type strain, respectively. To this end, these strains were grown to exponential phase and total RNAs were prepared from samples harvested at different time-points following the addition of Rifampicin and analyzed in Northern experiments using high resolution acrylamide gels.

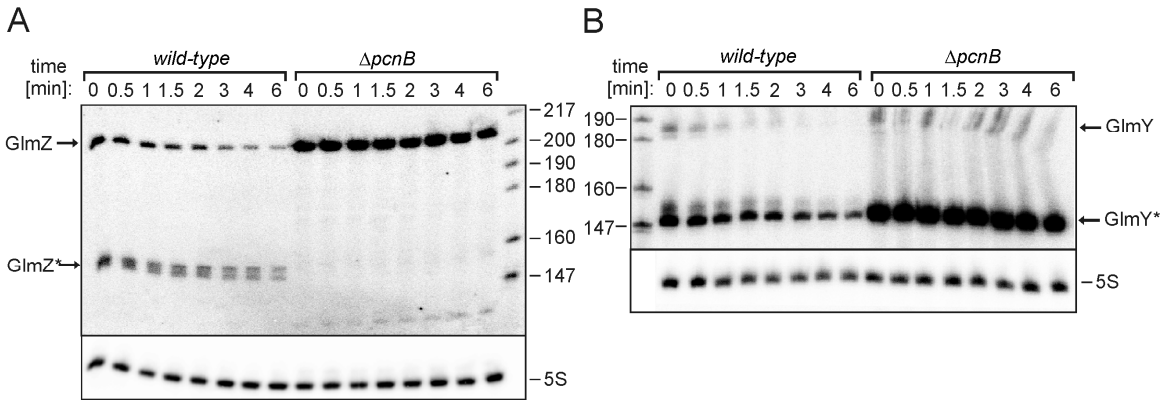

Figure 15: Mutation of *pcnB* strongly increases the half-lives of full-length GlmZ and GlmY*. Strains N3433 (wild-type) and IBPC903 (Δ*pcnB*) were treated with rifampicin for the inhibition of transcription initiation and subsequently samples were harvested at the indicated times and the total RNAs were isolated. The RNAs were analysed by Northern blotting using probes specific for GlmZ (A, top panel), GlmY (B, top panel) and 5S rRNA (bottom panels in A and B).

These experiments showed that the half-lifes of both GlmZ and GlmY were dramatically increased in the Δ*pcnB* background. Quantification and normalization of the signal intensities relative to the 5S rRNA signal revealed a half-life of 1.7 min +/- 0.1 min of full-length GlmZ in the wild-type strain (Fig. 15 A), which corresponds well with the previously reported half-life of ~2 min

for GlmZ observed in wild-type cells during the exponential phase (Vogel *et al.*, 2003). In contrast, in the Δ*pcnB* mutant the half-life of GlmZ increased to of 20.2 +/- 0.1 min (Fig. 15 A). The shorter GlmZ* species was not detectable in the Δ*pcnB* mutant confirming that processing of GlmZ is inhibited in the absence of PAP I. In contrast, a new shorter and low abundant GlmZ variant appeared in the Δ*pcnB* mutant and its amount slightly increased with time. Similarly to GlmZ, GlmY* was highly stabilized in the Δ*pcnB* mutant (Fig 15 B). In this case, the half-life increased from 1.4 min +/- 0.1 min in the wild-type to 6.7 min +/- 0.1 min in the *pcnB* mutant. Interestingly, in the wild-type strain but not in the Δ*pcnB* mutant, a smear of slightly larger transcripts running above GlmY* in the gel was detectable. Such a size heterogeneity could be caused by the presence of poly(A) tails of different length in GlmY, as also previously observed for another sRNA subject to polyadenylation (Viegas *et al.*, 2007). No such smear could be observed for GlmZ (Fig. 15 A). In sum, the data demonstrate that both GlmZ and GlmY* are stabilized by a *pcnB* mutation.

Poly (A) polymerase PAP-I polyadenylates and destabilizes the sRNA GlmY and thereby indirectly controls the GlmZ and *glmS* mRNA levels

Our data showed that the amount of GlmY positively controls the amount of full-length GlmZ and thereby up-regulates g*lmS*. Hence, the stabilization of the GlmZ and *glmS* RNAs in the *pcnB* mutant could be the indirect consequence of GlmY* stabilization alone. To test this idea, we compared the GlmZ and *glmS* amounts present in Δ*pcnB* and Δ*pcnB* Δ*glmY* mutants. Total RNAs were isolated from samples harvested at different time points during growth and analyzed in Northern experiments.

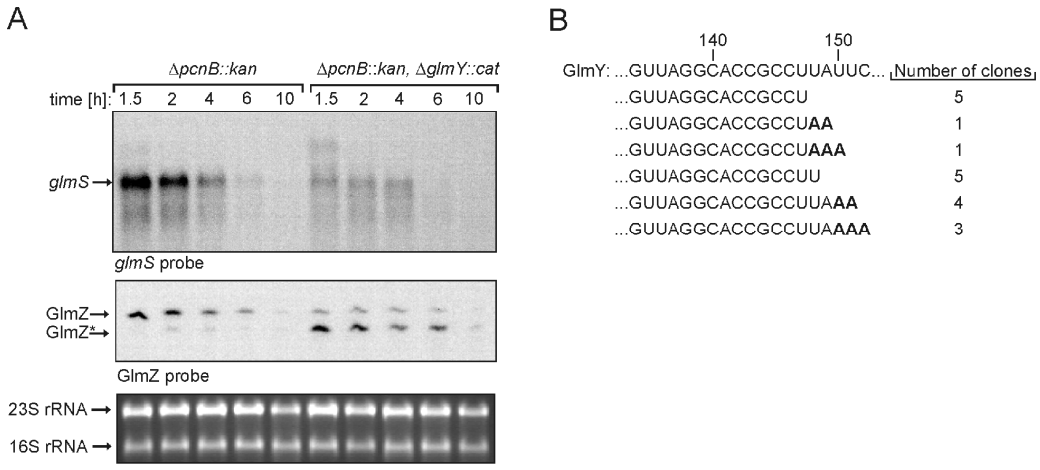

Figure 16: PAP I polyadenylates GlmY* and thereby indirectly destabilizes the *glmS* and GlmZ RNAs. A. Northern blot analysis of total RNA samples collected at various times during growth of strains Z129 (Δ*pcnB*) and Z152 (Δ*pcnB,* Δ*glmY*). The *glmS* (top panel) and GlmZ (second panel) RNAs were detected using specific probes, respectively. **B.** 3'RACE analysis of GlmY 3' ends in the wild-type. Total RNA of wild type strain R1279 was subjected to 3' RACE analysis. The obtained sequences and the frequency of their occurrence are shown. Adenosine residues unequivocally added by PAP I are depicted in bold.

These experiments showed that a *glmY* mutation prevents the accumulation of the *glmS* and full-length GlmZ RNAs in *pcnB* mutants (Fig. 16 A). In the Δ*pcnB* Δ*glmY* double mutant the *glmS* and GlmZ RNA amounts and patterns were very similar to those detectable in the wild-type strain (Fig. 14). This shows that GlmY is the target of PAP I and that the effects on GlmZ and *glmS* are indirect and the consequence of modulation of GlmY amounts. To obtain direct evidence that GlmY is polyadenylated by PAP I, we applied a 3'-RACE approach (Le Derout *et al.*, 2003) that allows to selectively amplify GlmY 3' ends by PCR and to determine their sequences after cloning. Of the altogether 19 clones analyzed, all corresponded to the shorter GlmY* variant encompassing 147, 148 or 149 nt of the *glmY* sequence (Fig. 16 B). Nine clones harbored at the 3' end short extensions of two or three A residues, which are added post-transcriptionally (Fig. 16 B). This result suggests that about half of the shorter GlmY* species are polyadenylated by PAP-I *in vivo*.

Discussion

In *E. coli*, the *glmS* gene encoding GlcN-6-P synthase, a central metabolic enzyme required for the synthesis of bacterial peptidoglycan, is subject to post-transcriptional regulation by the two small RNAs GlmY and GlmZ.

Overexpression of either of these sRNAs stabilizes the *glmS* monocistronic transcript and results in overproduction of GlmS protein ((Kalamorz *et al.*, 2007; Urban *et al.*, 2007); this work).

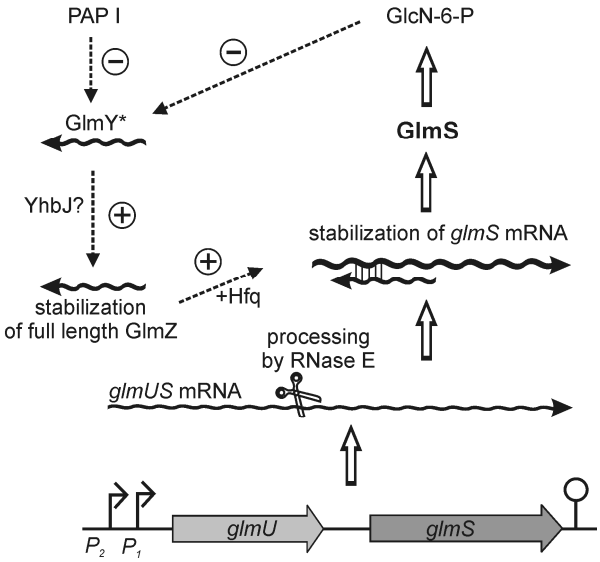

Figure 17: Model for the regulation of *glmS* expression by GlcN-6-P, PAP I, GlmY and GlmZ. PAP I polyadenylates and thereby destabilizes the sRNA GlmY. When the intracellular GlcN-6-P concentration drops, the short form of GlmY accumulates, which leads to stabilization of the full-length form of the sRNA GlmZ. Similarly, diminished PAP I activity causes accumulation of GlmY. Presumably, GlmY acts in concert with protein YhbJ to modulate processing of GlmZ by a still unknown mechanism. The accumulation of full-length GlmZ in turn stabilizes the *glmS* transcript that derives from processing of the *glmUS* primary transcripts by RNase E. GlmZ presumably base-pairs with the *glmS* mRNA, which may be assisted by Hfq.

In this work, we show that there is a hierarchical interdependence between the two sRNAs in the control of *glmS* expression: GlmY requires the presence of GlmZ to activate *glmS* expression. In contrast, GlmZ can activate *glmS* expression autonomously and does not require GlmY. Thirdly, a high cellular amount of GlmY prevents processing of GlmZ leading to accumulation of its full-length form. These findings suggest that GlmY acts indirectly on *glmS* by modulating the cellular amount of full-length GlmZ. Hence, unlike GlmZ, GlmY may not base-pair with the *glmS* mRNA, but act upstream of GlmZ in the signal cascade controlling *glmS* expression (see model in Fig. 17). Furthermore, we show that GlmY is also part of the GlcN-6-P dependent signaling cascade controlling *glmS* expression. In the wild-type, a decrease of the intracellular GlcN-6-P concentration causes accumulation of GlmY and concomitantly of full-length GlmZ and *glmS* mRNA. In a *glmY* mutant GlcN-6-P has no such effect: Full-length GlmZ and *glmS* mRNA do not anymore

accumulate. This suggests that GlcN-6-P controls *glmS* indirectly, via the GlmY-GlmZ signal cascade: Depletion of the GlcN-6-P level causes accumulation of GlmY, which stabilizes full-length GlmZ that finally activates *glmS* expression (Fig. 17). GlmY is conserved in the genomes of several *Enterobacteriaceae* (Urban *et al.*, 2007). As judged from blast analyses, all bacteria that possess the *glmY* gene also contain *glmZ* (data not shown). This suggests that these two sRNAs constitute an evolutionary conserved regulatory module.

How does GlmY control processing of GlmZ? One possibility is that GlmY negatively controls expression of a factor required for GlmZ processing. Our data obtained so far suggest that the function of GlmY may involve YhbJ, a putative RNA-binding protein encoded in the *rpoN* operon. In the *yhbJ* mutant, neither the absence nor the overproduction of GlmY had any effect on the already high amounts of full-length GlmZ and *glmS* mRNA (Figs. 11 and 12). This suggests that GlmY acts upstream or in concert with YhbJ in the same pathway to regulate GlmZ (Fig. 17). Hence, it is conceivable that GlmY controls the cellular amount of YhbJ, which in turn governs processing of GlmZ. However, so far our experiments did not detect any differences in *yhbJ* expression levels in *glmY* mutants or over-expressing strains (data not shown). In an alternative scenario, YhbJ may directly bind the sRNAs. Binding of GlmY could out-compete binding of GlmZ, which would automatically cause its accumulation in the active full-length form. Binding of GlmY by a protein like YhbJ would presumably also alter its accessibility to degrading RNAses like PNPase (see below) and could therefore explain the low GlmY amount present in *yhbJ* mutants (Fig. 10 and 11). Binding by a specific protein would require some similarities on the sequence and/or structural level of the two sRNAs. Interestingly, GlmY shares 63 % sequence identity with GlmZ and software analysis predicts strikingly similar overall secondary structures for both sRNAs (Fig. S4 in supplementary material). The structures consist of two large imperfect stem loops and an additional terminator stem loop at the 3' end. In addition, the second stem loop carries a characteristic pear-shaped bulge. A sequence alignment of GlmY and GlmZ sRNAs from 11 different species reveals a high degree of sequence identity in the 5' parts of the molecules preceding the processing sites (Fig. S4). This homology does not

extend into the putative base-pairing region within GlmZ. Taken together GlmY and GlmZ appear to be homologous sRNAs.

The clarification of the relationship between GlmY and GlmZ in the activation of *glmS* expression allowed us to address the role of PAP I in this regulatory circuit. In mutants defective for PAP I the GlmS protein strongly and specifically accumulates as a result of the accumulation of *glmS* mRNA (Joanny *et al.*, 2007). This drastic effect suggested a specific role for PAP I in *glmS* gene regulation. In this work, we show that PAP I exerts its destabilizing effect on *glmS* indirectly, by controlling the stability of sRNA GlmY: PAP I polyadenylates GlmY* and destabilizes it thereby. In PAP I mutants GlmY* accumulates, which induces accumulation of full-length GlmZ and *glmS*. The inactivation of PAP I has no effect in *glmY* mutants, demonstrating that PAP I acts exclusively via GlmY on *glmS* expression (Fig. 17).

As a result of 3' processing GlmY is present in two forms in wild-type strains, of which the shorter form GlmY* is much more abundant ((Vogel *et al.*, 2003); this work). It is this shorter variant that accumulates in *pcnB* mutants (Fig. 14) and that we detected as polyadenylated species in the 3'RACE experiments (Fig. 16 B). Therefore, it can be concluded that the shorter GlmY* variant is responsible for stabilization of full-length GlmZ, which causes activation of *glmS* expression. The 3' tail following the GlmY processing site should have no role in this process. Indeed, in close relatives of *E. coli*, the sequence of *glmY* corresponding to the shorter GlmY* variant is highly conserved, whereas the sequence downstream of the processing site is not. This is further supported by the finding that heterologous GlmY from *Erwinia carotovora* is able to activate expression of *E. coli glmS*, although the sequence of its 3' tail is completely different from that of *E. coli* GlmY (Urban *et al.*, 2007).

It is an accepted model that PAP I preferably polyadenylates RNA molecules that bear a 5'-monophosphate and a secondary structure at the 3' end and that may result from a preceding endonucleolytic processing event. Polyadenylation is thought to provide a toehold for RNAses like polynucleotide phosphorylase (PNPase) and RNAse R and may help them to get through the 3' secondary structures (Kushner, 2007; Condon, 2007). According to software analysis processed GlmY* carries an extensive secondary structure

at the 3' end, followed by only four or five unpaired nucleotides ((Urban *et al.*, 2007), (Fig. 16 B and Fig. S4 in supplementary material)). This stretch is presumably too short to make GlmY* accessible for subsequently degrading RNases and polyadenylation may overcome this barrier. In many cases PNPase is responsible for the degradation of polyadenylated RNAs and our further data show that GlmY also accumulates in PNPase mutants (data not shown). Hence, it is conclusive that polyadenylation makes GlmY more accessible for PNPase which subsequently degrades it to shorter oligoribonucleotides. It has been suggested that the Hfq protein may facilitate polyadenylation of RNAs by PAP I (Hajnsdorf and Regnier, 2000). However, mutation of *hfq* has no effect on GlmY amounts present in the cell (data not shown), making it unlikely that Hfq contributes to GlmY decay.

The way by which PAP I regulates activity of GlmY, a regulatory RNA, is not unprecedented. RNA I, the regulatory RNA that represses replication of ColEI-type plasmids is stabilized 10-fold in *pcnB* mutants. The form of RNA I that accumulates in *pcnB* mutants and which is active in repression has undergone a processing event that normally initiates RNA I decay by the PAP I/PNPase pathway (Xu *et al.*, 1993; He *et al.*, 1993b). Similar observations have been reported for CopA RNA regulating plasmid R1 replication and the Sok antisense RNA from plasmid R1 that inhibits translation of the *hok* mRNA (Xu and Cohen, 1995; Dam Mikkelsen and Gerdes, 1997; Soderbom *et al.*, 1997). Recently, the turnover of SraL, a small RNA of unknown function, has been reported to be regulated by PAP I (Viegas *et al.*, 2007). Half-life is a critical parameter for the function of regulatory RNAs since their activities unlike that of protein regulators usually cannot be reversibly switched on/off with the help of co-factors (Levine *et al.*, 2007). Therefore, to function appropriately, it is necessary that trans-encoded regulatory RNAs are consumed upon action (Masse *et al.*, 2003) or rapidly degraded. Taken together, it appears that another major domain of PAP I is the control of turnover of certain regulatory RNAs, which may provide the prerequisite for switching their amounts and thereby their activities in the cell.

Acknowledgments

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Supplementary material

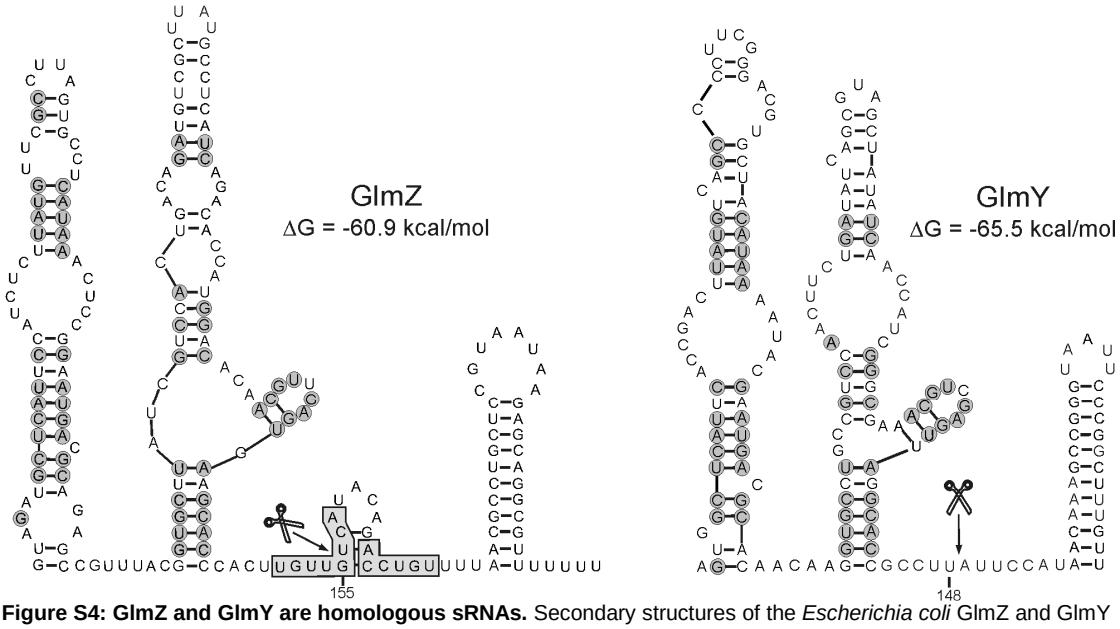

Figure S4: GlmZ and GlmY are homologous sRNAs. Secondary structures of the *Escherichia coli* GlmZ and GlmY sRNAs predicted by Mfold (Zuker, 2003). Residues, which are fully conserved in the alignment of GlmZ and GlmY sequences from 11 different bacterial species, are highlighted with grey circles. The region in GlmZ that putatively base-pairs with *glmS* is boxed. Scissors indicate the processing sites within GlmZ and GlmY. For the multiple sequence alignment (not shown) the *glmY* and *glmZ* sequences of the following species were used (accession numbers are in parentheses): *E. coli* K-12 (NC_000913), *Shigella flexneri* 5 str. 8401 (NC_008258), *Citrobacter koseri* ATCC BAA-895 (NC_009792), *Salmonella enterica* subsp. enterica serovar typhi str. CT18 (NC_003198), *Salmonella typhimurium* LT2 (NC_003197), *Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 (NC_009648), *Enterobacter sakazakii* ATCC BAA-894 (NC_009778), *Erwinia carotovora* subsp. atroseptica (NC_004547), *Photorabdus luminescens* subsp. laumondii (NC_005126), *Serratia marcescens* Db11 [http://www.sanger.ac.uk], *Yersinia pestis* CO92 (NC_003243). The alignment was compiled with Vector NTI Suite 9.0.

4. Dual control by perfectly overlapping σ^{54}- and σ^{70}-promoters adjusts small RNA GlmY expression to different environmental signals

The results described in this chapter have been submitted for publication to Mol. Microbiol..

Author contributions:

This study was designed by B.R. and B.G.. B.R. performed all 5'RACE analyses, northern blotting experiments and the *in vitro* transcription. Y.G. performed protein purification, EMSA's and constructed plasmids and strains for and performed β-galactosidase measurements with fusions bearing mutated activator binding sites. Y.G. performed the experiments under the supervision of B.R. during her internship. All other strains and plasmids were constructed by B.R. B.R. performed all other β-galactosidase measurements, partially with assistance by Sabine Lentes. Alignment analyses were performed by B.R.. The paper was written by B.R. and B.G..

Summary

In *Escherichia coli* synthesis of glucosamine-6-phosphate synthase GlmS is feedback-controlled by a regulatory cascade composed of small RNAs GlmY and GlmZ. When GlcN6P becomes limiting, GlmY accumulates and inhibits processing of GlmZ. Full-length GlmZ base-pairs with the *glmS* transcript and activates synthesis of GlmS, which re-synthesizes GlcN6P. Here we show that *glmY* expression is controlled by two overlapping promoters with the same transcription start site. A σ^{70}-dependent promoter contributes to *glmY* transcription during exponential growth. Alternatively, *glmY* can be transcribed from a σ^{54}-dependent promoter, which requires the YfhK/YfhA two-component system for activity. YfhK is a sensor kinase and YfhA is a response regulator that contains a σ^{54} interaction domain. YfhA binds to a DNA region located more than 100 base-pairs upstream of *glmY*. Three copies of the conserved sequence $TGTCN_{10}GACA$ contribute to binding, and the two sites next to *glmY* are essential for activation of the σ^{54}-dependent promoter by YfhA. YfhK and YfhA up-regulate GlmY when cells enter the stationary growth phase, whereas regulation by glucosamine-6-phosphate occurs post GlmY transcription. Target genes regulated by YfhK and YfhA were unknown so far. We propose to rename these proteins to GlrK and GlrR, for *glmY* regulating kinase and response regulator, respectively.

Introduction

In recent years, *trans*-encoded small RNAs (sRNAs) that act by base-pairing were shown to control a variety of important processes in bacteria, such as envelope stress response, stationary phase control, iron homeostasis, quorum sensing and others (Masse *et al.*, 2007; Gottesman, 2004; Svenningsen *et al.*, 2009; Vogel and Papenfort, 2006; Waters and Storz, 2009). Evidence emerges that fluxes through carbohydrate metabolic pathways are also extensively regulated by sRNAs (Görke and Vogel, 2008). In enteric bacteria glucose uptake is controlled by sRNA SgrS in response to sugar-phosphate stress, and galactose metabolism is regulated by sRNA Spot 42 (Horler and Vanderpool, 2009; Møller *et al.*, 2002; Vanderpool and Gottesman, 2004).

Whereas these classic examples are known for quite some while, recent research revealed that in *Escherichia coli* amino sugar metabolism is controlled at the post-transcriptional level by the small RNAs GlmY and GlmZ. Amino sugars are essential precursors for the biosynthesis of components of the cell wall and the outer membrane in Gram-negative bacteria. The pathway is initiated by synthesis of glucosamine-6-phosphate (GlcN6P), which can be derived from amino sugars available in the environment or from *de novo* synthesis by enzyme glucosamine-6-phosphate synthase (GlmS) (Durand *et al.*, 2008). GlmS is encoded together with enzyme GlmU in the bi-cistronic *glmUS* operon (Plumbridge, 1995). While GlmU is essential, GlmS is only required in the absence of external amino sugars. Differential expression is achieved post transcription of *glmUS*. The co-transcript is processed by RNase E at the *glmU* stop codon (Joanny *et al.*, 2007; Kalamorz *et al.*, 2007). The resulting *glmS*-specific mRNA is normally unstable and weakly translated due to an inhibitory stem loop structure, which buries the ribosomal binding site. However, base-pairing with sRNA GlmZ aided by protein Hfq unlocks this structure, which allows efficient translation and concomitant stabilization of the transcript (Kalamorz *et al.*, 2007; Urban and Vogel, 2008). The sRNA GlmZ is subject to processing, which removes most of the base-pairing site. Thus, exclusively full-length GlmZ is able to activate *glmS* mRNA. A second sRNA, GlmY, was shown to counteract GlmZ processing, thereby activating GlmS synthesis indirectly (Reichenbach *et al.*, 2008; Urban and Vogel, 2008). Hence, these two sRNAs act in a hierarchical cascade, which is a novel mechanism in bacteria. Sequence and structure comparison revealed that GlmY and GlmZ are homologous sRNAs. However, the homology is restricted to the 5'-part of these molecules and does not extend to the GlmZ base-pairing site, which is present between positions 151 to 169 in GlmZ. Similar to GlmZ, GlmY is processed in the 3'-half by a yet unidentified RNase. The physiological role of the GlmYZ regulatory cascade is to feedback-control GlmS synthesis in response to its product GlcN6P. A decreasing GlcN6P concentration in the cell induces the accumulation of sRNA GlmY, which stabilizes full-length GlmZ that finally activates GlmS synthesis to refill the GlcN6P pool (Kalamorz *et al.*, 2007; Reichenbach *et al.*, 2008).

GlmY acts at the top of the regulatory cascade controlling *glmS* expression. Therefore, one of the most urgent questions is to understand how the GlmY concentration is determined in the cell. One level of GlmY control involves the determination of its half-life by poly(A) polymerase (PAP-I)-dependent polyadenylation. PAP-I adds a short poly(A) stretch to the 3'-end of GlmY, which facilitates its subsequent 3'→5' exonucleolytic degradation by RNases such as polynucleotide phosphorylase PNPase (Reichenbach *et al.*, 2008; Urban and Vogel, 2008). Similar results have been obtained for sRNA SraL in *Salmonella typhimurium*. Therefore, PAP-I-dependent polyadenylation is perhaps a more widespread mechanism for sRNA decay, in particular of those sRNAs, which may not act by base-pairing with mRNAs and can therefore not be consumed and shut off upon action as it is the case for sRNAs RyhB, DsrA and OxyS (Masse *et al.*, 2003).

In addition to turn-over, transcription levels determine the cellular amount of a particular sRNA. As far as known, no principal mechanistic differences appear to exist between the control of transcription initiation at sRNA-genes and protein coding genes. sRNA promoters can be subject to regulatory protein dependent repression, activation or both ((for an overview, see: (Brantl, 2009)). Some sRNAs are expressed from promoters recognized by alternative σ-factors, e.g. MicA and RybB, which are members of the σ^{24}-dependent cell envelope stress response (Vogel and Papenfort, 2006). The Qrr sRNAS, which control quorum sensing in *Vibrio cholerae* are transcribed from σ^{54}-dependent promoters (Svenningsen *et al.*, 2009). Promoters recognized by σ^{54} are unique, because binding of the σ^{54}-RNA polymerase complex is not sufficient to initiate transcription (Rappas *et al.*, 2007). Open complex formation requires ATP hydrolysis catalyzed by transcriptional activators that usually bind DNA sites located far upstream of the promoter and contact the closed complex through DNA-looping. A sequence perfectly matching the -24/-12 consensus motif of σ^{54}-dependent promoters is present in front of the *glmY* gene. Indeed, binding of purified σ^{54} to a DNA-fragment containing the *glmY* promoter region was demonstrated suggesting that *glmY* might be transcribed from a σ^{54}-dependent promoter (Urban *et al.*, 2007).

In this work, we deciphered the regulatory network controlling *glmY* expression. We show that *glmY* transcription can be initiated either from a σ^{70}- or from a σ^{54}-dependent promoter. Both promoters overlap in a way that their transcription start sites map at the same position, which is an unprecedented case. The σ^{70}-dependent promoter is moderately active and provides a basal level of *glmY* transcription during exponential growth. However, *glmY* transcription can be increased by activation of the σ^{54}-dependent promoter. Activation of the latter requires the YfhK-YfhA two-component system (TCS), which is encoded downstream of *glmY*. Response regulator YfhA contains a σ^{54} interaction module and activates *glmY* transcription by binding to three conserved sites on the DNA located more than 100 bp upstream of the promoter. Target-genes regulated by the YfhK/YfhA-TCS were unknown so far and therefore we propose to rename the corresponding genes to *glrK* and *glrR* (for *glmY* regulating kinase and response regulator, respectively). Interestingly, GlrR-binding sites and σ^{54}-promoters also precede the *glmZ* gene in some enterobacterial species, but not in *E. coli* and *Shigella*. In the latter cases *glmZ* is transcribed from σ^{70}-dependent promoters suggesting an evolutionary drift of the *glmYZ* system losing σ^{54}-dependency in favor of σ^{70}-dependent transcription in these species.

Experimental procedures

Growth conditions and strains

Bacteria were routinely cultivated in LB medium under agitation (200 r.p.m.) at 37℃. When necessary, antibiotics were added to the medium (ampicillin 100µg/ml, kanamycin 30µg/ml, chloramphenicol 15µg/ml, spectinomycin 50µg/ml). The strains used in this study are listed in Table 1, including a description of their relevant genotypes. The *ΔglrK::kan*, *ΔglrR::kan* and *ΔrpoN::kan* alleles were moved between strains by transduction using bacteriophage T4GT7 (Wilson *et al.*, 1979). Subsequently, marker-less clean deletions were obtained by making use of the helper plasmid pCP20 as described previously (Datsenko and Wanner, 2000). The various *glmY-lacZ* reporter gene fusions used in Figs. 20, 21, 22 and 23 were first established

on plasmids and subsequently integrated into the *λattB*-site on the *E. coli* chromosome to yield the strains as indicated in Table 4.

Table 4: *E. coli* strains used in this study

Name	Genotype	Reference or construction
JW2538	Δ(*araD-araB*)567, Δ*lacZ*4787(::*rrnB*-3), λ-, Δ*glrR*728::*kan*, *rph-1*, Δ(*rhaD-rhaB*)568, *hsdR*514	(Baba *et al.*, 2006)
JW3169	Δ(*araD-araB*)567, Δ*lacZ*4787(::*rrnB*-3), λ-, Δ*rpoN730*::*kan*, *rph-1*, Δ(*rhaD-rhaB*)568, *hsdR*514	(Baba *et al.*, 2006)
JW5407	Δ(*araD-araB*)567, Δ*lacZ*4787(::*rrnB*-3), λ-, Δ*glrK*728::*kan*, *rph-1*, Δ(*rhaD-rhaB*)568, *hsdR*514	(Baba *et al.*, 2006)
R1279	CSH50 Δ(*pho-bgl*)201 Δ(*lac-pro*) *ara thi*	(Görke and Rak, 1999)
Z168	As R1279, but Δ*glrR::kan*	T4GT7(JW2538)→R1279; this work
Z171	As R1279, but Δ*glrK::kan*	T4GT7(JW5407)→R1279; this work
Z179	As R1279, but Δ*glrR*	Z168 cured from *kan*; this work
Z181	As R1279, but Δ*glrK*	Z171 cured from *kan*; this work
Z183	As R1279, but Δ*rpoN::kan*	T4GT7(JW3169)→R1279; this work
Z184	As R1279, but Δ*rpoN*	Z183 cured from *kan*; this work
Z190	As R1279, but *attB::*[*glmY'*(-238 to +22)-*lacZ,* -10 mutated]	pBGG209/BamHI→R1279; this work
Z196	As R1279, but Δ*glrR, attB::*[*glmY'*(-238 to +22)-*lacZ,* -10 mutated]	pBGG209/BamHI→Z179; this work
Z197	As R1279, but *attB::*[*glmY'*(-238 to +22)-*lacZ*]	pBGG201/BamHI→R1279; this work
Z198	As R1279, but *attB::*[*glmY'*(-208 to +22)-*lacZ*]	pBGG202/BamHI→R1279; this work
Z199	As R1279, but *attB::*[*glmY'*(-138 to +22)-*lacZ*]	pBGG226/BamHI→R1279, this work
Z200	As R1279, but *attB::*[*glmY'*(-108 to +22)-*lacZ*]	pBGG204/BamHI→R1279, this work
Z201	As R1279, but *attB::*[*glmY'*(-238 to +22)-*lacZ,* -24 mutated]	pBGG208/BamHI→R1279, this work
Z202	As R1279, but *attB::*[*glmY'*(-238 to +22)-*lacZ,* -10 and -24 mutated]	pBGG210/BamHI→R1279, this work
Z203	As R1279, but *attB::*[*glmY'*(-208 to +22)-*lacZ,* -10 mutated]	pBGG213/BamHI→R1279, this work
Z204	As R1279, but *attB::*[*glmY'*(-138 to +22)-*lacZ,* -10 mutated]	pBGG227/BamHI→R1279, this work
Z205	As R1279, but *attB::*[*glmY'*(-108 to +22)-*lacZ,* -10 mutated]	pBGG215/BamHI→R1279, this work
Z206	As R1279, but Δ*glrR, attB::*[*glmY'*(-238 to +22)-*lacZ*]	pBGG201/BamHI→Z179, this work
Z207	As R1279, but Δ*glrR, attB::*[*glmY'*(-208 to +22)-*lacZ*]	pBGG202/BamHI→Z179; this work
Z208	As R1279, but Δ*glrR, attB::*[*glmY'*(-138 to +22)-*lacZ*]	pBGG226/BamHI→Z179, this work
Z209	As R1279, but Δ*glrR, attB::*[*glmY'*(-108 to +22)-*lacZ*]	pBGG204/BamHI→Z179, this work
Z210	As R1279, but Δ*glrR, attB::*[*glmY'*(-238 to +22)-*lacZ,* -24 mutated]	pBGG208/BamHI→Z179, this work
Z211	As R1279, but Δ*glrR, attB::*[*glmY'*(-238 to +22)-*lacZ,* -10 and -24 mutated]	pBGG210/BamHI→Z179, this work
Z212	As R1279, but Δ*glrR, attB::*[*glmY'*(-208 to +22)-*lacZ,* -10 mutated]	pBGG213/BamHI→Z179, this work
Z213	As R1279, but Δ*glrR, attB::*[*glmY'*(-138 to +22)-*lacZ,* -10 mutated]	pBGG227/BamHI→Z179, this work
Z214	As R1279, but Δ*glrR, attB::*[*glmY'*(-108 to +22)-*lacZ,* -10 mutated]	pBGG215/BamHI→Z179, this work
Z215	As R1279, but Δ*glrK, attB::*[*glmY'*(-238 to +22)-*lacZ*]	pBGG201/BamHI→Z181, this work
Z219	As R1279, but Δ*glrK, attB::*[*glmY'*(-238 to +22)-*lacZ,* -24 mutated]	pBGG208/BamHI→Z181, this work
Z220	As R1279, but Δ*glrK, attB::*[*glmY'*(-238 to +22)-*lacZ,* -10 mutated]	pBGG209/BamHI→Z181, this work
Z221	As R1279, but Δ*glrK, attB::*[*glmY'*(-238 to +22)-*lacZ,* -10 and -24 mutated]	pBGG210/BamHI→Z181, this work
Z227	As R1279, but Δ*rpoN, attB::*[*glmY'*(-238 to +22)-*lacZ]*	pBGG201/BamHI→Z184, this work
Z228	As R1279, but Δ*rpoN, attB::*[*glmY'*(-238 to +22)-*lacZ,* -24 mutated]	pBGG208/BamHI→Z184, this work
Z229	As R1279, but Δ*rpoN, attB::*[*glmY'*(-238 to +22)-*lacZ,* -10 mutated]	pBGG209/BamHI→Z184, this work
Z240	As R1279, but *attB::*[*glmY'*(-238 to +22)-*lacZ*, ABS1 mutated]	pBGG305/BamHI→R1279, this work
Z241	As R1279, but *attB::*[*glmY'*(-238 to +22)-*lacZ*, ABS2 mutated]	pBGG306/BamHI→R1279, this work
Z242	As R1279, but *attB::*[*glmY'*(-238 to +22)-*lacZ*, ABS3 mutated]	pBGG307/BamHI→R1279, this work
Z243	As R1279, but *attB::*[*glmY'*(-238 to +22)-*lacZ,* ABS1+ABS2 mutated]	pBGG308/BamHI→R1279, this work
Z244	As R1279, but *attB::*[*glmY'*(-238 to +22)-*lacZ,* ABS1+ABS3 mutated]	pBGG309/BamHI→R1279, this work
Z245	As R1279, but *attB::*[*glmY'*(-238 to +22)-*lacZ,* ABS2+ABS3 mutated]	pBGG310/BamHI→R1279, this work
Z246	As R1279, but *attB::*[*glmY'*(-238 to +22)-*lacZ,* ABS1+ABS2+ABS3 mutated]	pBGG311/BamHI→R1279, this work
Z247	As R1279, but Δ*glrR, attB::*[*glmY'*(-238 to +22)-*lacZ*, ABS1 mutated]	pBGG305/BamHI→Z179, this work
Z248	As R1279, but Δ*glrR, attB::*[*glmY'*(-238 to +22)-*lacZ*, ABS2 mutated]	pBGG306/BamHI→Z179, this work
Z249	As R1279, but Δ*glrR, attB::*[*glmY'*(-238 to +22)-*lacZ*, ABS3 mutated]	pBGG307/BamHI→Z179, this work
Z250	As R1279, but Δ*glrR, attB::*[*glmY'*(-238 to +22)-*lacZ,* ABS1+ABS2 mutated]	pBGG308/BamHI→Z179, this work
Z251	As R1279, but Δ*glrR, attB::*[*glmY'*(-238 to +22)-*lacZ,* ABS1+ABS3 mutated]	pBGG309/BamHI→Z179, this work
Z252	As R1279, but Δ*glrR, attB::*[*glmY'*(-238 to +22)-*lacZ,* ABS2+ABS3 mutated]	pBGG310/BamHI→Z179, this work
Z253	As R1279, but Δ*glrR, attB::*[*glmY'*(-238 to +22)-*lacZ,* ABS1+ABS2+ ABS3 mutated]	pBGG311/BamHI→Z179, this work
Z266	As R1279, but Δ*rpoN, attB::*[*glmY'*(-238 to +22)-*lacZ,* -10 and -24 mutated]	pBGG210/BamHI→Z184, this work

Integration into the chromosome was achieved using helper plasmid pLDR8 as described previously (Diederich *et al.*, 1992; Dole *et al.*, 2002). Briefly,

origin-less DNA-fragments carrying the respective *glmY-lacZ* fusion, the *aadA* spectinomycin resistance cassette and the λ*attP*-site were isolated by BamHI restriction and agarose gel-electrophoresis. Subsequently, the fragments were self-ligated and transformed into target strains containing the temperature sensitive λ-integrase expression plasmid pLDR8. Selection on spectinomycin-containing plates at 42℃ resulted in integration of the fragments into the λ*attB*-site and concomitant loss of plasmid pLDR8. Correct integration was verified by PCR using appropriate primers.

Site directed mutagenesis and construction of plasmids

Cloning of DNA was carried out in strain DH5α. The oligonucleotides and plasmids used are listed in Table S2 and Table S3, respectively (see *Supplementary material*). For construction of plasmid pBGG219 carrying *glrR::His10* under *tacOP* control, *glrR* was amplified using primers BG484 and BG485. Subsequently, the PCR product was inserted between the NdeI- and XbaI-sites on plasmid pKES170. For construction of the reporter gene fusion carrying *glmY* (-238 to +22) fused to *lacZ* the *glmY*-5' region was amplified using primers BG377 and BG456 and the obtained PCR fragment was used to replace the SalI-XbaI fragment in plasmid pKES15 to yield plasmid pBGG201. To obtain isogenic constructs but carrying mutations in the -24 and -10 promoter sites, PCR reactions were performed with primer BG377 and a reverse primer carrying the desired mutations, respectively. The obtained PCR fragments were inserted between the SalI/XbaI-sites of plasmid pKES15 resulting in the following plasmids carrying the respective mutation by making use of the reverse primer as indicated in parentheses: pBGG208 (-24 mutated; BG481), pBGG209 (-10 mutated; BG482), pBGG210 (-24 and -10 mutated; BG483). Similarly, the GlrR binding site ABS1 was mutated by making use of the forward mutagenesis primer BG558 in combination with primer BG456, resulting in plasmid pBGG305.

To obtain constructs carrying mutations in the GlrR binding sites ABS2 and ABS3, we used the multiple mutation reaction protocol as described (Hames *et al.*, 2005). Briefly, 5'-phosphorylated oligonucleotides carrying the desired mutation were used in addition to the forward primers BG377 or BG558

(ABS1 mutated) and the reverse primer BG456 in PCR reactions containing thermo-stable Ampligase (Epicentre), which incorporates the mutagenesis primers during amplification. The PCR fragments were subsequently ligated to the SalI- and XbaI-treated vector pKES15 resulting in plasmids, which carried the desired mutations as follows. The used primer combinations are given in parentheses, respectively: pBGG306 (ABS2 mutated; BG377/BG456/BG559), pBGG307 (ABS3 mutated; BG377/BG456/BG582), pBGG308 (ABS1 + ABS2 mutated; BG558/BG456/BG559), pBGG309 (ABS1 + ABS3 mutated; BG558/BG456/BG582), pBGG310 (ABS2 + ABS3 mutated; BG377/BG456/BG559/BG583), pBGG311 (all ABS mutated; BG558/BG456/BG559/BG582).

To construct the plasmids carrying the successively 5'-truncated *glmY-lacZ* fusions, the respective *glmY* fragments were amplified and inserted between the SalI/XbaI-sites of plasmid pKES15. Thereby, the following plasmids were obtained by making use of the forward primers given in parentheses together with the reverse primer BG456: pBGG202 (BG378), pBGG226 (BG496), pBGG204 (BG480). The isogenic constructs, but carrying a mutated -10 promoter site, were obtained by substituting reverse primer BG456 by BG482 in the PCRs, respectively. These constructions resulted in plasmids pBGG213, pBGG227 and pBGG215, respectively.

5'RACE Analysis

5'RACE mapping of transcripts was performed essentially as described (Wagner and Vogel, 2005). Briefly, total RNA was isolated using the RNA protect RNeasy Mini kit (Qiagen). RNA samples were split and either treated with Tobacco pyrophosphatase (TAP) resulting in the removal of the 5' pyrophosphate group or this step was omitted. Subsequently, RNA adapter oligonucleotide BG618 was ligated to the mRNAs 5'-ends using T4 RNA Ligase (Fermentas). First strand cDNA synthesis was carried out using the RevertAid^{TM} H Minus First Strand cDNA Synthesis Kit (Fermentas) and the following gene specific oligonucleotides: BG622 for *glmY*, BG624 for *glrK* and BG620 for *lacZ*. Second strand synthesis and subsequent amplification was performed using *Taq* DNA polymerase and the oligonucleotide pairs BG619/BG623 for amplification of *glmY*-specific transcripts, BG619/BG649 for

lacZ-specific transcripts and BG619/BG625 for *glrK*-specific transcripts, respectively. The obtained DNA fragments were gel-purified and cloned into plasmid pBAD18-cm following appropriate restriction enzyme digestion. The inserts of several recombinant plasmid clones were sequenced.

Northern analysis

For northern analysis RNA was extracted using the RNeasy Mini kit (Qiagen). Five µg total RNA per lane was separated on 8% polyacrylamide/ 7M urea/ TBE-gels for analysis of GlmY and GlmZ, whereas the RNA was separated on 1% agarose formaldehyde gels for analysis of *glmS* transcripts. Subsequently, the RNA was transferred to a positively charged nylon membrane (Roche) and cross-linked by UV-radiation. The RNAs of interest were detected using specific Digoxigenin-labeled RNA probes as described previously (Reichenbach *et al.*, 2008). The RNA probe directed against 5S rRNA transcripts were obtained by *in vitro* transcription using the DIG-Labeling kit (Roche Diagnostics) and a DNA fragment as template generated by PCR using primers BG287/BG288. Hybridization and detection were carried out according to the supplier's instruction (DIG RNA Labeling kit, Roche Diagnostics).

Analysis of *glmY* transcription (β-Galactosidase assays)

Overnight cultures in LB were inoculated into fresh LB medium to an OD_{600} of 0.1 and grown at 37℃ to an OD_{600} of 0.5-0.8 and subsequently harvested, if not otherwise indicated. β-galactosidase activities were determined as described previously (Miller, 1972). Enzyme activities are expressed in Miller units and are the average of at least three measurements using independent cultures.

***In vitro* transcription assay**

In vitro transcription using 1 unit σ^{70} RNA polymerase holoenzyme (Epicentre Biotechnologies) was carried out in 25 µl volume containing buffer (40 mM Tris-HCl (pH 7.5), 150 mM KCl, 10 mM $MgCl_2$, 0.01% Triton X-100, 1 mM DTT), 0.5 mM NTPs each and 200 ng of a DNA-template obtained by PCR

using oligos BG377 and BG472. *In vitro* transcriptions using 40 units T7 RNA polymerase (Roche) were carried in 20 µl containing buffer (40 mM Tris-HCl (pH 8.0), 6 mM $MgCl_2$, 10 mM DTT, 2 mM spermidine), 1 mM NTPs and 200 ng DNA-template obtained by PCR using primer pairs BG527/BG528 (*P_{T7}::sraC*) and BG446/BG472 (*P_{T7}::glmY*), respectively. Transcription assays were incubated at 37℃ for 2 h and RNAs were subsequently purified by LiCl/Ethanol precipitation. GlmY transcripts were analyzed and detected by northern blotting.

Purification of GlrR

For the purification of C-terminally His-tagged GlrR protein, strain DH5α carrying plasmid pBGG219 was grown in 1 l LB-ampicillin to an OD_{600} = 0.5. GlrR synthesis was induced by the addition of 1 mM IPTG and after an additional 1 h of growth cells were harvested and washed in ZAP-buffer (50 mM Tris-HCl, 200 mM NaCl, pH 7.5). The crude lysate was prepared using a one shot cell disrupter (Constant systems Ltd) and subsequently cleared by ultracentrifugation. The cleared lysate was loaded onto a pre-equilibrated Ni-NTA Superflow column (Qiagen) and proteins were eluted with a gradient of imidazol solved in ZAP buffer (See Fig. S6). The 250 mM fraction containing pure GlrR-His_{10} was dialyzed against buffer (20 mM Tris-HCl pH 7.5, 100 mM KCl, 2 mM DTT, 25% (v/v) glycerol) and used for EMSAs.

Electro mobility shift assays (EMSAs)

EMSAs were carried out essentially as described previously (Stratmann *et al.*, 2008). Briefly, DNA fragments were amplified by PCR by making use of the oligonucleotides that were used used for construction of the various *glmY-lacZ* gene fusions (Table S3). DNA concentrations were determined using the NanoDrop Spectrometer ND-1000 (Peqlab). Binding assays were carried out in binding buffer (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 2mM DTT, 10% glycerol) and contained in 10 µl volume 25 ng of each DNA-fragment and the protein amounts as indicated in Figs. 22 and 23. The mixtures were incubated at 30℃ for 20 min and subsequently separated at 4℃ alongside with a DNA size marker by non-denaturing gel-electrophoresis

using 8% acrylamide in 0.5×TBE. The gels were stained with ethidium bromide for visualization of the DNA.

Results

Transcription of *glrK* initiates in the *glmY-glrK* intergenic region

Initiation of transcription by the σ^{54}-RNA polymerase complex requires interaction of the closed complex with a transcriptional activator protein. GlmY is encoded in the *purL-glrK* intergenic region and genes *glrK* and *glrR*, which are located downstream of *glmY* (Fig. 18 A), encode a histidine kinase and a response regulator of a two-component system (TCS).

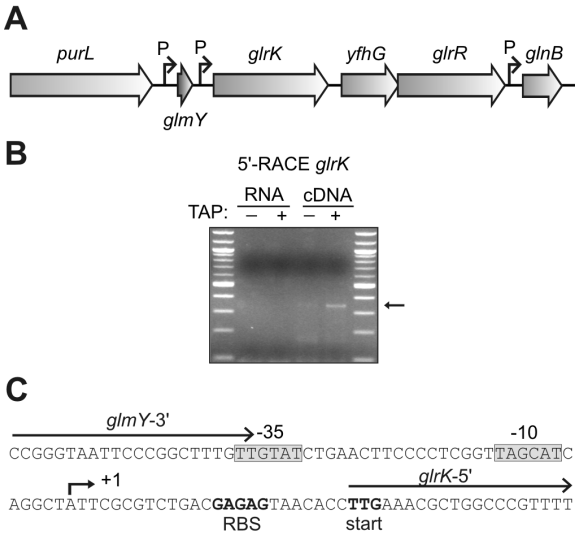

Figure 18: Mapping of the transcriptional start site of *glrK*. A. Organization of the *glmY-glrK-yfhG-glrR* chromosomal region. Overlapping σ^{54}- and σ^{70}-dependent promoters direct transcription of *glmY* (this work). Genes *glrK-yfhG-glrR* putatively form an operon and are independently transcribed from a promoter located in the *glmY-glrK* intergenic region (this study). Three transcriptional start sites of *glnB* have been mapped previously, which are located in the 3' end of *glrR* and the *glrR-glnB* intergenic region, respectively (He *et al.*, 1993a). **B.** 5' RACE mapping of the *glrK* mRNA 5' end. An increased PCR signal is obtained upon treatment of the RNA with tobacco acid pyrophosphatase (cDNA samples, lane +) indicating 5' triphosphate ends. No signal was obtained when the RNA was directly used in PCR, ruling out contamination with genomic DNA (RNA samples). **C.** Location of the *glrK* transcriptional start point as revealed by 5' RACE. Putative -35 and -10 sequence motifs of a σ^{70}-promoter are boxed. The 3' end of *glmY* and the 5' end of the *glrK* coding sequence are indicated by arrows. The ribosomal binding site and the start codon of *glrK* are in bold.

Recent work has demonstrated that GlrK is the cognate kinase for response regulator GlrR (Yamamoto *et al.*, 2005). Intriguingly, GlrR contains a putative σ^{54}-interaction module (Studholme and Dixon, 2003) raising the possibility that it could be the activator protein of the putative σ^{54}-promoter preceding *glmY*. The transcription start site of *glrK* has not been mapped so far. It was

predicted that transcription of *glrK* is initiated at the putative σ^{54}-promoter site in front of *glmY* (Reitzer and Schneider, 2001). This would generate a co-transcript containing *glmY* as well as *glrK*. In order to map the transcription start site of *glrK* unequivocally, we performed a 5'-RACE analysis of *glrK* transcripts. A single PCR product was obtained upon treatment of the RNA with Tobacco pyrophosphatase (TAP) prior to ligation to an RNA oligonucleotide, whereas no product was obtained when omitting this step (Fig. 18 B). This demonstrates that the *glrK* mRNA detected by this approach is a primary transcript rather than a cleavage product. Cloning of the PCR fragment and subsequent sequencing of nine independent recombinant plasmids revealed in all cases a transcription start site located at an adenine 25 bp upstream of the *glrK* start codon (Fig. 18 C). Putative -35 and -10 sequences of a σ^{70}-dependent promoter are present upstream at appropriate positions. In conclusion, *glmY* and *glrK* are independently transcribed from different promoters.

In mutants lacking σ^{54}, GlrR or GlrK, expression of GlmY is reduced but not abolished

In order to test whether transcription of *glmY* might be controlled by σ^{54} and the GlrK/GlrR TCS, we analyzed the GlmY amount in mutants lacking these proteins. To this end, we isolated total RNAs of the wild-type strain and of mutants defective in *rpoN*, *glrR* or *glrK* and analyzed them in Northern experiments using a probe specific for GlmY.

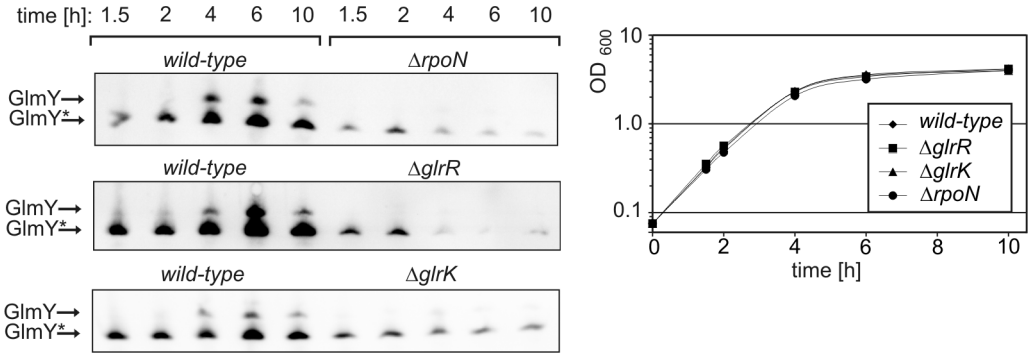

Figure 19: Absence of σ^{54} or the GlrR/GlrK-TCS reduces the GlmY amount in the cell. Northern blot analysis of RNA samples collected at various time points during growth of strains R1279 (wild-type), Z184 (*ΔrpoN*), Z179 (*ΔglrR*) and Z181 (*ΔglrK*). The corresponding growth curves are shown at the top. GlmY was detected using a specific RNA-probe. The processed variant of GlmY is designated with an asterisk. Re-probing of the blots with a probe directed against 5S rRNA confirmed that similar amounts of total RNA were analyzed (see Fig. S5).

As expected from previous data, two species corresponding to full-length and processed GlmY were detectable in the wild-type strain and both forms accumulated during transition to stationary phase (Fig. 19). In contrast, in the three mutants the GlmY amounts were drastically reduced: In particular, GlmY was barely detectable when cells entered the stationary growth phase. On the other hand, during exponential growth the amount of GlmY appeared only slightly reduced in these mutants when compared to the wild-type (Fig. 19). Re-probing of the blots with a probe specific for 5S rRNA confirmed that comparable amounts of total RNA were subjected to blotting (Fig. S5). In conclusion, σ^{54}, GlrK and GlrR have clearly positive effects on the GlmY amount, but they are not absolutely required for *glmY* expression.

Evidence for two promoters controlling transcription of *glmY*

Our results concerning the roles of σ^{54} and of the GlrK/GlrR TCS for *glmY* expression were surprising. It is a characteristic of σ^{54}-dependent promoters that σ^{54} and the corresponding activator protein are absolutely required for transcription initiation (Reitzer and Schneider, 2001). Hence, the *glmY* expression detected in the mutant lacking σ^{54} cannot be explained by residual σ^{54}-promoter activity. Therefore, the possibility had to be considered that an additional promoter contributes to expression of *glmY*. Inspection of the sequence of the DNA region upstream of *glmY* reveals the presence of putative -35 (TTTTCT) and -10 (CATAAT) sequence motifs, which are separated by 18 base-pairs and could represent a σ^{70}-dependent promoter that overlaps with the σ^{54}-dependent promoter (Fig. 20 A). To explore whether this is indeed the case, we constructed a *glmY-lacZ* reporter fusion comprising positions -238 to +22 relative to the *glmY* transcription start site. Next, mutations were introduced into the -24 sequence and the putative -10 sequence, respectively, as indicated in Fig. 20 A. These mutations should abolish the corresponding promoter activity leaving the respective second promoter unaffected. Finally, the various *glmY-lacZ* fusion constructs were integrated into the *λattB*-site on the chromosome and the β-galactosidase activities were determined from exponentially growing cells.

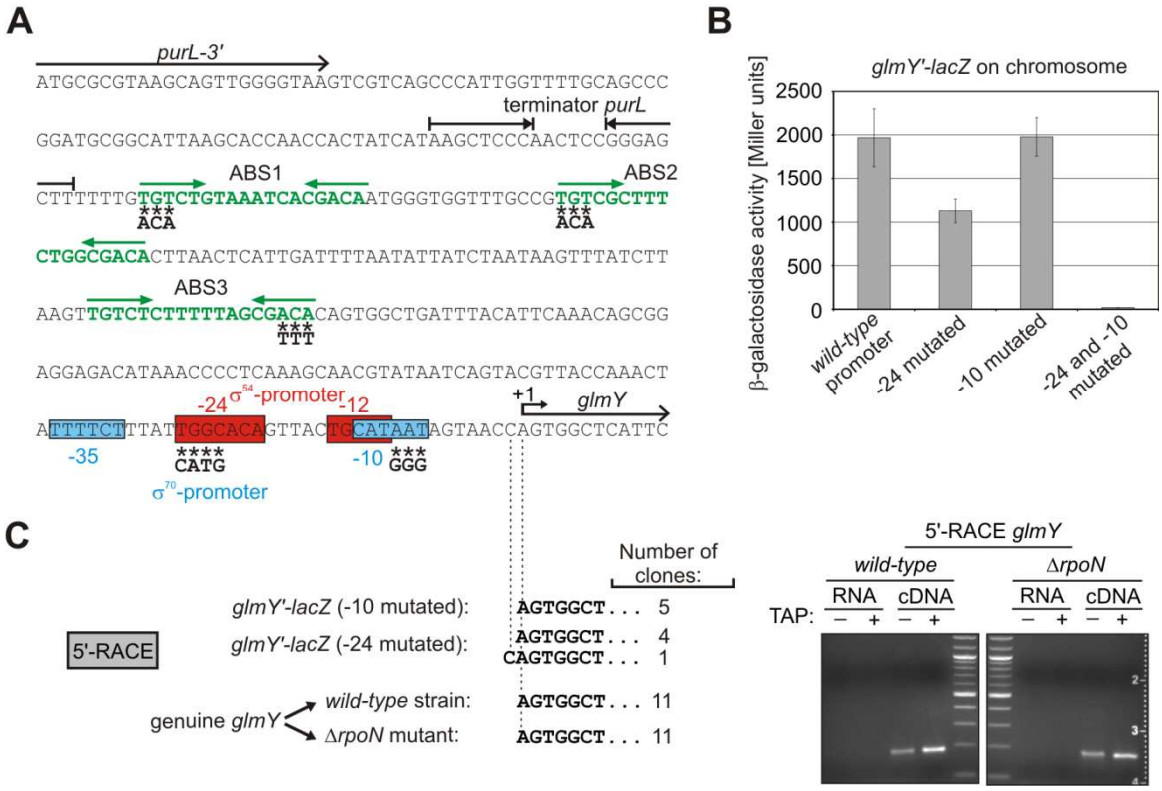

Figure 20: Overlapping σ^{54}- and σ^{70}-dependent promoters start *glmY* transcription at the same position
A. Sequence of the *purL-glmY* intergenic region. The -24/-12 sequence motifs of the σ^{54}-dependent promoter are highlighted by red boxes. The putative -35/-10 sequences of an overlapping σ^{70}-dependent promoter are boxed in blue. The putative GlrR binding sites (designated ABS) as revealed by searching for conserved sequences in the *purL-glmY* IGS (Fig. S3) are depicted in green. The positions mutated in the ABS and in the -24 and -10 sequence motifs, respectively, are marked with asterisks and the introduced nucleotide exchanges are shown below in bold. **B.** Simultaneous mutation of the -24 and the -10 sequence motifs is required to abolish *glmY* expression. β-galactosidase activities of strains carrying a *glmY'-lacZ* fusion in the *λattB*-site on the chromosome. In strain Z197 this fusion is transcribed from the *glmY* wild-type promoter, whereas the -24 sequence motif is mutated in strain Z201 and the -10 sequence is mutated in strain Z190. Strain Z202 carries both mutations. **C.** 5' RACE analyses of *glmY* transcriptional start points. The results of the 5' RACE mappings of the *glmY'-lacZ* fusion mRNAs transcribed from the differently mutated *glmY* promoters in strains Z190 and Z201, respectively, are shown. In addition, authentic *glmY* transcripts were analyzed in strains R1279 (wild-type) and Z184 (*ΔrpoN*). The agarose gels depicting the PCR products obtained in this latter case are shown at the right.

As can be seen from the data, the *glmY-lacZ* reporter was readily expressed when the wild-type promoter region was present upstream of the fusion (Fig. 20 B, column 1). Mutation of the -24 sequence reduced *glmY-lacZ* expression only two-fold (Fig. 20 B, column 2), supporting the idea that a second promoter is present upstream of *glmY*. Mutation of the putative -10 sequence had no effect on expression of the *glmY-lacZ* fusion (Fig. 20 B, column 3). Intriguingly, *glmY-lacZ* expression was completely abolished when both mutations were combined (Fig. 20 B, column 4). These results can only be

explained by the presence of two promoters, which both can trigger transcription of *glmY*.

To obtain direct evidence that *glmY* is also transcribed from a σ^{70}-dependent promoter, in *vitro* transcription assays were carried out. A linear DNA fragment covering the *glmY* region (-238 to + 204) was used as template for "cold" *in vitro* transcription using σ^{70}-RNA polymerase holoenzyme and un-labeled nucleotides. As a size-control *glmY* was transcribed from a PCR fragment carrying a promoter for T7 RNA-Polymerase in front of *glmY*. This control assay generates *glmY* RNA (184 nt) and a slightly longer run off transcript (204 nt) due to read-through at the *glmY* terminator. The reactions were separated on polyacrylamide gels. Gels were subsequently blotted and *glmY* was detected using a specific Digoxigenin-labeled RNA probe (Fig. 21 A). Indeed, the *in vitro* transcription assay containing σ^{70}-RNAP generated GlmY RNA as revealed by comparison with the transcripts obtained from T7-RNAP-dependent transcription (Fig. 21 A, compare lanes 2 and 3). The slower migrating band represents the DNA fragment used as template in the assay (Fig. 21, compare lanes 2 and 4).

The σ^{70}-glmY promoter is active during exponential growth whereas activity of the σ^{54}-glmY promoter increases during transition to the stationary growth phase

The Northern blot experiments (Fig. 19) suggested that σ^{54} is in particular important for expression of *glmY* during transition to the stationary phase, whereas activity of the σ^{70}-dependent promoter should be restricted to the exponential growth phase. To see, whether this is indeed the case, we determined the β-galactosidase activities produced by the various *glmY-lacZ* fusion constructs at different times during growth (Fig. S6). The β-galactosidase activities produced by the *glmY-lacZ* fusion transcribed from the wild-type promoter remained more or less constant during exponential growth and activities increased up to 3-fold during transition to the stationary growth phase. The *glmY-lacZ* fusion started from the σ^{54}-promoter (-10 sequence mutated) produced a very similar expression pattern. In contrast, there was only a negligible increase in activities when the *glmY-lacZ* fusion was

transcribed from the σ^{70}-promoter (-24 sequence mutated). In conclusion, the σ^{70}-promoter contributes to *glmY* expression during exponential growth, whereas the σ^{54}-promoter ensures expression of the sRNA during transition to the stationary growth phase, at least under the conditions tested.

Perfectly overlapping σ^{70}- and σ^{54}-dependent promoters for transcription of *glmY*

The next question was whether the two promoters in front of *glmY* start transcription at the same or at different positions. In the latter case, GlmY species possessing different 5'-ends would be generated, which could have functional consequences. Therefore, we carried out 5' RACE analyses of the *glmY-lacZ* fusion mRNAs, which were either transcribed from the σ^{54}-promoter (-10 sequence mutated) or from the σ^{70}-promoter (-24 sequence mutated). The obtained PCR fragments were cloned into a plasmid and 5 independent clones were sequenced, respectively. This analysis revealed that the *glmY-lacZ* mRNAs, which were transcribed from the promoter carrying the mutations in the -10 sequence, all started at an adenine located 13 bp downstream of the center of the -12 sequence motif (Fig. 20 C, left). This is the known transcriptional start point of *glmY* (Vogel *et al.*, 2003). Four of the clones derived from the *glmY-lacZ* mRNAs that were transcribed from the σ^{70}-promoter (-24 sequence mutated) exhibited the same start position, while one transcript was started at the cytosine adjacent to the left (Fig. 20 C, left). To confirm these results, we also analyzed the 5' ends of genuine GlmY in the wild-type strain and in a *ΔrpoN* mutant, in which σ^{54}-dependent promoters are inactive. Hence, in the latter case transcription can only be started from the σ^{70}-dependent promoter. Sequence analysis of 11 plasmid clones each revealed that *glmY* transcription started in all cases at the adenine already mapped before (Fig. 20 C left). In these 5' RACE analyses single PCR products were obtained, which were much more abundant when the RNAs were treated with TAP prior to ligation to the RNA oligonucleotide (Fig. 20 C, right). This confirms that primary *glmY* transcripts were detected. Taken together, the data suggest that *glmY* can be transcribed from a σ^{54}-dependent

as well as from a σ^{70}-dependent promoter. These two promoters perfectly overlap in a way that GlmY species with the same 5'-ends are generated.

Activity of the σ^{54}-*glmY* promoter is completely dependent on σ^{54} and the GlrK/GlrR TCS, whereas the σ^{70}-*glmY* promoter is not affected by these proteins

The next question was to clarify whether the activities of the two promoters directing expression of *glmY* are controlled by σ^{54} and the GlrK/GlrR TCS. Therefore, we studied expression of the various chromosomal *glmY-lacZ* fusion constructs in mutants lacking GlrK, GlrR or σ^{54}. To this end, the bacteria were grown to exponential phase and the β-galactosidase activities were determined.

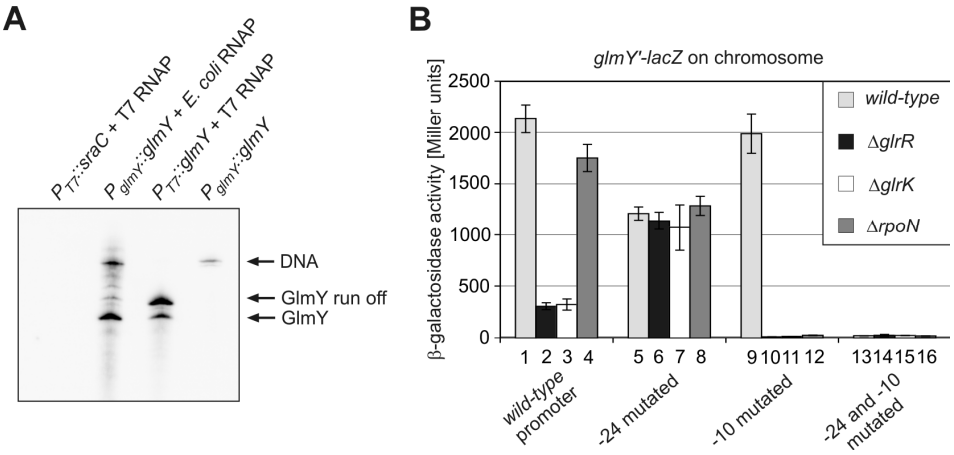

Figure 21: A. *In vitro* transcription of *glmY* by σ^{70}-RNA polymerase. The amounts corresponding to 10 ng RNA were separated by polyacrylamide gel electrophoresis and subsequently nucleic acids were detected by Northern blotting using a probe specific for *glmY*. Lane 1: sraC-RNA generated by *in vitro* transcription using T7-RNAP was loaded as control for probe-specificity. Lane 2: Assay using σ^{70}-RNAP and a DNA fragment encompassing the *glmY* region (-238 to + 204). Lane 3: Assay using T7-RNAP and a PCR-fragment (*glmY*: +1 to +204) carrying a T7-promoter in front of *glmY*. Lane 4: 1 ng of the DNA-template used in lane 2 was directly loaded. **B.** Roles of σ^{54} and the GlrK/GlrR-TCS for the activities of the σ^{54}- and σ^{70}-dependent *glmY* promoters. β-galactosidase activities of strains carrying a *glmY'-lacZ* fusion in the *λattB*-site on the chromosome. The wild-type promoter or the mutated promoter versions were present in front of the *glmY* gene as depicted in the figure. In addition, *glrR*, *glrK* or *rpoN* were deleted as indicated in the legend. The following strains were tested (corresponding to the columns from left to right): Z197, Z206, Z215, Z227, Z201, Z210, Z219, Z228, Z190, Z196, Z220, Z229, Z202, Z211, Z221 and Z266.

Transcription of the *glmY-lacZ* fusion from the wild-type promoter was 7-fold reduced in *ΔglrR* and in *ΔglrK* mutants when compared to the wild-type strain (Fig. 21 B, columns 1-3). However a certain level of transcription was retained in these cases as revealed by comparison with the construct in which both

promoters were mutated (Fig. 21 B, columns 13-16). Deletion of *rpoN* had just a weak negative effect on transcription of the *glmY-lacZ* fusion started from the wild-type promoter (Fig. 21 B, columns 1 and 4). When *glmY-lacZ* was exclusively transcribed from the σ^{70}-promoter (-24 sequence mutated), no differences in expression could be detected between the wild-type and the mutant strains (Fig. 21 B, columns 4-8). In contrast, when only σ^{54}-promoter activity was retained (-10 sequence mutated) *glmY-lacZ* expression was completely abolished in the strains lacking GlrR, GlrK or σ^{54} (Fig. 21 B, columns 9-12). These data demonstrate that (I) activity of the σ^{54}-promoter completely depends on σ^{54} and the GlrK/GlrR TCS, whereas (II) the σ^{70}-promoter is constitutive and not regulated by GlrK and GlrR. Moreover, the data suggest that binding of σ^{54} represses the σ^{70}-promoter to some extent. Expression from the wild-type promoter is relatively low, when *glrR* or *glrK* are deleted, i.e. when σ^{54} can still bind (Fig. 21 B, columns 2 and 3). In contrast, activity of the wild-type promoter is much higher in the absence of σ^{54} and comparable with the activities produced by the construct carrying the mutated -24 sequence to which binding of σ^{54} is prevented (Fig. 21 B, compare columns 4 and 5-8).

GlrR regulates *glmY* transcription by binding to sequences upstream of its promoter

Activator proteins controlling σ^{54}-dependent promoters bind to DNA sequences usually located at least 100 bp upstream of the promoter site. Sometimes more than one binding site is present in front of the controlled gene as it is the case for several target genes regulated by the nitrogen regulator NtrC (Reitzer and Schneider, 2001; Studholme and Dixon, 2003).

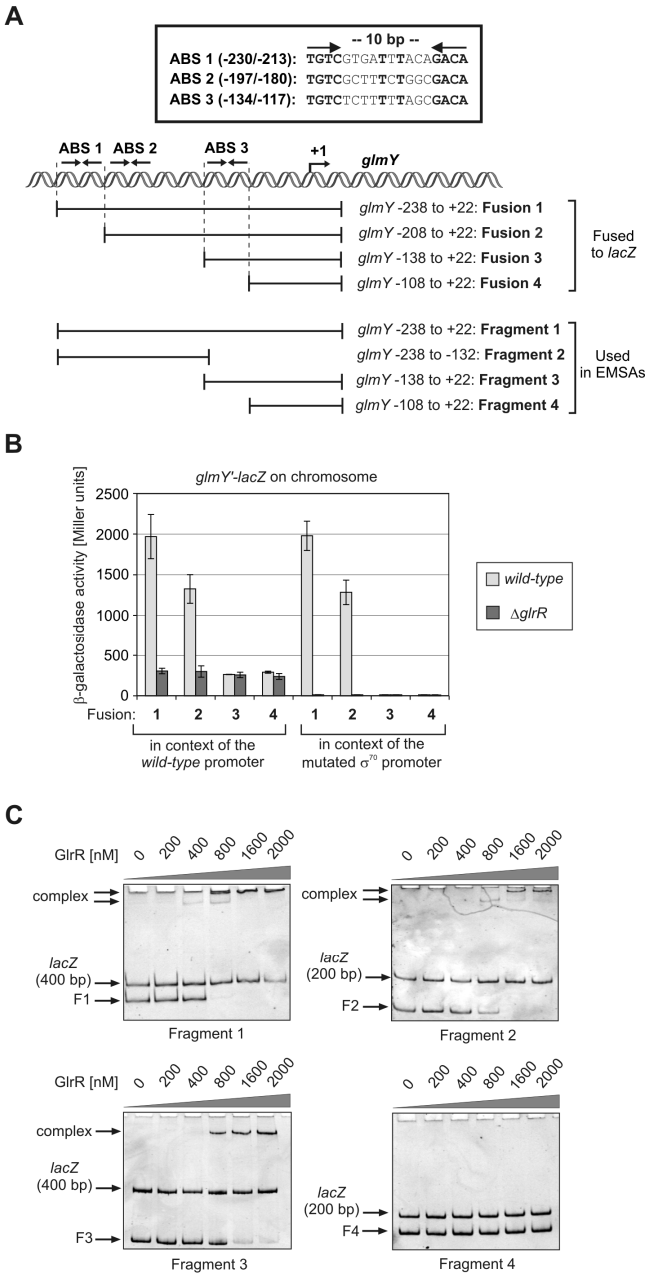

Figure 22: Regulation of the σ^{54}-dependent *glmY* promoter by binding of GlrR to sites upstream of *glmY*. A. An alignment of the putative GlrR binding sites ("ABS") and their positions relative to the *glmY* transcription start is shown at the top. Below: Schematic representation of the *purL-glmY* IGS and of the positions of the gradually shortened fragments, which were fused to *lacZ* and tested in Fig. 22 B. Bottom: Schematic representation of the positions of the DNA fragments tested in the EMSAs in Fig. 22 C. **B.** 5'→3' deletion analysis of the *glmY* promoter region. β-galactosidase activities of strains carrying the stepwise 5'-truncated *glmY-lacZ* fusions (as depicted in Fig. 22 A) on the chromosome. These fusions were tested in the context of the *glmY* wild-type promoter as well as in the context of the mutated -10 sequence motif (left and right half-sites of the graph respectively). Where indicated, gene *glrR* has been deleted. The following strains were tested (corresponding to the columns from left to right): Z197, Z206, Z198, Z207, Z199, Z208, Z200, Z209, Z190, Z196, Z203, Z212, Z204, Z213, Z205 and Z214. **C.** Binding of GlrR to fragments 1 to 4 (as depicted in Fig. 22 A) in comparison to the *lacZ* control fragments as revealed by EMSA.

In order to identify the putative binding site(s) of GlrR, we performed a sequence alignment of the *purL-glmY* intergenic regions from different

Enterobacteriaceae (Fig. S7). This analysis revealed the presence of a highly conserved palindromic sequence whose half-sites are separated by ten base-pairs ($TGTCN_{10}GACA$) and which occurs three times in this DNA region (Fig. 20 A and Fig. 22 A). To get first insight, whether these sites are important for regulation of *glmY* expression by GlrR, we carried out a 5'→3' deletion analysis of the *purL-glmY* intergenic region in the context of the chromosomal *glmY-lacZ* fusion (Fig. 22 A). Removal of the first putative activator binding site ("ABS1") located most distal relative to *glmY* had just a slight negative impact on expression of the reporter fusion and its regulation by GlrR (Fig. 22 B, compare fusions 1 and 2). The additional elimination of the DNA region containing the central putative binding site ("ABS2") reduced *glmY-lacZ* expression 8-fold (Fig. 22 B, compare fusions 1 and 3) and very similar results were obtained when all three putative binding sites were absent (Fig. 22 B, compare fusions 1 and 4). To confirm that the residual expression of these fusions in the *ΔglrR* mutant background is due to the activity of the σ^{70}-promoter in front of *glmY*, we repeated these experiments using *glmY-lacZ* fusion constructs, which were exclusively expressed from the σ^{54}-dependent promoter (-10 sequence mutated). Indeed, in this case expression of the *glmY-lacZ* fusion was completely shut off upon truncation of ABS 2 and none of the fusion constructs was expressed in the absence of *glrR* (Fig. 22 B, right half of the graph).

To analyze the binding of GlrR to the region upstream of *glmY*, His-tagged GlrR protein was purified to homogeneity (Fig. S8) and EMSAs were performed by using DNA fragments covering the *purL-glmY* intergenic region to different extents (Fig. 22 A). In addition, a 400 bp or a 200 bp fragment encompassing the *lacZ* promoter was used as a negative control. Mixtures of the different *purL-glmY* fragments with the *lacZ* control fragment were incubated with increasing amounts of GlrR and subsequently separated by native polyacrylamide gel electrophoresis. As shown in Fig. 22 C, fragment 1 covering all three ABS and the *glmY* promoter was shifted efficiently, whereas the *lacZ* control fragment was not. Similarly, fragment 2 covering ABS1 and ABS2 and fragment 3 covering ABS3 and the *glmY* promoter were specifically shifted by GlrR albeit with somewhat lower efficiencies in comparison to

fragment 1. In contrast, fragment 4, which lacks any ABS but still carries the *glmY* promoter, was not bound by GlrR. Taken together, these data show that GlrR binds at least two different sites located more than 100 bp upstream of the *glmY* transcriptional start site. These sites must be present between positions -138 and -108 and between positions -238 and -132, respectively.

Each of the three palindromic sequences present in the *purL-glmY* intergenic region contributes to binding by GlrR and the two binding sites located proximal to *glmY* are essential for regulation of *glmY* expression by GlrR

The genetic analysis suggested that the region extending to position -208 upstream of *glmY* is essential for regulation of *glmY* expression by GlrR, while the most distal palindromic sequence ("ABS1") is dispensable. This suggested that ABS2 and ABS3 are required for activation of the σ^{54}-promoter by GlrR. In order to see whether this is indeed the case, we mutated each of the three putative GlrR binding sites by introducing nucleotide exchanges into one of the half-sites of each of these palindromes (see Fig. 20 A for location of the mutations). The mutations were introduced into the chromosomal *glmY-lacZ* reporter fusion individually as well as in all possible combinations and subsequently the β-galactosidase activities produced by the various strains were determined (Fig. 23 A). These experiments revealed that mutation of ABS2 or ABS3 is sufficient to abolish regulation of *glmY-lacZ* expression by GlrR. In the corresponding strains *glmY-lacZ* expression was decreased to the level brought about by the σ^{70}-dependent promoter alone and no differences between the wild-type strain and the Δ*glrR* mutant were detectable. In contrast, mutation of ABS1 had only a minor impact on expression of *glmY-lacZ* and its regulation by GlrR.

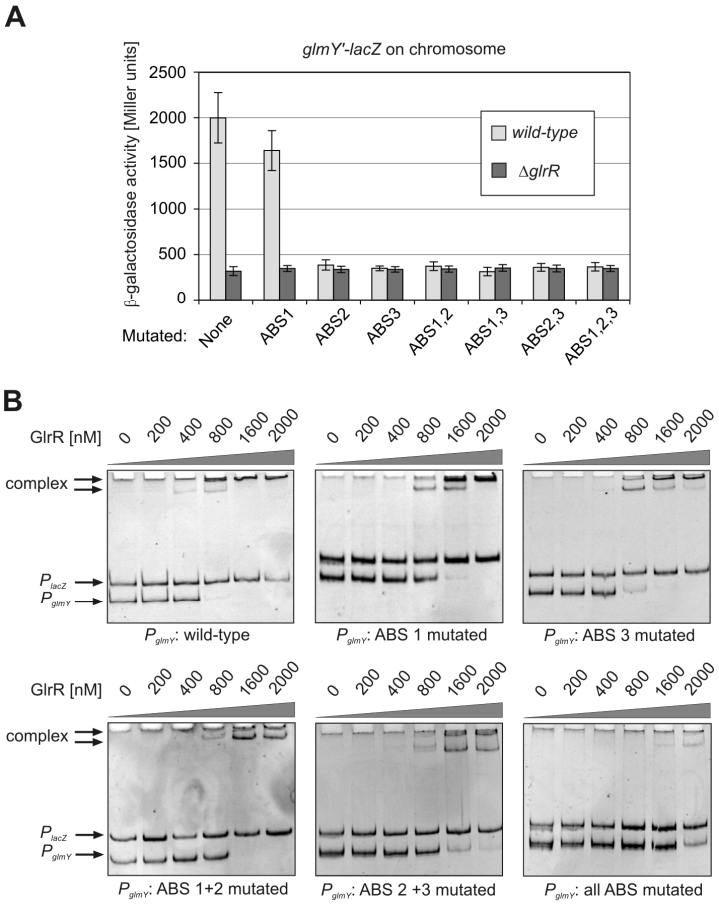

Figure 23: Regulation of *glmY* transcription by binding of GlrR to three conserved sites in the *purL-glmY* IGS. A. β-galactosidase activities of strains carrying mutated activator binding sites (ABS) in various combinations in the chromosomal *glmY-lacZ* fusion. Expression of the various fusions was tested in the wild-type as well as in the *glrR* deletion background, as indicated in the legend. The following strains were tested (corresponding to the columns from left to right): Z197, Z206, Z240, Z247, Z241, Z248, Z242, Z249, Z243, Z250, Z244, Z251, Z245, Z252, Z246 and Z253. **B.** Binding of GlrR to DNA fragments, which cover the *purL-glmY* IGS (-238 to +22 relative to *glmY*) and carry mutated ABS in various combinations. EMSAs using a 400 bp fragment covering the *lacZ* promoter as control. Refer to Fig. 20 A for the individual nucleotide exchanges that were introduced into the ABS.

Next, EMSA experiments were carried out using DNA fragments, which encompassed the *glmY* control region (-238 to +22) and carried the various mutations analyzed before in the reporter gene assays, respectively. As can be seen from the data, higher GlrR concentrations were required to completely shift the DNA fragments carrying mutations in ABS1 or ABS3 in comparison to the wild-type fragment (Fig. 23 B, upper panels). Very similar results were obtained when ABS2 was mutated (data not shown). When two ABS were simultaneously mutated, the respective DNA-fragments were still bound by GlrR, but the binding efficiencies appeared further decreased in comparison to the fragments possessing a single mutated ABS (Fig. 23 B,

lower panel and data not shown). In contrast, mutation of all three ABS almost completely abolished binding of GlrR (Fig. 23 B, last panel). Taken together, the data demonstrate that GlrR specifically binds to each of the three ABS. However, ABS2 and ABS3 are essential for regulation of *glmY* expression by GlrR, while ABS1 is not.

Up-regulation of the GlmY-GlmZ-*glmS* regulatory cascade by GlcN6P-depletion does not involve the GlrK/GlrR-TCS and occurs post-transcriptional of *glmY*

We have previously shown that expression of the *glmS* gene is feedback-regulated by GlcN6P at the post-transcriptional level by a mechanism that involves the hierarchical action of the small RNAs GlmY and GlmZ. Upon GlcN6P-depletion GlmY accumulates and counteracts processing of GlmZ, which is then able to up-regulate the *glmS* mRNA (Kalamorz *et al.*, 2007; Reichenbach *et al.*, 2008). In this respect it appeared feasible that decreasing GlcN6P-concentrations are sensed by the GlrK/GlrR-TCS, which in turn increases *glmY* expression by activation of its σ^{54}-dependent promoter. To address this possibility, we used Nva-FMDP, a compound that selectively inhibits GlmS enzymatic activity *in vivo* (Marshall *et al.*, 2003), which leads to GlcN6P depletion in the cell and thus to induction of *glmS* expression via the GlmY-GlmZ regulatory cascade (Reichenbach *et al.*, 2008). The wild-type and mutant strains lacking σ^{54}, GlrK or GlrR, were grown to early exponential phase. Subsequently, the cultures were split and growth was continued either in the absence or presence of Nva-FMDP for 1 h. Thereafter, cells were harvested and total RNAs were isolated and subjected to Northern analysis using probes specific for GlmY, GlmZ and *glmS*, respectively (Fig. 24).

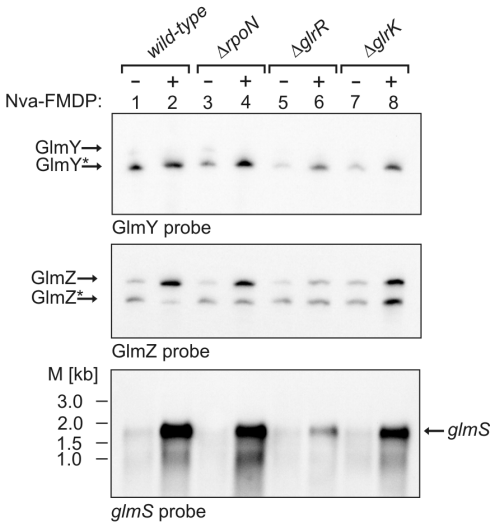

Figure 24: σ^{54} and the GlrR/GlrK-TCS are dispensable for up-regulation of the GlmY-GlmZ-*glmS* regulatory cascade by glucosamine-6-phosphate depletion. Northern blot analyses to determine the effect of the GlmS inhibitor Nva-FMDP on GlmY (GlmY probe, top panel), GlmZ (GlmZ probe, central panel) and on the *glmS* transcript (*glmS* probe, bottom panel) in strains R1279 (wild-type), Z184 (*ΔrpoN*), Z179 (*ΔglrR*) and Z181 (*ΔglrK*).

As expected from previous analyses (Reichenbach *et al.*, 2008), Nva-FMDP caused the accumulation of GlmY, full-length GlmZ and *glmS* (Fig. 24, lanes 1 and 2). Interestingly, very similar results were obtained in the mutant strains (Fig. 24, lanes 3-8). This shows that activation of the GlmY-GlmZ-*glmS* regulatory cascade by GlcN6P-depletion does not require GlrR, GlrK and σ^{54}. Hence, the GlrK/GlrR-TCS is not the sensor of GlcN6P.

So far, the data do still not exclude that activity of the σ^{70}-promoter in front of *glmY* is triggered by GlcN6P. To decide once and for all whether increased transcription rates account for the higher GlmY levels in response to GlcN6P depletion, we studied the effect of Nva-FMDP on transcription of the *glmY-lacZ* reporter fusion, started either from the wild-type promoter, the σ^{70}-promoter (-24 sequence mutated) or the σ^{54}-promoter (-10 sequence mutated). The strains carrying these fusions on the chromosome were grown to early exponential phase and the cultures were subsequently split. Growth was continued either in the presence or absence of Nva-FMDP and following different time-intervals samples were harvested and the β-galactosidase activities were determined. As can be seen from the data, the presence of Nva-FMDP had no significant effect on *glmY-lacZ* expression, irrespective whether the fusion was expressed from the wild-type promoter, the σ^{70}-

promoter or the σ^{54}-promoter (Fig. S9). Taken together, these data demonstrate that GlcN6P controls the GlmY amount by a post-transcriptional mechanism (see model in Fig. 25).

Discussion

Control of transcription by multiple promoters recognized by alternative σ-factors is a widespread principle in bacteria. One example is provided by the *E. coli rpoH* gene which is transcribed from two σ^{70}-dependent promoters and at least from one σ^{32}- and one σ^{24}-dependent promoter (Yura *et al.*, 1993). Multiple promoters allow the cell to trigger gene expression in response to different environmental signals. However, these promoters have different transcriptional start sites and generate transcripts of different length. Whereas this is without consequence for the proteins encoded by mRNAs, it has a large impact on sRNAs since species with different 5'-ends are generated, which may have functional consequences. Indeed, in the case of the IstR-1 and IstR-2 sRNAs, which are transcribed from two consecutive promoters and therefore differ in the 5'-end, exclusively the shorter IstR-1 variant is able to repress toxic peptide TisB production (Darfeuille *et al.*, 2007). In this respect, it was an open question whether one and the same sRNA can be generated by transcription from different promoters in response to different cellular signals.

In this work, we demonstrate that two promoters, which are recognized by different σ-factors, overlap in a manner to start transcription of the sRNA *glmY* gene at the same position (see model in Fig. 25). To the best of our knowledge such an arrangement is an unprecedented case. A previous work suggested GlmY to be expressed from a σ^{54}-dependent promoter (Urban *et al.*, 2007). To our surprise, GlmY was still detectable in the absence of σ^{54}. In particular, elimination of σ^{54} strongly reduced the GlmY-level during transition to the stationary growth phase, while it had only a minor impact on *glmY* transcription and accumulation during exponential growth (Figs. 19 and 21).

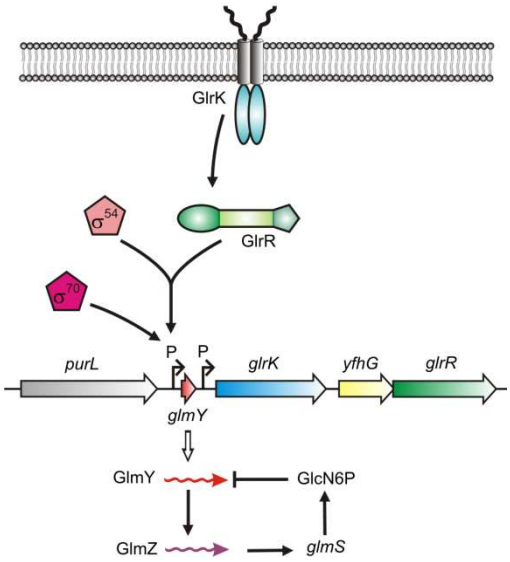

Figure 25: Model for control of small RNA *glmY* transcription as revealed by this study. Gene *glmY* can be transcribed from two different promoters, which perfectly overlap and start transcription at the same position. Response regulator GlrR binds to three conserved sites in the *purL-glmY* IGS and thereby activates transcription initiation at the σ^{54}-dependent *glmY* promoter. The DNA binding activity of GlrR is presumably triggered by phosphorylation catalyzed by the cognate membrane-bound sensor kinase GlrK. The genes encoding GlrR and GlrK are present downstream of *glmY* and separated by gene *yfhG* whose function is still unknown. These three genes putatively form an operon, which is transcribed from a σ^{70}-promoter in the *glmY-glrK* IGS. In the absence of the signal activating GlrR, *glmY*-transcription can still proceed from the σ^{70}-*glmY* promoter. This basal transcription level is sufficient to allow activation of the GlmY-GlmZ-*glmS* regulatory cascade in response to GlcN6P depletion. Hence, GlcN6P deprivation induces accumulation of GlmY by a post-transcriptional mechanism.

Inspection of the *glmY* promoter region revealed putative -35/-10 sequence motifs of a σ^{70}-dependent promoter whose -10 sequence partially overlaps with the -12 sequence motif of the predicted σ^{54}-promoter (Fig. 20 A). Neither mutation of the -24 sequence of the σ^{54}-promoter nor mutation of the -10 sequence had a large negative impact on *glmY* transcription during exponential growth, while promoter activity was completely lost when both mutations were combined (Fig. 20 B). Mutation of the -10 sequence rendered transcription of *glmY* fully dependent on σ^{54}, whereas it was σ^{54}-independent when the -24 sequence was mutated (Fig. 21 B). Hence, there are two overlapping promoters controlling *glmY* expression and 5'-RACE analyses showed that they start transcription at the same position (Fig. 20 C). In agreement with the Northern blot data (Fig. 19), reporter gene assays showed that the σ^{70}-promoter is predominantly active during exponential growth whereas activity of the σ^{54}-promoter strongly increases during transition to stationary phase (Fig. S6). This is in agreement with the known properties of

σ^{70}- and σ^{54}-dependent transcription. Interestingly, overlapping σ^{70}- and σ^{54}-promoter sequences are also detectable in front of *glmY* in other enterobacterial species, suggesting that this is a conserved feature (Fig. S7).

Open complex formation at σ^{54}-dependent promoters requires ATP hydrolysis catalyzed by an activator protein that interacts with σ^{54}. Usually these activator proteins bind as oligomers at DNA-sites located far upstream of the promoter they control. Interaction between σ^{54} and the activator protein occurs via DNA-looping, which is often supported by DNA-bending proteins, such as the integration host factor (IHF) (Wigneshweraraj *et al.*, 2008). Three copies of a highly conserved sequence motif ($TGTCN_{10}GACA$) reside in the *purL-glmY* intergenic (designated ABS1 to 3). A further conserved sequence motif exhibiting similarity to an IHF-binding site (Swinger and Rice, 2004), is present five base-pairs downstream of ABS2 (Fig. S7). Downstream of *glmY*, genes *glrK-yfhG-glrR* are present and GlrK and GlrR (formerly designated YfhK and YfhA) were shown to form a TCS. Response regulator GlrR consists of an N-terminal receiver domain containing the phosphorylated aspartate residue, a central domain with homology to the σ^{54} interaction module and a C-terminal helix-turn-helix DNA-binding domain. Transcription of *glrK* starts 34 bp downstream of the *glmY* gene (Fig. 18) and is thus not driven from the σ^{54}-promoter in front of *glmY* as previously proposed (Reitzer and Schneider, 2001). Putative -35/-10 sequence motifs of a σ^{70}-dependent promoter are present (Fig. 18 C). Using EMSA, specific binding of response regulator GlrR to the DNA region located between the *purL* terminator and the *glmY* promoters was observed (Fig. 22). Each of the three ABS present in this region contributes to GlrR-binding and presence of at least one ABS is essential for binding (Fig. 23 B). Genetic analysis revealed that the GlrK/GlrR-TCS is essential for activity of the σ^{54}-*glmY* promoter whereas it is dispensable for activity of the σ^{70}-*glmY* promoter (Fig. 21 B). In agreement, activity of the σ^{54}-promoter in front of *glmY* completely relies on the presence of GlrR binding sites ABS 2 and 3 (Fig 22 B and Fig. 23 A). Taken together, a model for *glmY* transcription is proposed as depicted in Fig. 25: A basal level of *glmY* transcription is provided from the σ^{70}-dependent promoter. Expression of *glmY* can be further increased by activation of the overlapping

σ^{54}-dependent promoter. This takes place upon activation of response regulator GlrR by sensor kinase GlrK, which allows binding of GlrR to its sites on the DNA located more than 117 bp upstream of *glmY*. Interaction of GlrR with σ^{54} allows open complex formation and transcription initiation at the σ^{54}-promoter. It should be noted that both promoters do not operate independently from each other. Mutations that prevent binding of σ^{54}, e.g. by deleting σ^{54} or by mutating the -24 sequence motif, result in higher σ^{70}-promoter activities than those observed upon mutation of *glrK* or *glrR* (Fig. 21 B). This suggests that binding of σ^{54} to some degree limits access of the σ^{70}-RNA polymerase to the *glmY* promoter.

The sRNAs GlmY and GlmZ are homologous and form a functional unit to control expression of the *glmS* gene. In this respect, it is an interesting question how transcription of *glmZ* is controlled. A sequence alignment of the DNA region upstream of *glmZ* reveals a σ^{70}-promoter preceding the *glmZ* gene in *E. coli*, *Shigella* and in *Klebsiella pneumoniae* (Fig. S10). A 5'→3' deletion analysis of the *aslA-glmZ* intergenic region in *E. coli*-K12 confirmed that *glmZ* is transcribed from this σ^{70}-promoter and no regulatory upstream sequences required for promoter activity were detectable (D. Lüttmann and B. Görke, unpublished). In contrast, in *Salmonella* and *Yersinia* species and other *Enterobacteriaceae* a σ^{54}- rather than a σ^{70}-dependent promoter is present in front of *glmZ* (Fig. S10). Intriguingly, GlrR binding sites including the putative IHF-site precede the promoter in these cases, reminiscent of the organization of the *glmY* control region. These observations let us speculate that (I) the GlrK/GlrR TCS regulates *glmZ* in addition to *glmY* transcription in several enteric bacteria but not in *E. coli*, *Shigella* and *Klebsiella* and (II) that the GlmY/GlmZ system has been evolved by gene duplication in an ancestor of *Enterobacteriaceae* and (III) that in a subset of these bacteria including *E. coli* and *Shigella glmYZ* partially lost σ^{54}-dependency being in transition to a σ^{70}-dependent system.

What is the biological function of the GlrK/GlrR-exerted control of *glmY* expression? We demonstrated that this system is not concerned with the GlcN6P-dependent feedback-control of *glmS* synthesis. In mutants lacking σ^{54}, GlrK or GlrR up-regulation of the GlmY/GlmZ/*glmS* regulatory cascade in

response to GlcN6P depletion is still possible (Fig. 24). Furthermore, reporter gene assays indicate that neither the σ^{54}- nor the σ^{70}-promoter of *glmY* responds to the GlcN6P concentration (Fig. S9). Hence GlcN6P exerts its effect at the post-transcriptional level, obviously by slowing down GlmY degradation (Fig. 25). Indeed, the GlrK/GlrR TCS strongly up-regulates *glmY* transcription when cells enter the stationary growth phase (Fig. 19 and Fig. S8), i.e. when cell wall and outer membrane syntheses stop and ongoing GlcN6P synthesis is not required. Under these conditions GlmZ and *glmS* RNAs do not accumulate, although GlmY-levels are fairly high. This is presumably due to the reduced activities of the σ^{70}-promoters driving expression of GlmZ and *glmS*. Hence, GlmY must have additional regulatory roles, which become most relevant in the stationary growth phase and the GlrK/GlrR-TCS senses and transduces the corresponding signal. These topics provide the future road to be travelled in the field of GlmY research.

Acknowledgements

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Supplementary material

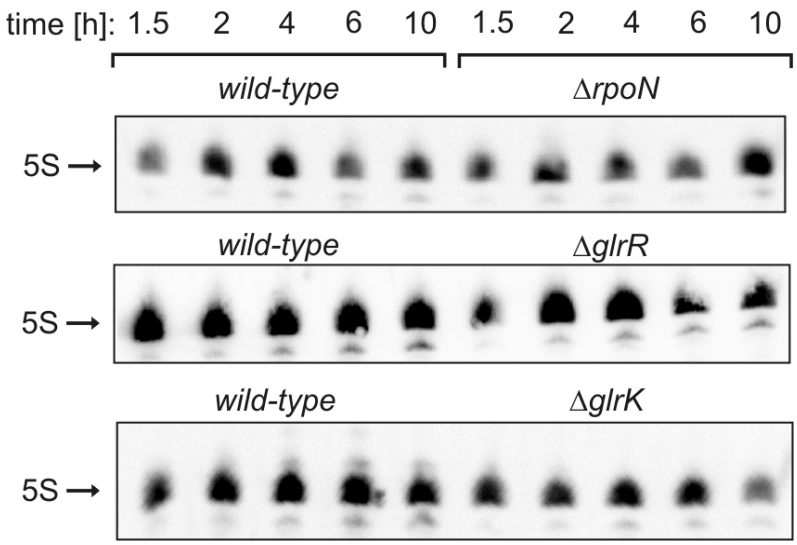

Figure S5: Loading control for Northern blots presented in Fig. 18. The blots presented in Fig. 18 were re-probed using an RNA-probe specific for 5S rRNA. The 5S rRNA probe was obtained by *in vitro* transcription using the DIG-Labeling kit (Roche Diagnostics) and a DNA fragment as template, which was generated by PCR using primers BG287 and BG288.

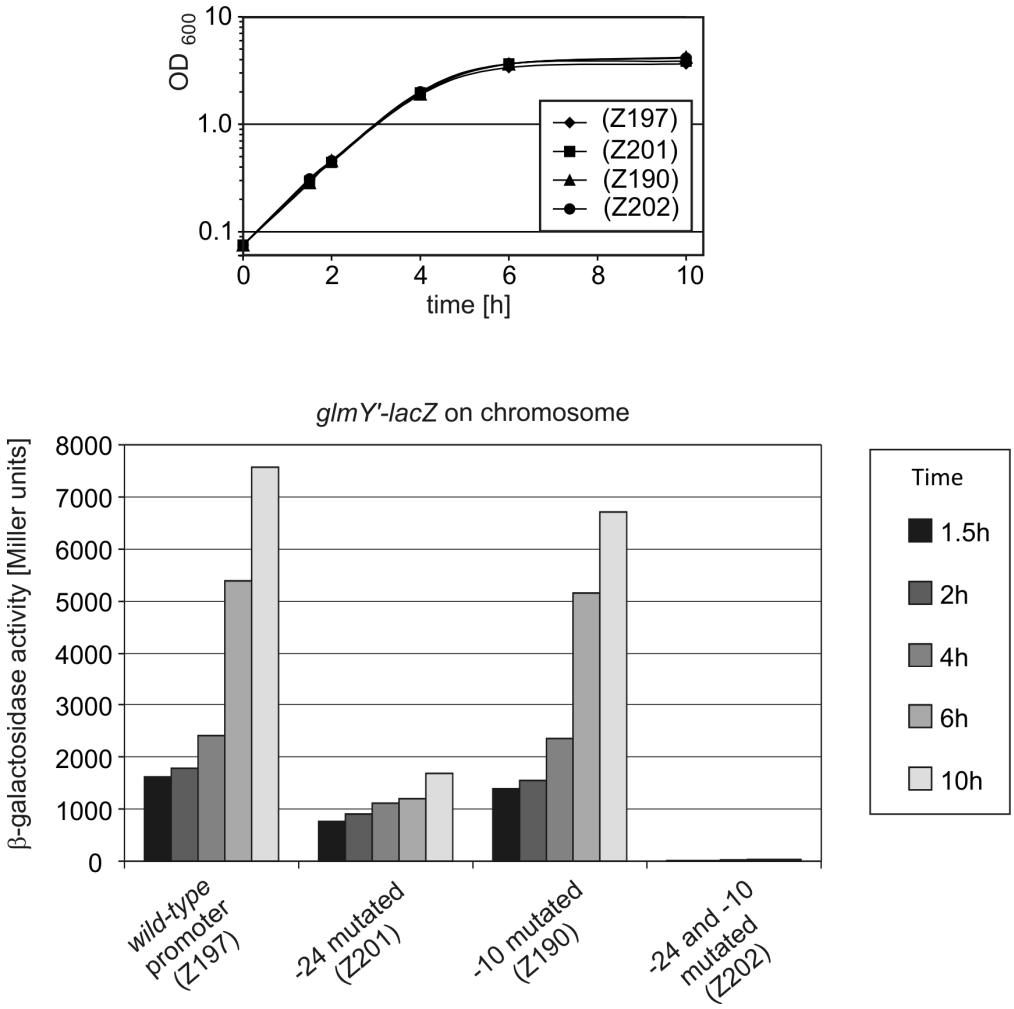

Figure S6: Activities of the σ^{54}- and σ^{70}-dependent *glmY* promoters at various time points during growth. The same strains as tested in Fig. 20 B were used. Strain Z197: *glmY'-lacZ* transcribed from the *glmY* wild-type promoter; strain Z201: -24 sequence motif mutated; Z190: -10 sequence mutated; strain Z202: -24 and -10 sequences mutated. Samples were harvested at different time points during growth as indicated in the legend and the β-galactosidase activities were determined. The corresponding growth curves are shown at the top.

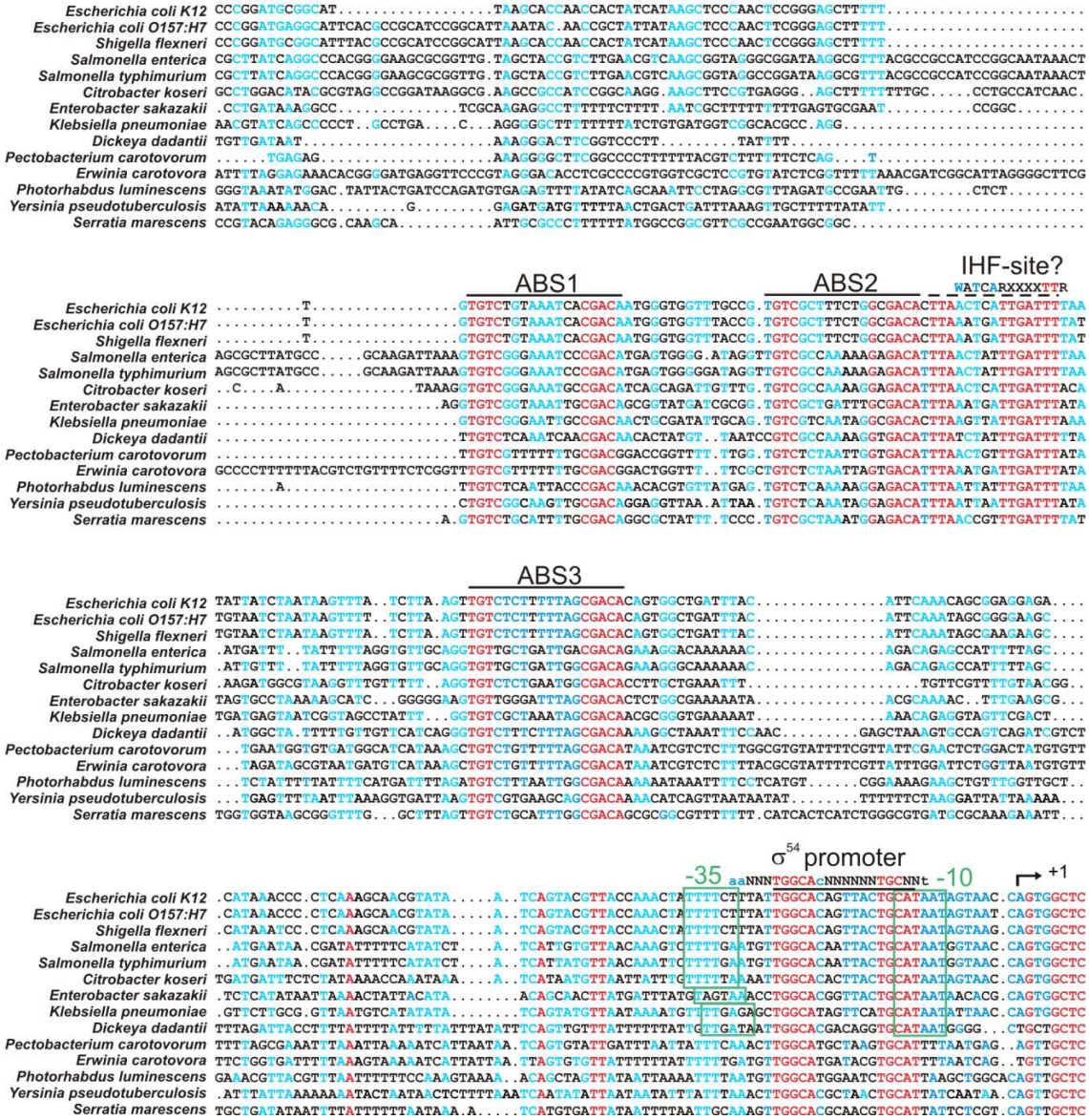

Figure S7: Sequence alignment of the *purL-glmY* intergenic region from 14 enterobacterial species. The *glmY* transcriptional start site is marked with an arrow. Fully conserved nucleotide positions are highlighted in red and positions conserved in the majority of these sequences are in blue. The putative GlrR activating binding sites (ABS) and the -24/-12 sequence motifs of putative σ^{54} promoters are marked with continuous lines. An additional conserved region present downstream of ABS2 (indicated by a dashed line) has similarity to the consensus sequence of integration host factor IHF (Swinger and Rice, 2004): WATCARXXXXTTR (W = A or T; R = A or G; X = A, T, C or G). The σ^{54}- and IHF-consensus sequences are shown above the alignment. The -35 and -10 sequence motifs of putative σ^{70}-promoters are boxed in green. Sequences were compiled from the following genomes (NCBI accession numbers are in parentheses): *Escherichia coli* K12 (NC_000913), *Escherichia coli* O157:H7 str. EC4486 (NZ_ABHS00000000), *Shigella flexneri* 5 str. 8401 (NC_008258), *Salmonella enterica* subsp. enterica serovar typhi str. CT18 (NC_003198), *Salmonella typhimurium* LT2 (NC_003197), *Citrobacter koseri* ATCC BAA-895 (NC_009792), *Enterobacter sakazakii* (NC_009778), *Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 (NC_009648), *Dickeya dadantii* 3937 [http://www.microbesonline.org], *Pectobacterium carotovorum* subsp. carotovorum WPP14 (NZ_ABVY00000000), *Erwinia carotovora* subsp. atroseptica (NC_004547), *Photorhabdus luminescens* subsp. laumondii TT01 (NC_005126), *Yersinia pseudotuberculosis* IP31758 (NC_009708), *Serratia marescens* Db11 [http://www.sanger.ac.uk]. The alignment was compiled using the AlignX tool from Vector NTI Suite 10.3.0.

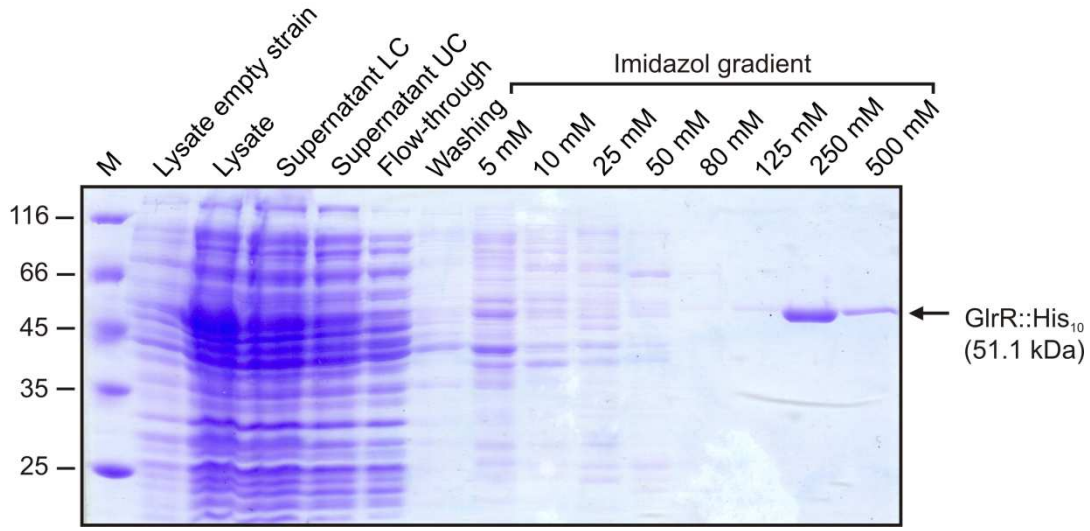

Figure S8: Overproduction and purification of recombinant GlrR::His$_{10}$ protein. Recombinant GlrR::His$_{10}$ was purified as described under "Experimental procedures". Aliquots of the lysate and the purification steps were analyzed by SDS-PAGE and Coomassie staining. An aliquot of a crude lysate of a transformant, which carried the empty expression vector was analyzed for comparison (Lane 2). The sizes [kDa] of the protein molecular weight marker (lane 1; Fermentas) are shown at the left.

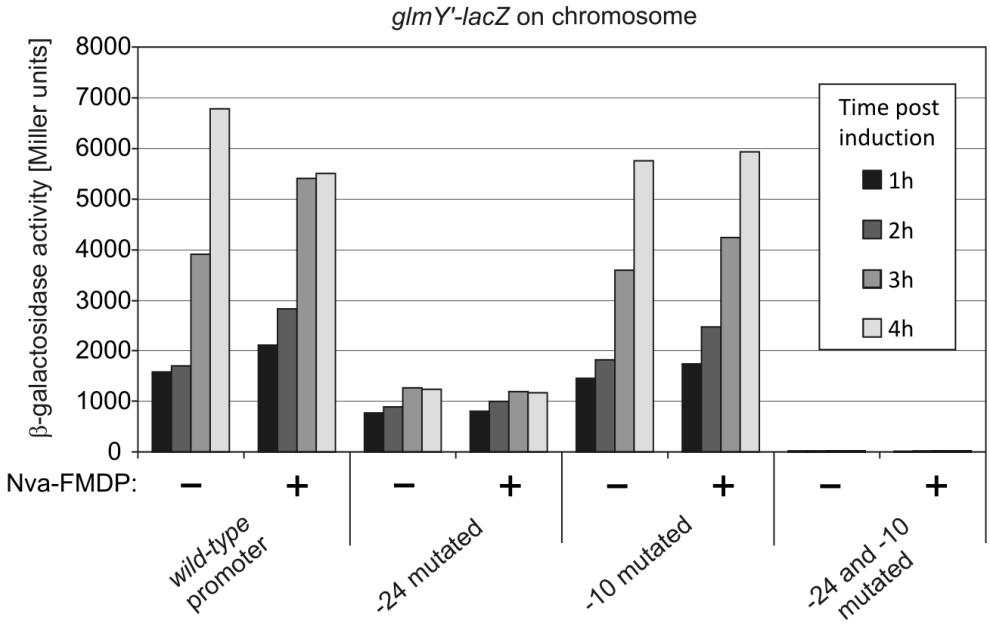

Figure S9: Glucosamine-6-phosphate does not affect initiation of *glmY* transcription neither at the σ^{70}- nor at the σ^{54}-promoter. Cultures of strains carrying the *glmY'-lacZ* gene fusion on the chromosome were grown to exponential phase and split and growth was continued in the absence or presence of Nva-FMDP [100 µg/ml], which inhibits GlmS enzymatic activity. Subsequently samples were harvested at various time-points as indicated in the legend and the β-galactosidase activities were determined. The following strains were used: Z197 (*glmY'-lacZ* transcribed from the wild-type promoter), Z201 (-24 sequence motif mutated), Z190 (-10 sequence mutated) and strain Z202 (-24 and -10 sequences mutated).

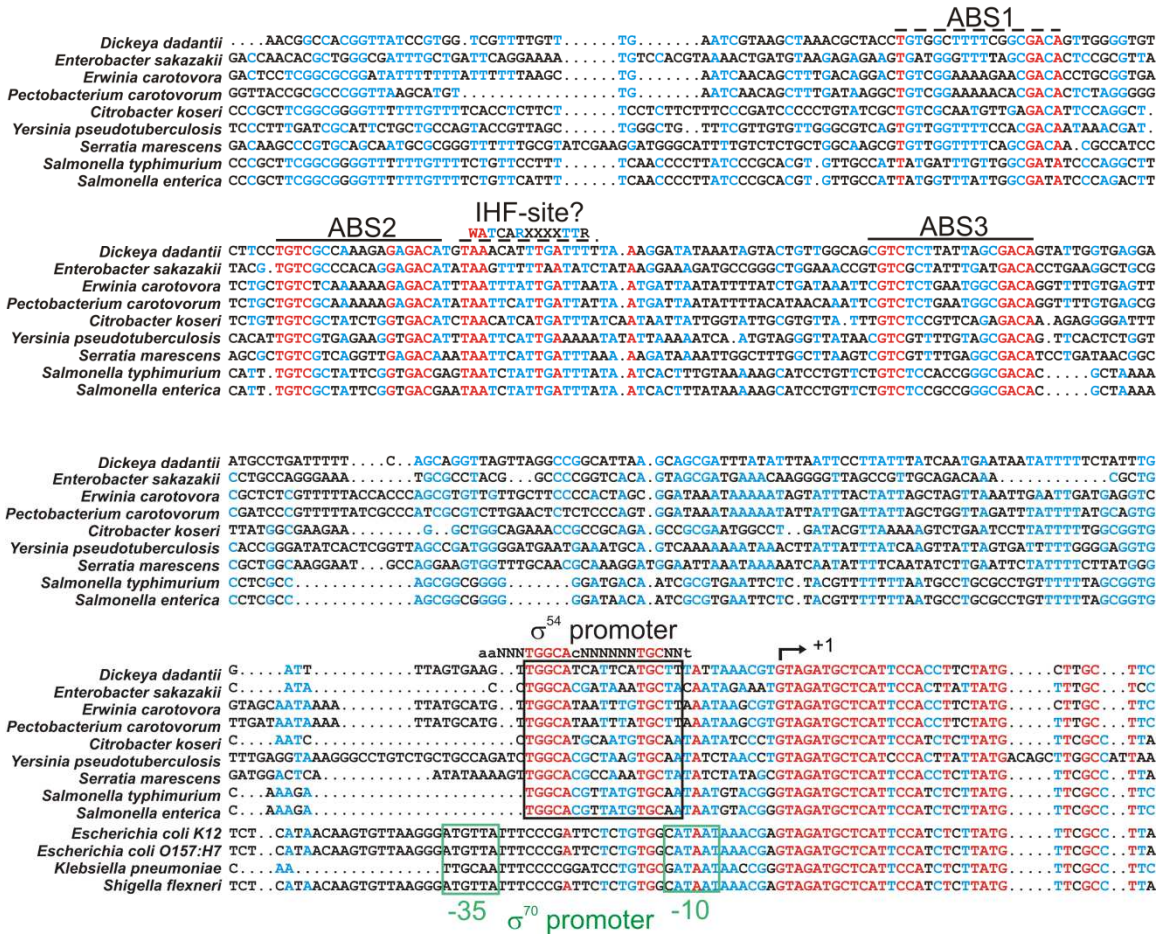

Figure S10: Sequence alignment of the *glmZ* upstream region from 13 enterobacterial species. The *glmZ* transcriptional start site is marked with an arrow. See legend to Fig. S7 for further information. The used genome sequences were the same as in Fig. S7 except for *Photorhabdus luminescens*, which was omitted.

Table S2: Plasmids used in this study

Name	Genotype or relevant structures	Reference or construction
pBAD18-cm	P_{ara}, MCS, *cat*, ori pBR322	(Guzman *et al.*, 1995)
pBGG201	Fusion of *glmY*' (-238 to +22) to *lacZ*	this work
pBGG202	Fusion of *glmY*' (-208 to +22) to *lacZ*	this work
pBGG204	Fusion of *glmY*' (-108 to +22) to *lacZ*	this work
pBGG208	Fusion of *glmY*' (-238 to +22) to *lacZ,* -24 region mutated	this work
pBGG209	Fusion of *glmY*' (-238 to +22) to *lacZ,* -10 region mutated	this work
pBGG210	Fusion of *glmY*' (-238 to +22) to *lacZ,* -10 and -24 region mutated	this work
pBGG213	Fusion of *glmY*' (-208 to +22) to *lacZ,* -10 region mutated	this work
pBGG215	Fusion of *glmY*' (-108 to +22) to *lacZ,* -10 region mutated	this work
pBGG219	*glrR*:: His_{10} in pKES170	this work
pBGG226	Fusion of *glmY*' (-138 to +22) to *lacZ*	this work
pBGG227	Fusion of *glmY*' (-138 to +22) to *lacZ,* -10 region mutated	this work
pBGG305	Fusion of *glmY*' (-238 to +22) to *lacZ*, ABS1 mutated	this work
pBGG306	Fusion of *glmY*' (-238 to +22) to *lacZ*, ABS2 mutated	this work
pBGG307	Fusion of *glmY*' (-238 to +22) to *lacZ*, ABS3 mutated	this work
pBGG308	Fusion of *glmY*' (-238 to +22) to *lacZ,* ABS1+ABS2 mutated	this work
pBGG309	Fusion of *glmY*' (-238 to +22) to *lacZ,* ABS1+ABS3 mutated	this work
pBGG310	Fusion of *glmY*' (-238 to +22) to *lacZ,* ABS2+ABS3 mutated	this work
pBGG311	Fusion of *glmY*' (-238 to +22) to *lacZ,* ABS1+ABS2+ABS3 mutated	this work
pCP20	FLP recombinase gene, *bla*, *cat,* ori pSC101-rep^{TS}	(Datsenko and Wanner, 2000)
pKES15	*bgl'-lacZ,kan, attP, aadA,* ori pACYC177	(Nagarajavel *et al.*, 2007)
pKES170	$lacI^q$, *Ptac*, T7gene10-RBS, NdeI, XbaI, *rrnBT1/T2*, *bla*,pBR322-ori	Karin Schnetz
pLDR8	λ *int* under control of λP_R, λcI_{857}, *kan*, ori pSC101-rep^{TS}	(Diederich *et al.*, 1992)

Table S3: Oligonucleotides used in this study.

Primer	Sequence[a]	Res. sites	Position[b]
BG287	TGCCTGGCGGCCGTAG		*rrfD* +1 to +16
BG288	CTAATACGACTCACTATAGGGAGAGCCTGGCAGTTCCCTAC		*rrfD* +118 to +102
BG377	GCACGCGTCGACCTTTTTTGTGTCTGTAAATCACG	SalI	*glmY* -238 to -213
BG378	GCACGCGTCGACGTGGTTTGCCGTGTCGCTTTC	SalI	*glmY* -208 to -187
BG446	ctaatacgactcactatagggagaAGTGGCTCATTCACCGACTTATGTC		*glmY* +1 to +25
BG456	GCTCTAGAATAAGTCGGTGAATGAGCCAC	XbaI	*glmY* +22 to +2
BG472	CCGAGGGGAAGTTCAGATAC		*glmY* +204 to +185
BG480	GCACGCGTCGACGATTTACATTCAAACAGCGGAG	SalI	*glmY* -108 to -86
BG481	GCTCTAGAATAAGTCGGTGAATGAGCCACTGGTTACTATTATGCA GTAACTGT**CATG**ATAAAGAAAATAG	XbaI	*glmY* +22 to -40
BG482	GGCTCTAGAATAAGTCGGTGAATGAGCCACTGGTTACT**CCC**ATGC AGTAACTGTGC	XbaI	*glmY* +22 to -25
BG483	GCTCTAGAATAAGTCGGTGAATGAGCCACTGGTTACT**CCC**ATGCA GTAACTGT**CATG**ATAAAGAAAATAG	XbaI	*glmY* +22 to -40
BG484	CTCGTACTCATATGAGCCATAAACCTGCGC	NdeI	*glrR* +1 to +19
BG485	GGCTCTAGATTCCTTGAAATCGTTTGCATC	XbaI	*glrR* +1332 to +1312
BG496	GCACGCGTCGACAAGTTGTCTCTTTTTAGCGACACAG	SalI	*glmY* -138 to -113
BG527	ctaatacgactcactatagggagaAAGTCAGCGAAGGAAATG		*sraC* +1 to +19
BG528	ATCACCAGAACGGGCGG		*sraC* +250 to +233
BG558	GCACGCGTCGACCTTTTTTG**ACA**CTGTAAATCACGACAATGGG	SalI	*glmY*-238 to -208
BG559	[P]-GTCGCCAGAAAGCG**TGT**CGGCAAACCACCC		*glmY* -181 to -210
BG560	GACAACTTAAGATAAACTTATTAG		*glmY* -131 to -154
BG578	CGGTGAAGGGCAATCAGCTG		*lacZ* -271 to -252
BG579	GGCCTCTTCGCTATTACGCC		*lacZ* +129 to +110
BG580	ATTAATGCAGCTGGCACGACAG		*lacZ* -171 to -150
BG581	ACGGCCAGTGAATCCGTAATC		*lacZ* +29 to + 9
BG582	[P]-GTTGTCTCTTTTTAGCG**TTT**CAGTGGCTGATTTAC		*glmY* -136 to -102
BG618	RNA[AUAUGCGCGAAUUCCUGUAGAACGAACACUAGAAGAAA]		
BG619	CGCGAGCTCGCGCGAATTCCTGTAGA	SacI	BG618 +5 to +21
BG620	GTTACGTTGGTGTAGATG		*lacZ* +314 to +296
BG622	GTGCCTAACTCGACG		*glmY* +142 to +127
BG623	GCGTCTAGACTCGACGTTTCGCCCG	XbaI	*glmY* +134 to +119
BG624	CTGGCTTTGATAAACCTTC		*glrK* +270 to +251
BG625	GCGTCTAGACTTTGATAAACCTTCGCCAG	XbaI	*glrK* +266 to +247
BG649	GCACGCGTCGACGTTGGTGTAGATGGGCG	SalI	*lacZ* +309 to +292

[a]Restriction sites are underlined; The promoter of T7-RNA polymerase is in small type; Nucleotide positions that differ from the wild-type sequence are in boldface; [P] indicates 5'-phosphorylation of the oligonucleotide. [b]Positions are relative to the first nucleotide of the respective gene. Gene names are according to http://ecocyc.org/ except for *glrR* and *glrK*, which were formerly named *yfhA* and *yfhK*, respectively.

5. YhbJ is involved in modulation of activity of the *glmY* σ^{54} dependent promoter in *Escherichia coli*

Summary

Gene *yhbJ* encodes a protein of unknown function, which has been recently implicated in the small RNA (sRNA) dependent activation of glucosamine-6-phosphate synthase (GlmS) expression. Translation of the *glmS* mRNA is activated by a sRNA cascade consisting of GlmY and GlmZ in a feed-back loop that is activated upon a decrease of the intracellular glucosamine-6-phosphate concentration. We previously showed that deletion of *yhbJ* abrogates processing of GlmZ thereby leading to accumulation of GlmZ and activating *glmS* expression. At the same time the amounts of the upstream acting sRNA GlmY are strongly reduced in the cell. Here we report that this reduction of GlmY amounts in the Δ*yhbJ* mutant is not due to a decreased stability of the GlmY sRNA as previously proposed. Instead, it is due to a strongly reduced activity of the *glmY* σ^{54}-dependent promoter in a Δ*yhbJ* mutant strain, which is independent of the GlmZ dependent activation of *glmS* expression. Further analyses showed that not only the *glmY* σ^{54}-dependent promoter is modulated by YhbJ, but that also the *argT* and *rtcB* σ^{54}-dependent promoters are affected upon deletion of *yhbJ*. We therefore propose that YhbJ might be a modulator of σ^{54}-promoter activity of at least some σ^{54}-dependent promoters.

Introduction

Most organisms live in variable environments and have adapted to cope with difficulties, which might arise from changes in environmental conditions. Cells employ various strategies to adapt to environmental cues such as changed nutrient supply, changed osmolarity or various potentially life threatening stress conditions such as heat stress, oxidative stress or attack by antibiotics. One possibility to respond to an altered condition is to adjust the transcription rates of specific genes by activating or repressing promoter activities through the action of DNA-binding proteins. Another option is the use of alternative sigma factors, which are able to recognize promoter consensus sequences

that diverge from the promoter sequences, which are recognized by the general house keeping sigma factor σ^{70}. The use of alternative sigma factors allows cells to turn on or turn off a large set of genes in response to a signal. *Escherichia coli* possesses altogether seven different sigma factors, including the housekeeping sigma factor σ^{70} (Reitzer and Schneider, 2001). Most sigma factors are homologous to σ^{70}. In contrast, the σ^{54} factor does not share significant sequence homology with σ^{70} (Merrick, 1993; Merrick and Gibbins, 1985). σ^{54} is special among sigma factors because it absolutely depends on binding of activator proteins to the DNA and subsequent ATP hydrolysis to form an open complex and initiate transcription (Reitzer and Schneider, 2001). This specialty allows the cell to completely shut off transcription of σ^{54}-dependent genes that are not required at a certain time. The σ^{54}-factor is encoded by gene *rpoN* within the *rpoN-hpf-ptsN-yhbJ-npr* operon (Reitzer and Schneider, 2001). This operon encodes pleiotropic regulatory proteins, including IIANtr, which regulates potassium transport (Lüttmann *et al.*, 2009) and Hpf, which regulates stationary phase storage of ribosomes (Ueta *et al.*, 2005). Interestingly, both σ^{54} and YhbJ are involved in regulation of factors that in turn regulate *glmS* expression (Kalamorz *et al.*, 2007; Urban *et al.*, 2007; Reichenbach *et al.*, 2008; Reichenbach *et al.*, 2009).

Gene *glmS* encodes the enzyme glucosamine-6-phosphate synthase (GlmS), which catalyzes the first dedicated step of amino sugar biosynthesis: the synthesis of glucosamine-6-phosphate (GlcN6P) and glutamate from fructose-6-phosphate and glutamine (Milewski, 2002). GlcN6P is subsequently converted to UDP-*N*-acetylglucosamine by the action of enzymes GlmM and GlmU, which are encoded by *glmM* and *glmU*, respectively (Mengin-Lecreulx and van Heijenoort, 1993; Mengin-Lecreulx and van Heijenoort, 1994; Mengin-Lecreulx and van Heijenoort, 1996). Both *glmU* and *glmS* are encoded in the bi-cistronic *glmUS* operon (Plumbridge, 1995). While the activity of GlmU is needed all the time, GlmS is only needed, when no external amino sugars are available. Therefore, differential expression of *glmU* and *glmS* is necessary. This differential expression is achieved post-transcriptionally by RNase E dependent processing of the *glmUS* transcript and the action of the small regulatory RNAs (sRNAs) GlmY and GlmZ (Görke

and Vogel, 2008). GlmY and GlmZ are homologous in sequence and secondary structure (Urban and Vogel, 2008; Reichenbach *et al.*, 2008) and act in a GlmS translation activating feed-back cascade in response to low intracellular GlcN6P levels (Kalamorz *et al.*, 2007; Reichenbach *et al.*, 2008; Urban and Vogel, 2008). The GlcN6P signal is sensed by GlmY and then transduced to GlmZ, which accumulates in its active unprocessed form and then activates GlmS translation by base-pairing with the *glmS* transcript. Binding of GlmZ to the *glmS* transcript resolves an inhibitory stem loop structure that buries the RBS. Hence, *glmS*-GlmZ base-pairing allows increased translation of GlmS. Despite its sequence homology with GlmZ, GlmY does not possess the sequence elements, which are necessary for binding to the *glmS*-transcript (Urban and Vogel, 2008; Reichenbach *et al.*, 2008). Therefore, a factor must exist, which facilitates signal transduction from GlmY to GlmZ. Currently, all available data indicate that YhbJ might be this factor. Deletion of gene *yhbJ* leads to a total abrogation of GlmZ processing, which is more stable in its unprocessed form and therefore accumulates (Kalamorz *et al.*, 2007). Accumulation of unprocessed GlmZ then results in activation of *glmS* expression (Kalamorz *et al.*, 2007). Interestingly, deletion of *yhbJ* has contrary effects on GlmY and GlmZ. While GlmZ accumulates, GlmY becomes destabilized and GlmY amounts are diminished as compared to the wild type (Reichenbach *et al.*, 2008). This led us to hypothesize that YhbJ might be a RNA-binding protein that is able to bind GlmY and GlmZ. Under non-inducing conditions it might preferentially bind to GlmZ and mediate its processing. When GlcN6P levels become low in the cell, GlmY accumulates. Accumulation of GlmY might titrate YhbJ away from GlmZ by a mimicry-mechanism, which would then lead to reduced GlmZ processing and accumulation of the active unprocessed form of GlmZ. This would then activate GlmS translation (Görke and Vogel, 2008).

YhbJ is not the only factor controlling the amount of GlmY. Other factors also govern intracellular GlmY levels. Deletion of the poly(A)polymerase I encoding gene *pcnB* results in increased expression of *glmS* (Joanny *et al.*, 2007). Poly(A)polymerase I adds poly(A) tails to RNAs, which possess stable stem loop structures at their 3'end. Polyadenylation makes these stable RNA species better substrates for the RNA degrading enzyme PNPase (Khemici

and Carpousis, 2004). It was first assumed that poly(A)polymerase I acts directly on the *glmS* mRNA (Joanny *et al.*, 2007), but later it was shown that actually GlmY is the target of poly(A)polymerase I (Reichenbach *et al.*, 2008; Urban and Vogel, 2008). Deletion of *pcnB* results in accumulation of GlmY, which prevents GlmZ processing and thereby induces accumulation of *glmS*. In addition to mutation of *pcnB* also mutation of *yhbJ* alters the amounts of GlmY in the cell. Upon deletion of *yhbJ*, intracellular levels of GlmY are strongly decreased during stationary phase as compared to the wild type (Reichenbach *et al.*, 2008). Interestingly, GlmY is not only regulated post transcription but also transcription of GlmY is regulated. Gene *glmY* is transcribed from two overlapping promoters, which generate identical GlmY species. One promoter is a σ^{70}-dependent promoter, while the other one depends on σ^{54} (Urban *et al.*, 2007; Reichenbach *et al.*, 2009). The σ^{70}-dependent promoter is active mainly during exponential growth and does not seem to be regulated (Reichenbach *et al.*, 2009). The σ^{54}-dependent promoter is activated by the DNA-binding protein GlrR. GlrR binds to three activator binding sites, which are located upstream of the σ^{54}-dependent promoter. It is part of a two component system consisting of the response regulator GlrR and its cognate sensor kinase GlrK. Genes *glrK* and *glrR* are encoded immediately downstream of *glmY* within the *glrK-yfhG-glrR* operon, which is transcribed independently of *glmY* from a σ^{70}-dependent promoter (Yamamoto *et al.*, 2005; Reichenbach *et al.*, 2009). The signal, which is sensed by GlrK and activates the σ^{54}-promoter in a GlrR-dependent manner, is unknown. This signal should be present in normally growing cells, since the σ^{54}-dependent promoter is highly active under all tested conditions (Reichenbach *et al.*, 2009).

In this work we investigated the function of YhbJ in more detail. We find that YhbJ modulates the activity of the σ^{54}-dependent promoter that is located upstream of *glmY* and that also the activities of other σ^{54}-dependent promoters are modulated by YhbJ. It is likely that the effects of a Δ*yhbJ* mutation on GlmY and GlmZ are independent from each other.

Material and Methods

Growth conditions, strains and plasmids

Cells were routinely grown in LB medium or M9 minimal medium at 37℃ (unless otherwise indicated) under agitation (200 rpm). When necessary the medium was supplemented with antibiotics (spectinomycin [50 µg/ml], ampicillin [100 µg/ml], chloramphenicol: [15 µg/ml], tetracycline [12.5 µg/ml], kanamycin [30 µg/ml]) or with IPTG [1 mM]. Minimal medium was supplemented with proline [40 µg/ml] and thiamine [1 µg/ml]. For analysis of *rne*^{TS} strains, cells were grown at 30℃ until early exponential phase (OD_{600} = 0.3). At this point, cultures were split and growth was continued at 44℃ or at 30℃. All strains and plasmids used in this study and the characteristics and genotypes are listed in tables 5 and 6, respectively. Integration of *lacZ* fusions into the λ attachment site (*attB*) of the *E. coli* chromosome was achieved as described previously (Diederich *et al.*, 1992). Antibiotic resistance cassettes were removed from strains via pCP20 as described previously (Datsenko and Wanner, 2000). All strain constructions were checked by colony PCR using appropriate primers. Oligonucleotides used in this study are listed in table 7. For construction of plasmids pBGG325 and pBGG396, SalI/XbaI digested PCR fragments, which were amplified by PCR from *E. coli* W3110 chromosomal DNA using oligos BG598/BG599 and BG702/BG703, respectively, were inserted between the SalI and XbaI sites of pKES15.

Table 5: Strains used in this study.

Strain	Genotype	Source or reference
IBPC633	As N3433, but *rnc*105 *nadB*51 ::*Tn10* (*tet*)	(Régnier and Hajnsdorf, 1991)
IBPC935	As N3433, but *rng::cm*^{R}	(Bardey *et al.*, 2005)
JC357	F-, *argG*6, *metB*1, *his*-1, *leu*-6, *mtl*-2, *xyl*-7, *malA*1, *gal*-6, *lacY*1, *tonA*2, *tsx*-1, *supE*44, *rpsL*, *pnp::Tn5* (*Kan*^{R}), λ^{R}, λ^{-}, *recA*1	(Portier *et al.*, 1981)
MG1693	*thyA715*	(Arraiano *et al.*, 1988)
N3431	*lacZ43 (Fs) rne3071(ts) relA1 spoT1 thi1*	(Goldblum and Apririon, 1981)
N3433	as N3431 but wild type *rne*	(Goldblum and Apririon, 1981)
R1279	CSH50 Δ(*pho-bgl*)201 Δ(*lac-pro*) *ara thi*	(Görke and Rak, 1999)
S3768	As N3431, but *rnc*105 *nadB*51 ::*Tn10* (*tet*)	Karin Schnetz
SK5691	as MG1693 but *pnp-7 rph-1 thyA715*	(Arraiano *et al.*, 1988)
Z37	As R1279, but Δ*yhbJ*	(Kalamorz *et al.*, 2007)
Z45	As R1279, but Δ*glmZ*	(Kalamorz *et al.*, 2007)
Z96	As R1279, but Δ*glmY*	(Reichenbach *et al.*, 2008)
Z115	As R1279, but Δ*yhbJ*, Δ*glmY*	(Reichenbach *et al.*, 2008)
Z116	As R1279, but Δ*yhbJ*, Δ*glmZ*	(Reichenbach *et al.*, 2008)
Z184	As R1279, but Δ*rpoN*	(Reichenbach *et al.*, 2009)
Z189	As R1279, but Δ*yhbJ* Δ*glmZ*	Z116 cured from *cat*; this work
Z190	As R1279, but *attB*::[*glmY*-5' (-238 to +22) *lacZ,* 10 region mutated]	(Reichenbach *et al.*, 2009)
Z197	As R1279, but *attB*::[*glmY*-5' (-238 to +22) *lacZ*]	(Reichenbach *et al.*, 2009)
Z201	As R1279, but *attB*::[*glmY*-5' (-238 to +22) *lacZ,* -24 region mutated]	(Reichenbach *et al.*, 2009)
Z202	As R1279, but *attB*::[*glmY*-5' (-238 to +22) *lacZ,* -10 and -24 mutated]	(Reichenbach *et al.*, 2009)

Z206	As R1279, but Δ*glrR, attB::*[*glmY*-5' (-238 to +22) *lacZ*]	(Reichenbach *et al.*, 2009)
Z225	As R1279, but Δ*yhbJ, attB::*[*glmY*-5' (-238 to +22) *lacZ*]	pBGG201/BamHI->Z37, this work
Z226	As R1279, but Δ*yhbJ, attB::*[*glmY*-5' (-238 to +22) *lacZ,* -10 mutated]	pBGG209/BamHI->Z37, this work
Z235	As R1279, but Δ*yhbJ, attB::*[*glmY*-5' (-238 to +22) *lacZ,* -24 mutated]	pBGG208/BamHI->Z37, this work
Z236	As R1279, but Δ*yhbJ, attB::*[*glmY*-5' (-238 to +22) *lacZ,* -10 and -24 mutated]	pBGG210/BamHI->Z37, this work
Z254	As R1279, but Δ*yhbJ,* Δ*glmZ, attB::*[*glmY*-5' (-238 to +22) *lacZ*]	pBGG201/BamHI->Z189, this work
Z258	As R1279, but Δ*yhbJ,* Δ*glmZ, attB::*[*glmY*-5' (-238 to +22) *lacZ,* -24 mutated]	pBGG208/BamHI->Z189, this work
Z259	As R1279, but Δ*yhbJ,* Δ*glmZ, attB::*[*glmY*-5' (-238 to +22) *lacZ,* -10 mutated]	pBGG209/BamHI->Z189, this work
Z260	As R1279, but Δ*yhbJ,* Δ*glmZ, attB::*[*glmY*-5' (-238 to +22) *lacZ,* -10 and -24 mutated]	pBGG210/BamHI->Z189, this work
Z277	As R1279, but *attB::*[*argT*-5' (-287 to +53) *lacZ*]	pBGG325/BamHI->R1279, this work
Z278	As R1279, but Δ*yhbJ, attB::*[*argT*-5' (-287 to +53) *lacZ*]	pBGG325/BamHI->Z37, this work
Z279	As R1279, but Δ*rpoN, attB::*[*argT*-5' (-287 to +53) *lacZ*]	pBGG325/BamHI->Z184, this work
Z283	As R1279, but Δ*glmY, attB::*[*glmY*-5' (-238 to +22) *lacZ*]	pBGG201/BamHI->Z96, this work
Z284	As R1279, but Δ*glmY, attB::*[*glmY*-5' (-238 to +22) *lacZ,* -24 mutated]	pBGG208/BamHI->Z96, this work
Z285	As R1279, but Δ*glmY, attB::*[*glmY*-5' (-238 to +22) *lacZ,* -10 mutated]	pBGG209/BamHI->Z96, this work
Z286	As R1279, but Δ*glmY, attB::*[*glmY*-5' (-238 to +22) *lacZ,* -10 and -24 mutated]	pBGG210/BamHI->Z96, this work
Z296	As R1279, but Δ*yhbJ*, Δ*glmY*	Z115 cured from *cat*, this work
Z303	As R1279, but Δ*glmZ, attB::*[*glmY*-5' (-238 to +22) *lacZ*]	pBGG201/BamHI->Z45, this work
Z304	As R1279, but Δ*glmZ, attB::*[*glmY*-5' (-238 to +22) *lacZ,* -24 mutated]	pBGG208/BamHI->Z45, this work
Z305	As R1279, but Δ*glmZ, attB::*[*glmY*-5' (-238 to +22) *lacZ,* -10 mutated]	pBGG209/BamHI->Z45, this work
Z306	As R1279, but Δ*glmZ, attB::*[*glmY*-5' (-238 to +22) *lacZ,* -10 and -24 mutated]	pBGG210/BamHI->Z45, this work
Z307	As R1279, but Δ*yhbJ,* Δ*glmY, attB::*[*glmY*-5' (-238 to +22) *lacZ*]	pBGG201/BamHI->Z296, this work
Z308	As R1279, but Δ*yhbJ,* Δ*glmY, attB::*[*glmY*-5' (-238 to +22) *lacZ,* -24 mutated]	pBGG208/BamHI->Z296, this work
Z309	As R1279, but Δ*yhbJ,* Δ*glmY, attB::*[*glmY*-5' (-238 to +22) *lacZ,* -10 mutated]	pBGG209/BamHI->Z296, this work
Z310	As R1279, but Δ*yhbJ,* Δ*glmY, attB::*[*glmY*-5' (-238 to +22) *lacZ,* -10 and -24 mutated]	pBGG210/BamHI->Z296, this work

Table 6: Plasmids used in this study.

plasmid	relevant structures	Source or reference
pBGG201	Transcriptional fusion of *glmY*-5' (-238 to +22) to *lacZ*	(Reichenbach *et al.*, 2009)
pBGG208	Transcriptional fusion of *glmY*-5' (-238 to +22) to *lacZ,* -24 mutated	(Reichenbach *et al.*, 2009)
pBGG209	Transcriptional fusion of *glmY*-5' (-238 to +22) to *lacZ,* -10 mutated	(Reichenbach *et al.*, 2009)
pBGG210	Transcriptional fusion of *glmY*-5' (-238 to +22) to *lacZ,* -10 and -24 mutated	(Reichenbach *et al.*, 2009)
pBGG325	Transcriptional fusion of *argT*-5'(-287 to +53) to *lacZ*	This work
pBGG396	Transcriptional fusion of *rtcB*-5'(-199 to +51) to *lacZ*	This work
pCP20	FLP recombinase gene, *bla*, *cat*, ori pSC101-*rep*^{TS}	(Datsenko and Wanner, 2000)
pFDX4291	pSC101-ori, *cat*, operator-less P_{tac}, BglII, *sacB*-RBS, NdeI, XbaI, HincII	(Kalamorz *et al.*, 2007)
pFDX4324	*yhbJ* under P_{tac}-control in pFDX4291	(Kalamorz *et al.*, 2007)
pKES15	*bgl'-lacZ, kan, attP, aadA,* ori pACYC177	(Nagarajavel *et al.*, 2007)
pKES170	*bla*, *lacI*, *Ptac*, SD T7 gene 10, NdeI, XbaI, pBR322-ori	Karin Schnetz
pLDR8	λ *int* under control of λP_R, λcI_{857}, *kan*, ori pSC101-*rep*^{TS}	(Diederich *et al.*, 1992)

Table 7: Oligonucleotides used in this study.

Primer	Sequence[a]	Res. Sites	position
BG377	GCACGCGTCGACCTTTTTTGTGTCTGTAAATCACG	SalI	*glmY* -238 to -213
BG456	GCTCTAGAATAAGTCGGTGAATGAGCCAC	XbaI	*glmY* +22 to +2
BG578	CGGTGAAGGGCAATCAGCTG		*lacZ* -271 to -252
BG579	GGCCTCTTCGCTATTACGCC		*lacZ* +129 to +110
BG598	GCACGCGTCGACTGCCCGCTGGCAGGGCG	SalI	*argT* -287 to -271
BG599	CGTCTCTAGAGCCGCTGTGGAGAGACCG	XbaI	*argT* +53 to +36
BG702	GCACGCGTCGACGTTTTACGCATCTTAGATATCC	SalI	*rtcB* -199 to -178
BG703	CGTCTCTAGAGGTCCACATTTTTACCGGGG	XbaI	*rtcB* +51 to +32

[a]Restriction sites are underlined.

β-galactosidase assays

When not otherwise indicated, overnight cultures in LB were inoculated into fresh LB medium to an OD_{600} of 0.1. Growth was continued at 37℃ and 200 r.p.m. until an OD_{600} of 0.5-0.8 (exponential growth phase) was reached and cells were subsequently harvested. β-galactosidase activities were determined as described previously (Miller, 1972). Enzyme activities are given in Miller units. They are the average of at least two measurements from independent cultures.

Electro mobility shift assays (EMSAs)

In general, EMSAs were carried out as described (Stratmann *et al.*, 2008). Briefly, *glmY* and *lacZ* promoter fragments were amplified from chromosomal DNA using oligos BG377/BG456 and BG578/BG579, respectively. Purified YhbJ::Strep protein was obtained from Denise Lüttmann. Binding assays were conducted in binding buffer (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 2 mM DTT, 10% glycerol) in a total volume of 10 µl containing 20 ng of each DNA fragment for 20 min at 30℃. Where indicated, ATP was added at 25 mM final concentration. Protein amounts were as indicated in Fig. 28. Subsequently, the mixtures were separated on 8% polyacrylamide/TBE native gels at 4℃ by electrophoresis. The DNA was visualized by ethidium bromide staining of gels.

RNA extraction and northern analysis

RNA was extracted from cells, which were harvested in exponential growth phase, using the RNeasy Mini kit (Qiagen). For analysis of sRNAs GlmY and GlmZ, 5 µg of total RNA were separated on 8% polyacrylamide/7M urea/ TBE gels. The RNA was subsequently transferred to positively charged nylon membranes (Roche) by electroblotting. For analysis of *glmS* transcripts, 5 µg of total RNA were separated on 1% agarose formaldehyde gels and subsequently transferred to positively charged nylon membranes (Roche) by vacuum blotting. In both cases, the RNA was fixed to the membrane by UV cross-linking. GlmY and GlmZ RNAs and *glmS* transcripts were detected using digoxigenin labeled RNA-probes as described (Reichenbach *et al.*,

2008). RNAs were hybridized and detected following the manufacturer's instructions (DIG RNA labeling kit, Roche).

Results

YhbJ stimulates activity of P_{glmY}

Deletion of *yhbJ* has opposite effects on the amounts of the two small RNAs GlmY and GlmZ in the cell. While the active unprocessed form of GlmZ accumulates upon deletion of *yhbJ*, the amount of GlmY is strongly reduced as compared to the wild type (Reichenbach *et al.*, 2008). Since both GlmY and GlmZ share a very similar secondary structure (Reichenbach *et al.*, 2008; Urban and Vogel, 2008) we reasoned that YhbJ might be a RNA binding protein, which transduces the signal between GlmY and GlmZ. This might be achieved by altering the stability of both GlmY and GlmZ through titration effects. It is conceivable that upon induction of GlmY YhbJ is titrated away from GlmZ allowing accumulation of the unprocessed form of GlmZ and subsequent activation of its *glmS* target. However, EMSA's showed that YhbJ does not specifically bind GlmY or GlmZ (Kalamorz, 2009). The amounts of GlmY determined at different growth stages are reduced in a Δ*yhbJ* strain as compared to the wild type, especially in stationary phase. Interestingly, we observed similar GlmY amounts in Δ*glrR*, Δ*glrK* and Δ*rpoN* strains (compare (Reichenbach *et al.*, 2008) and (Reichenbach *et al.*, 2009)). GlrR, GlrK and σ^{54} affect GlmY amounts on the level of *glmY* promoter activity. This raised the possibility that YhbJ might also influence *glmY* promoter activity. Therefore, we checked whether *glmY* promoter activity is altered in a Δ*yhbJ* strain as compared to the wild type. To this end, we measured β-galactosidase activities of *glmY-lacZ* fusions. These fusions were inserted into the *attB*-site of the chromosomes of the wild type, the Δ*glrR* and the Δ*yhbJ* mutants. Indeed, deletion of *yhbJ* resulted in a four-fold reduced activity of P_{glmY} as compared to the wild type (Fig. 26 A, compare left and right columns), but the promoter activity was not as low as in the Δ*glrR* strain (Fig. 26 A, compare middle and right columns).

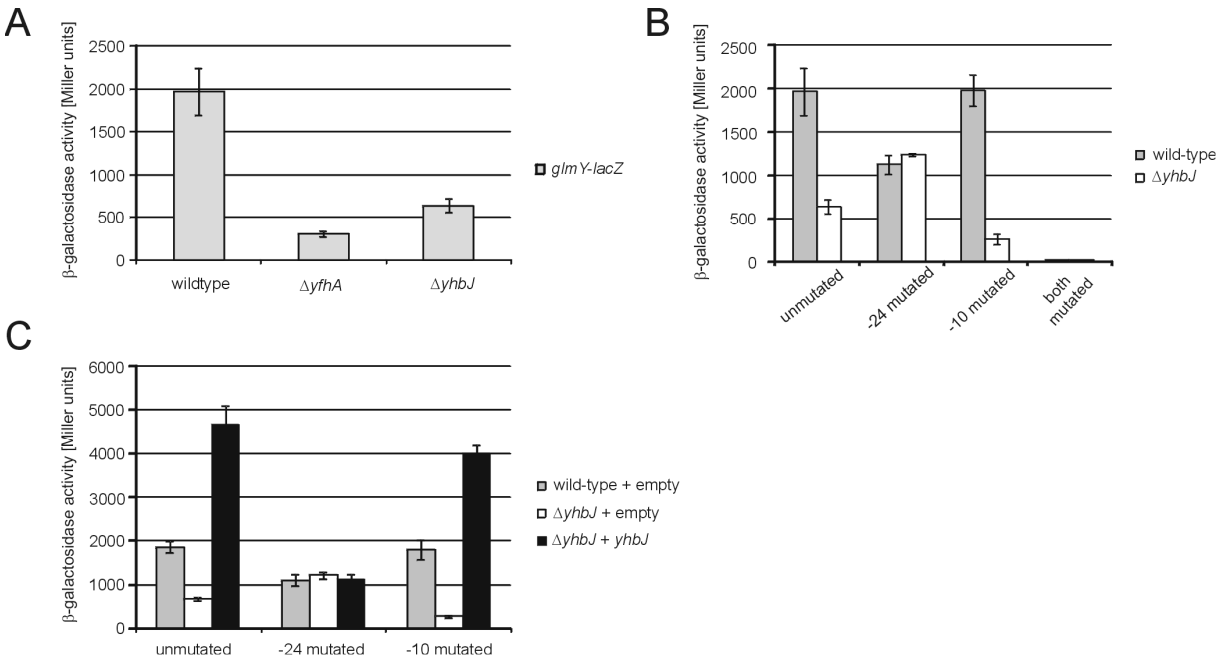

Figure 26: YhbJ modulates the activity of the σ^{54} dependent promoter, which is located upstream of *glmY*. A. β-galactosidase activities of *glmY-lacZ* (-238 to +22) on the chromosome. Strains Z197 (wild type), Z206 (Δ*yfhA*) and Z225 (Δ*yhbJ*) were grown to exponential phase and β-galactosidase activities were determined. **B.** β-galactosidase activities of *glmY-lacZ* (-238 to +22) transcribed from selectively inactivated promoters on the chromosome. Strains Z197 (wild type, unmutated), Z225 (Δ*yhbJ*, unmutated), Z201 (wild type, -24 mutated), Z235 (Δ*yhbJ*, -24 mutated), Z190 (wild type, -10 mutated), Z226 (Δ*yhbJ*, -10 mutated), Z202 (wild type, both mutated) and Z236 (Δ*yhbJ*, both mutated) were grown to exponential phase and β-galactosidase activities were determined. **C.** Complementation analysis demonstrating that plasmid driven overexpression of *yhbJ* restores *glmY*-expression levels. Transformants Z197/pFDX4291, Z225/pFDX4291, Z225/pFDX4324, Z201/pFDX4291, Z235/pFDX4291, Z235/pFDX4324, Z190/pFDX4291, Z226/pFDX4291 and Z226/pFDX4324 were induced with 1mM IPTG, grown to exponential phase and β-galactosidase activities were determined. The values are the average of three independent measurements.

Since *glmY* is transcribed from two overlapping σ^{70}- and σ^{54}-dependent promoters (Reichenbach *et al.*, 2009), we wanted to determine which promoter is affected by the Δ*yhbJ* mutation. Therefore, we mutationally inactivated each of both promoters. These mutations have no impact on the activity of the respective second promoter (Reichenbach *et al.*, 2009). We obtained the activities of these promoters in the wild type and the Δ*yhbJ* strain (Fig. 26 B). While deletion of *yhbJ* had no impact on the activity of the σ^{70}-dependent promoter (see second column), activity of the σ^{54}-dependent promoter was reduced 10-fold in the Δ*yhbJ* strain as compared to the wild type (Fig. 26 B, column 3). Interestingly, σ^{54}-dependent promoter activity was not fully abrogated as it is the case in *glrR*- or *rpoN*-mutants (Reichenbach *et al.*, 2009). Since *yhbJ* is encoded in one operon with *rpoN*, we reasoned that the reduced activity of the σ^{54}-promoter of *glmY* might be due to a polar effect

on *rpoN*. Deletion of *yhbJ* might reduce transcript stability of the *rpoN-hpf-ptsN-yhbJ-npr* co-transcript, thereby reducing σ^{54}-levels in the cell. Reduced σ^{54}-levels could lead to reduced activities of σ^{54}-dependent promoters. To exclude this possibility we complemented the Δ*yhbJ* strain with a plasmid carrying *yhbJ* under control of an IPTG-inducible promoter and determined the β-galactosidase activity of the complemented strain (Fig. 26 C). While the activity of the σ^{70}-dependent promoter is altered neither by deletion nor by overexpression of *yhbJ*, the activity of the σ^{54}-dependent promoter is clearly reduced upon deletion and again increased upon overexpression of *yhbJ* (Fig. 26 C, compare second and third columns). Surprisingly, when compared to the wild type carrying the empty plasmid the activity of the σ^{54}-dependent promoter is even increased approximately 2-fold upon overexpression of *yhbJ*. These results show that YhbJ modulates the activity of the *glmY* σ^{54}-dependent promoter.

Modulation of *glmY* σ^{54}-promoter activity by YhbJ is independent of GlmY and GlmZ

YhbJ controls the amounts of GlmY and GlmZ. Therefore, one can imagine that either GlmY or GlmZ autoregulates GlmY amounts by a feedback mechanism, e.g. by regulation of *glrR*- or *rpoN*-transcript stability or translation of GlrR or σ^{54}. In addition, deletion of *yhbJ* results in overproduction of GlmS, which in turn results in increased conversion of fructose-6-P and glutamine to glucosamine-6-P. As a result, intracellular concentrations of several compounds are altered. In turn, this might alter the concentration(s) of the unknown signal molecule(s), which is (are) sensed by GlrK. This would then affect *glmY* promoter activity. To investigate these possibilities, *glmY* promoter activities were determined by β-galactosidase measurements in Δ*glmY*, Δ*glmZ*, Δ*glmY* Δ*yhbJ* and Δ*glmZ* Δ*yhbJ* mutants in comparison to the wild type and the Δ*yhbJ* mutant (Fig. 27).

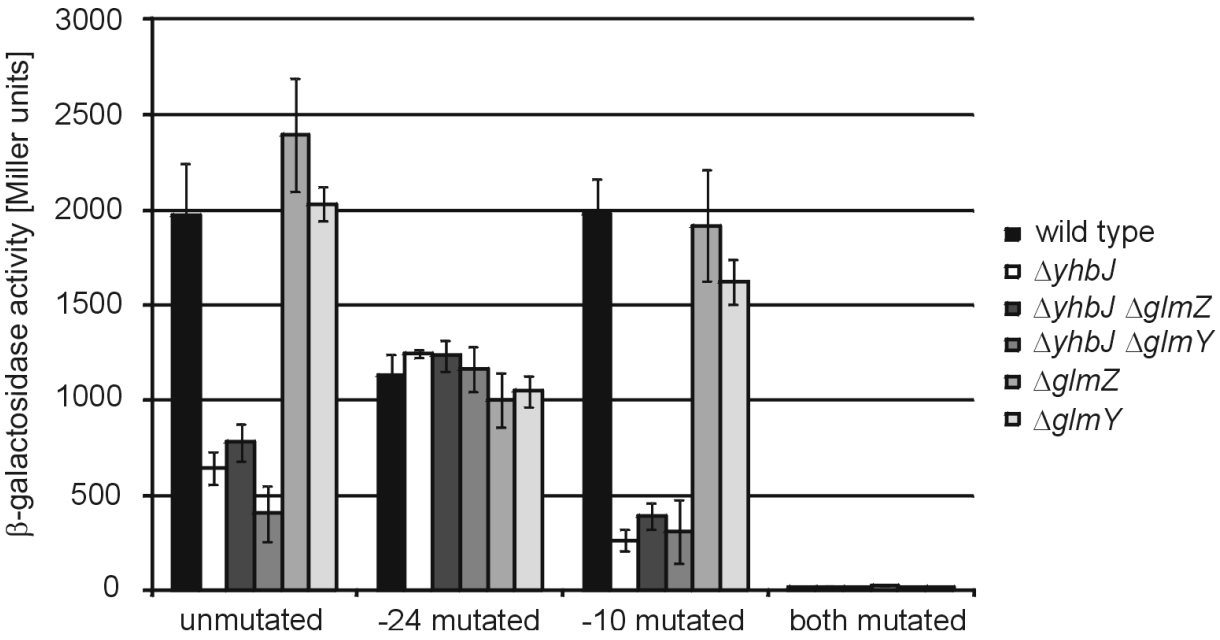

Figure 27: YhbJ affects *glmY* promoter activity independently of GlmY or GlmZ. β-galactosidase activities of *glmY-lacZ* (-238 to +22) with selectively inactivated promoters on the chromosome. Strains (from left to right) Z197, Z225, Z254, Z307, Z303, Z283, Z201, Z235, Z258, Z308, Z304, Z284, Z190, Z226, Z259, Z309, Z305, Z285, Z202, Z236, Z260, Z310, Z306 and Z286 were grown to exponential phase and β-galactosidase activities were determined. Deletion of either *glmY* or *glmZ* has no impact on *glmY* promoter activity. YhbJ dependent modulation of *glmY* promoter activity is likewise unaffected by deletion of *glmY* or *glmZ*. The values are the average of three independent measurements.

In all tested mutant strains, activity of the σ^{70}-dependent *glmY* promoter was unaffected (Fig. 27, -24 mutated). Therefore, one can conclude that the activity of this promoter is not regulated by GlmY or GlmZ. The σ^{54}-dependent promoter is reduced in its activity whenever *yhbJ* is deleted as compared to strains where *yhbJ* is intact. Deletion of *glmY* or *glmZ* has no effect on the activity of the σ^{54}-depedent promoter, since *glmY* σ^{54}-promoter activity in the Δ*glmY* and the Δ*glmZ* mutants is as in the wild type. In the Δ*yhbJ* Δ*glmY* and the Δ*yhbJ* Δ*glmZ* mutants β-galactosidase activities are as in the Δ*yhbJ* mutant (Fig. 27). Therefore, one can conclude that the effect on the *glmY* σ^{54}-dependent promoter in Δ*yhbJ* mutant strains is not a secondary effect resulting from YhbJ-dependent modulation of GlmY and GlmZ amounts. YhbJ modulates *glmY* σ^{54} promoter activity either directly or via another unknown factor.

YhbJ does not bind directly to the *glmY* promoter region

YhbJ has a function in regulation of σ^{54}-dependent *glmY* expression. Therefore, it appeared conceivable that YhbJ is a DNA binding protein, which directly activates the σ^{54}-dependent *glmY* promoter by binding to the *glmY* promoter region. To see whether this is the case, we performed *in vitro* electro mobility shift assays with purified YhbJ::Strep-tag and DNA-fragments encompassing the *glmY* promoter region (Fig. 28). As a control we used a *lacZ* promoter fragment. Since it has been reported that YhbJ possesses an ATP binding P-loop motif and ATP- and GTP-hydrolyzing activity has been observed for YhbJ (Luciano *et al.*, 2009), we conducted EMSAs in the absence and presence of ATP.

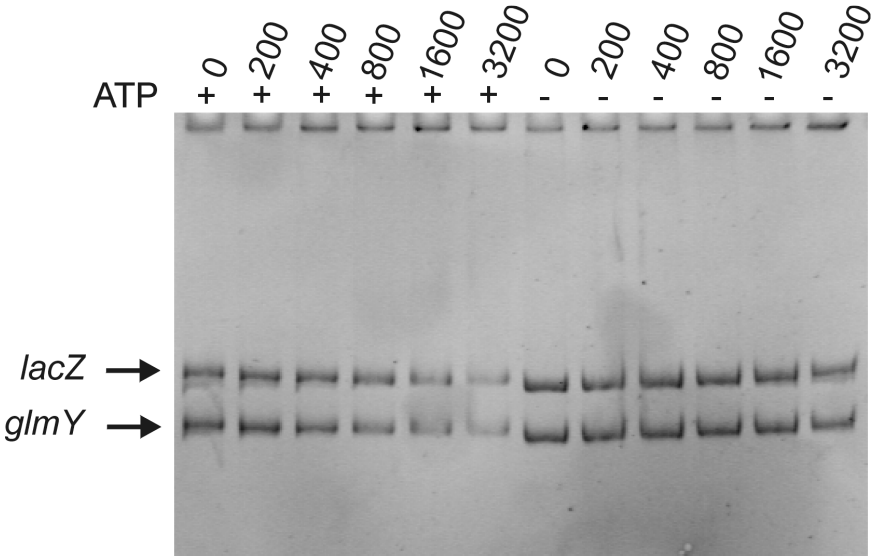

Figure 28: YhbJ does not bind to the *glmY* promoter region. EMSAs of the *glmY* promoter region and purified YhbJ::Strep using a 400 bp *lacZ* fragment as control. DNA fragments were incubated in the absence or presence of 25 mM ATP and the indicated protein concentration [nM]. YhbJ::Strep does not bind to any of the DNA fragments.

It can be clearly seen from the data that YhbJ::Strep does not possess binding affinity towards either the *glmY*- or the *lacZ*-promoter region. Co-incubation with 25 mM ATP does not induce binding of YhbJ to the DNA. Instead, both *glmY*- and *lacZ*-promoter fragments appear to be degraded in a protein concentration dependent manner upon addition of ATP to the reaction. This observation raised the possibility that YhbJ might have ATP-dependent DNase activity. For this reason the experiment was repeated employing YhbJ::His. In this case no ATP-dependent DNA degradation was observed (Yvonne Göpel, unpublished data). Therefore, it must be concluded that the

ATP-dependent effect that was observed with YhbJ::Strep-tag was due to a DNase contamination of the purified protein.

YhbJ modulates the activity of another σ^{54}-dependent promoter

Since we could not observe direct binding of YhbJ to the *glmY* promoter region, we wondered whether YhbJ might be a general modulator of σ^{54}-dependent promoters. Such a general function in regulation of σ^{54}-dependent promoter activity would fit to the highly conserved localization of *yhbJ* within the *rpoN* operon. To see whether also other σ^{54}-dependent promoters are altered in their activity in a Δ*yhbJ* strain as compared to the wild type, we fused the promoter region of *argT* to *lacZ*. It was previously suggested that *argT* expression might be under control of a σ^{54}-dependent promoter of the NtrC regulon (Reitzer and Schneider, 2001; Schmitz *et al.*, 1987). The β-galactosidase activity of the *argT'-lacZ* fusion integrated into the chromosome was measured in the wild type and Δ*yhbJ*- and Δ*rpoN*-mutant strains grown in M9 minimal medium (Fig. 29).

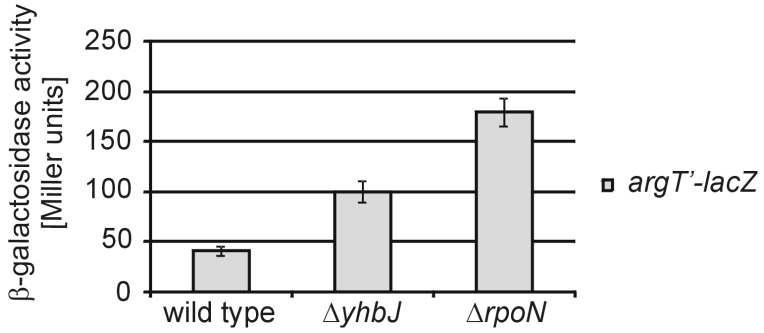

Figure 29: Deletion of *yhbJ* and *rpoN* alters promoter activity of P_{argT}. β-galactosidase activities of *argT'-lacZ* on the chromosome in wild type, Δ*yhbJ* and Δ*rpoN* strains. Strains Z277, Z278 and Z279 (from left to right) were grown in M9 minimal medium to exponential phase and β-galactosidase activities were determined. Deletion of *yhbJ* and *rpoN* both leads to increased β-galactosidase activity of the *argT'-lacZ* fusion. The values are the average of three independent measurements.

Interestingly, promoter activity of P_{argT} was affected by both the deletion of *yhbJ* and the deletion of *rpoN*. Surprisingly, the promoter activity was increased both in the Δ*rpoN* (3- to 4-fold) and the Δ*yhbJ* (2-fold) mutant strain as compared to the wild type. This result was very surprising, because the activity of σ^{54}-dependent promoters is fully dependent on the presence of the σ^{54}-factor (Reitzer and Schneider, 2001). Therefore, one has to speculate that the σ^{54}-dependent promoter upstream of *argT* is inactive under the conditions

tested, but that a second promoter is present upstream of the σ^{54}-promoter sequence within the region that was fused to *lacZ*. As previously observed for the σ^{54}-dependent *glmY*-promoter (Reichenbach *et al.*, 2009), it is likely that σ^{54} RNA polymerase holoenzyme binds to the *argT*-σ^{54} promoter, forms an inactive closed complex and acts as a repressor of the proposed second promoter. If this is the case, deletion of *rpoN* would derepress the second promoter. The derepression, which is observed upon deletion of *yhbJ*, could be due to its proposed function as a modulator of σ^{54}-activity. It is interesting to note that YhbJ does not only influence the σ^{54}-dependent *glmY* promoter, but also at least one additional σ^{54}-dependent promoter.

GlmZ is processed by RNase E

The deletion of *yhbJ* has opposing effects on the two small RNAs GlmY and GlmZ. While GlmZ accumulates in its unprocessed form in a Δ*yhbJ* mutant strain, GlmY amounts are reduced in this strain as compared to the wild type (Reichenbach *et al.*, 2008; Kalamorz *et al.*, 2007). Previously we proposed that YhbJ might be an RNA binding protein which functions as a switch in between GlmY and GlmZ by binding of both RNAs (Reichenbach *et al.*, 2008). This hypothesis cannot stand up to the data presented above. Deletion of *yhbJ* clearly reduces the activity of the σ^{54}-dependent *glmY* promoter and this effect is independent of possible GlmY- or GlmZ-binding functions of YhbJ (Fig. 27). Therefore we wondered how YhbJ can be both a modulator of σ^{54}-promoter activity and a regulator of GlmZ processing. We reasoned that a σ^{54}-dependent factor might be necessary for the processing of GlmZ and that YhbJ might directly regulate the expression or the activity of this factor. First, we wanted to verify that GlmZ is really processed by RNase III, as claimed by (Argaman *et al.*, 2001). To this end we analyzed processing of GlmZ by northern blotting in several RNase-mutant strains (Fig. 30). At the same time, we checked for accumulation of *glmS*, since unprocessed GlmZ is more stable then processed GlmZ (Kalamorz *et al.*, 2007; Reichenbach *et al.*, 2008). Therefore, the inactivation of the RNase responsible for GlmZ processing should also result in accumulation of *glmS*-transcript. GlmY

processing was monitored, because the RNase responsible for processing of GlmY is unknown.

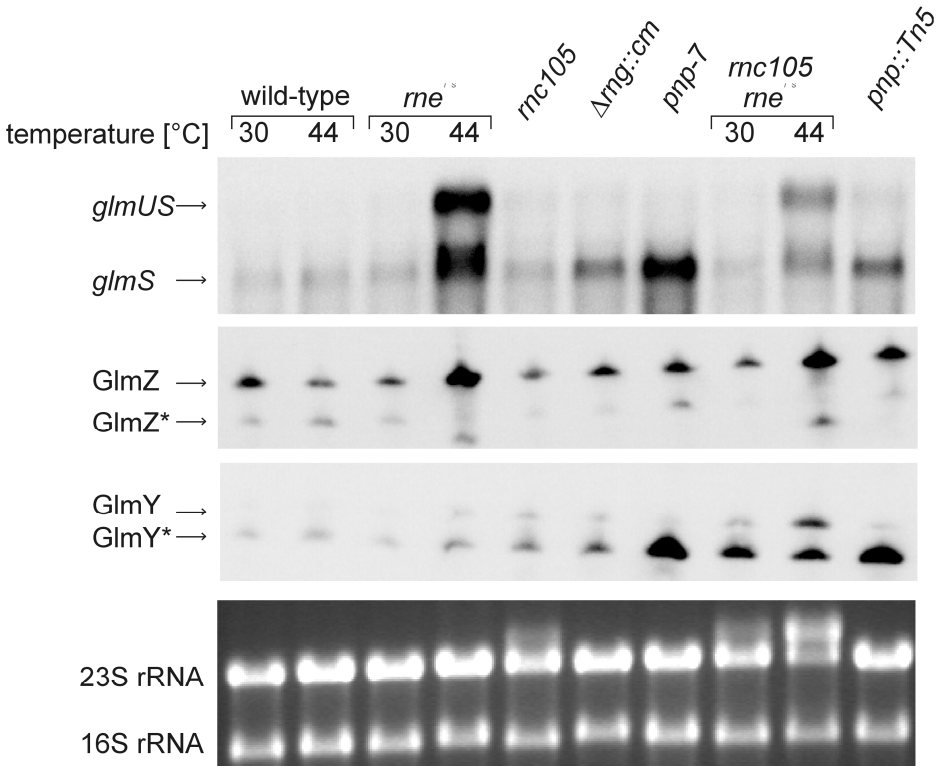

Figure 30: Processing of GlmZ is dependent on RNase E. Northern blot analysis of *glmS* and *glmUS* transcripts, GlmZ and GlmY using probes directed against *glmS*-mRNA, GlmZ and GlmY, respectively. Total RNAs of the following strains were used: N3433 (lanes 1 and 2), N3431 (lanes 3 and 4), IBPC633 (lane 5), IBPC935 (lane 6), SK5691 (lane 7), S3768 (lanes 8 and 9) and JC357 (lane 10). Cells were grown at 37℃ unless otherwise indicated. At the bottom the ethidium bromide stained gel of the *glmS*-blot is shown as loading control.

The data show that, surprisingly, mutation of RNase III (*rnc105*) does not abrogate processing of the GlmZ sRNA. This is not consistent with a previous report, which claimed that GlmZ is processed by RNase III (data not shown in (Argaman *et al.*, 2001)). In contrast our data clearly show that processing of GlmZ depends on the activity of RNase E (middle panel, compare lanes 3 and 4 in Fig. 30). We employed a temperature sensitive RNase E mutant. At non-restrictive temperatures (30℃) the RNase E enzyme is active, while at restrictive temperatures (44℃) the enzyme becomes misfolded and inactive. Upon inactivation of RNase E, GlmZ accumulates in its unprocessed form. At the same time, the *glmUS* co-transcript, which is normally processed by RNase E (Joanny *et al.*, 2007; Kalamorz *et al.*, 2007), is no longer processed and the full-length transcript accumulates (Fig. 30, lane 4). The remaining processed *glmS*-transcript is stabilized by the accumulating full length GlmZ sRNA and likewise accumulates. It is interesting to note that, although we

could not identify the RNase responsible for processing of GlmY, GlmY* accumulates in *pnp* mutants. This ultimately leads to accumulation of *glmS* transcript (Fig. 30 lanes 7 and 10). Gene *pnp* encodes for PNPase. PNPase is a RNA-degrading enzyme with 3' to 5' exonuclease activity. PNPase activity is inhibited, if stable stem loop structures are present at the 3' end of the substrate. In such cases it acts in concert with poly(A)polymerase I, which adds poly(A) tails to the 3' end of the substrate. These poly(A) tail then enables PNPase to break up the secondary structure and to degrade the substrate (Khemici and Carpousis, 2004). We have previously demonstrated that deletion of *pcnB* encoding poly(A)polymerase I leads to accumulation of GlmY* and subsequently to accumulation of *glmS* transcript (Reichenbach *et al.*, 2008). Considering this, the observed effect of *pnp* inactivation on GlmY, GlmZ and *glmS* RNAs in agreement with our previous observations.

Candidates for YhbJ regulated GlmZ processing factors

We were able to show that GlmZ is processed by RNase E. Therefore, possible candidates that might mediate the YhbJ-dependent regulation of GlmZ processing should be restricted to (I) proteins, which are involved in RNase E dependent RNA processing, (II) proteins, which have been shown to interact with RNase E, (III) proteins, which (might) have a general role in RNA metabolism that might interfere with RNase E function, and (IV) σ^{54}-dependently expressed genes of unknown function. A list of such candidates is presented in table 8.

Table 8: Genes whose products might be involved in YhbJ dependent regulation of GlmZ processing

gene	promoter and regulation	function	references
rtcB	σ^{54}, activated by RtcR and IHF	highly conserved protein of unknown function	(Genschik *et al.*, 1997; Okada *et al.*, 2006; Genschik *et al.*, 1998)
rtcA	σ^{54}, activated by RtcR and IHF	RNA 3'-terminal phosphate cyclase	(Genschik *et al.*, 1997; Genschik *et al.*, 1998)
rppH	Very low ranked σ^{54}	RNA 5' pyrophosphohydrolase, initiates RNase E dependent RNA decay	(Reitzer and Schneider, 2001; Deana *et al.*, 2008)
yaiS	σ^{54}	conserved protein of unknown function	(Reitzer and Schneider, 2001)

pnp	2 σ^{70} promoters, posttranscriptional regulation by RNase III	polynucleotide phosphorylase, component of the degradosome	(Régnier and Portier, 1986; Carpousis, 2007)
pcnB	3 promoters, 1 known σ^{70}	Poly(A)polymerase I, component of the degradosome	(Jasiecki and Wegrzyn, 2006; Carpousis, 2007)
rhlB	unknown	ATP-dependent RNA helicase, component of the degradosome	(Carpousis, 2007; Py *et al.*, 1996)
eno	7 promoters, 3 are repressed by FruR	enolase, component of the degradosome	(Gama-Castro *et al.*, 2008; Py *et al.*, 1996; Shimada *et al.*, 2005; Weng *et al.*, 1986)
rraA	σ^{S} dependet promoter, 2 additional promoter for upstream gene (1 σ^{70}) post-transcriptional regulation by RNase E	Inhibitor or RNase E, overexpression has pleiotropic effects on RNase E substrates	(Gama-Castro *et al.*, 2008; Zhao *et al.*, 2006; Gao *et al.*, 2006; Lee *et al.*, 2003; Suvarna *et al.*, 1998)
rraB	1 σ^{70}-dependent promoter, that is upregulated in response to low intracellular amino sugar concentrations	Inhibitor or RNase E	(Gao *et al.*, 2006; Zhou *et al.*, 2009)
dnaK	3 σ^{32}-dependent promoters	Hsp70 chaperone, associated with the degradosome	(Carpousis, 2007; Nonaka *et al.*, 2006; Cowing *et al.*, 1985)
groL	4 promoters, 1 is σ^{32}-dependent	Hsp60 chaperone, associated with the degradosome	(Carpousis, 2007; Gama-Castro *et al.*, 2008; Cowing *et al.*, 1985)
ppk	unknown	Polyphosphate kinase, associated with the degradosome	(Carpousis, 2007)

Genes *pcnB* and *pnp* can be excluded as candidates with high confidence. Results, which have been reported previously (Joanny *et al.*, 2007; Reichenbach *et al.*, 2008; Urban and Vogel, 2008) and in this work (Fig. 30) are strongly contradictory to a PAP I- or PNPase-dependent abrogation of GlmZ processing. While mutation of *pnp* or *pcnB* results in increased levels of unprocessed GlmZ in a GlmY-dependent manner, the processed form of GlmZ is still observed in these mutants excluding reduced *pcnB* or *pnp* expression levels as the cause for the Δ*yhbJ* effect on GlmZ. Keeping this in mind, it is also not conceivable how overexpression of *pnp* or *pcnB* might abrogate processing of GlmZ.

Activity of P_{rtcB} is increased upon deletion of *yhbJ*

For first analysis candidates *rtcB* and *rtcA* were chosen from the list in table 8. Considering all requirements for the modulator of GlmZ processing, *rtcB* and

rtcA are the best candidates: They are transcribed in one operon from a σ^{54}-dependent promoter. Both genes are highly conserved in bacteria and even in eukaryotes (Genschik *et al.*, 1997; Okada *et al.*, 2006; Genschik *et al.*, 1998) and one of the encoded proteins, RctA, possesses an enzymatic activity, which makes an involvement in RNA metabolism probable. Due to the high degree of conservation of RtcB and its co-localization with the equally well conserved gene encoding RtcA, also RtcB might have a function in RNA metabolism. Therefore we decided to analyze whether expression of *rtcBA* is altered upon deletion of *yhbJ*. To this end, we constructed a *rtcB'-lacZ* fusion and tested its β-galactosidase activity in the wild type, a Δ*yhbJ* strain and a Δ*rpoN* strain (Fig. 31).

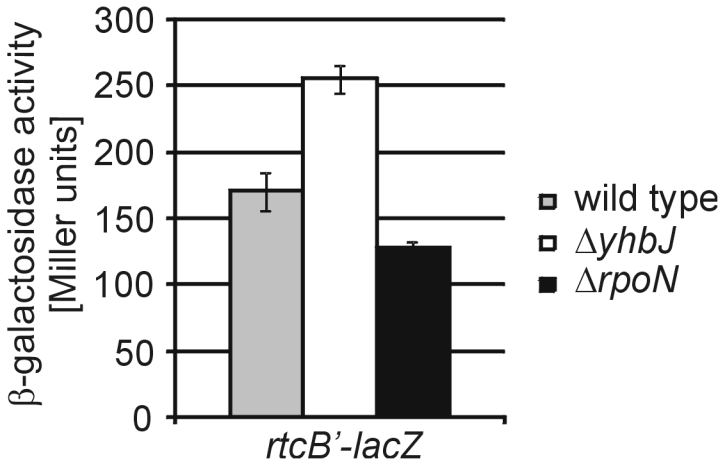

Figure 31: Deletion of *yhbJ* activates expression of *rtcBA*. β-galactosidase activities of *rtcB'-lacZ* in the multi copy situation in wild type, Δ*yhbJ* and Δ*rpoN* strains. Transformants R1279/pBGG396 (wild type), Z37/pBGG396 (Δ*yhbJ*) and Z184/pBGG396 (Δ*rpoN*) were grown in LB medium to exponential phase and subsequently β-galactosidase activities were determined. The values are the average of at least two independent measurements.

The data show that *rtcB* promoter activity is very low in the wild type, because the values, which were measured in the wild type strain, are only slightly increased as compared to the Δ*rpoN* mutant, where the σ^{54}-dependent promoter should be inactive. Interestingly, deletion of *yhbJ* results in activation of P_{rtcB}-promoter activity. Since *rtcB* promoter activities were determined on the plasmid in a multi copy situation, back ground β-galactosidase activities are relatively high. For this reason it is not possible to estimate the degree of transcriptional activation that is exerted by deletion of *yhbJ*. In a next step, it is necessary to repeat this experiment in the single copy situation and to show that the effect of the Δ*yhbJ* mutation is a direct effect of the absence of YhbJ

and not due to a polar effect on the *rpoN* operon. In addition it is necessary to analyze whether GlmY or GlmZ are responsible for the observed alteration of *rtcB* promoter activity.

Discussion

In this work, we demonstrate that YhbJ is a modulator of σ^{54}-dependent promoter activity of *glmY*, *argT* and *rtcBA*. At least in the case of P_{glmY}, this modulation of σ^{54}-promoter activity does not depend on the sRNAs GlmY or GlmZ and it is also not an indirect effect of activation of *glmS* expression. The effects of deletion of *yhbJ* on GlmY and GlmZ appear to be absolutely independent from each other. We therefore wondered how deletion of *yhbJ* can affect both the processing of GlmZ and the transcription of GlmY from its σ^{54}-dependent promoter. The most likely explanation seems to be that YhbJ is a modulator of σ^{54}-promoter activity of at least some σ^{54}-dependent promoters and that the effect of a Δ*yhbJ* mutation on processing of GlmZ is an indirect effect via an unknown factor that is also regulated by YhbJ. To get a better insight, which factors might be involved in regulation of processing of GlmZ, we decided to find the RNase, which processes GlmZ, and identified RNase E as this RNase. See Fig. 32 for a model of the roles of YhbJ and RNase E in regulation of *glmS* expression. A factor, which is responsible for the Δ*yhbJ*-dependent abrogation of RNase E dependent processing of GlmZ could not be identified, although a preliminary analysis showed that RtcB and RtcA are good candidates for this factor (Fig. 31). Another good candidate is RraB. It was recently reported that expression of *rraB* is significantly upregulated in response to reduced GlmS activity (Zhou *et al.*, 2009). Since RraB is an inhibitor of RNase E activity, activation of RraB in response to reduced intracellular amino sugar concentrations is suspicious in respect to GlmZ- and GlmY-dependent regulation of *glmS* expression. The observed upregulation of rraB expression nicely fits with the prerequisites for an additional regulator that regulates RNase E dependent GlmZ processing in response to GlcN6P levels in conjunction with GlmY.

RNase E plays an important role in sRNA dependent gene regulation. Often, sRNAs act by base-pairing to the Shine-Dalgarno region of their target

mRNAs. This results in inhibition of translation of the mRNA. The sRNA along with its bound target mRNA, which is no longer protected from degradation by ribosomes, is subsequently degraded. The first step of this RNA degradation is usually conducted by RNase E (Aiba, 2007). In the case of GlmZ, the RNase E dependent processing of GlmZ should be independent of the *glmS* target mRNA. Only unprocessed GlmZ species are able to bind to the *glmS* mRNA. Therefore, a processing event that follows the binding to the target seems possible. However, in the case of *glmS* the prerequisites differ from those cases where processing and degradation follow the binding of the sRNA to its target. Upon binding to the target mRNA, GlmZ activates translation of the *glmS* transcript and as a result the *glmS* transcript becomes stabilized (Kalamorz *et al.*, 2007; Urban and Vogel, 2008). Therefore, it is clear that no RNA degradation follows the binding of GlmZ to its *glmS* target. Nevertheless it is interesting to note that RNase E is involved in regulation of *glmS* expression at two independent steps. First, it processes the *glmUS* co-transcript, which ultimately enables the cell to regulate *glmS* expression independently from *glmU* expression. Secondly, it catalyzes the processing of the active GlmZ species into its inactive form. This processing step is the major determinant of whether *glmS* expression is activated or not. In sum, RNase E fulfills two very important roles in the regulation of *glmS* expression and is indispensable for this regulation. Recently, RNase E has been shown to be responsible for the processing and subsequent modulation of sRNA regulatory activity for several sRNAs. For example RNase E dependent processing of MicX in *Vibrio cholerae* leads to stabilization and increased regulatory activity of MicX (Davis and Waldor, 2007). For *Salmonella typhimurium* it has been shown that RNase E is responsible for processing of several sRNAs (Viegas *et al.*, 2007). Therefore, GlmZ should be one of several sRNAs, which are regulated by RNase E dependent processing in their activity and more such cases are awaiting their discovery.

In the case of GlmZ it is unusual that the processing is absolutely dependent on the presence of an additional, so far uncharacterized protein, YhbJ. So far only one other, similar case is known. In *E. coli*, the CsrD protein controls the RNase E dependent degradation of the CsrB/CsrC sRNAs (Suzuki *et al.*, 2006), which regulate the activity of the global carbon storage regulator CsrA

by protein sequestration (Weilbacher *et al.*, 2003; Liu *et al.*, 1997; Dubey *et al.*, 2005). Therefore, it is possible that YhbJ functions analogously to CsrD. But to our surprise, we observed that YhbJ also modulates the activity of σ^{54}-dependent promoters, in particular that of the σ^{54}-dependent promoter of *glmY*. Since the YhbJ-dependent modulation of the P_{glmY} σ^{54}-dependent promoter activity is independent of GlmY and GlmZ (Fig. 27), and also other σ^{54}-dependent promoters are affected, it is unlikely that YhbJ functions analogously to CsrD as an auxiliary factor for regulated RNase E processing of GlmZ.

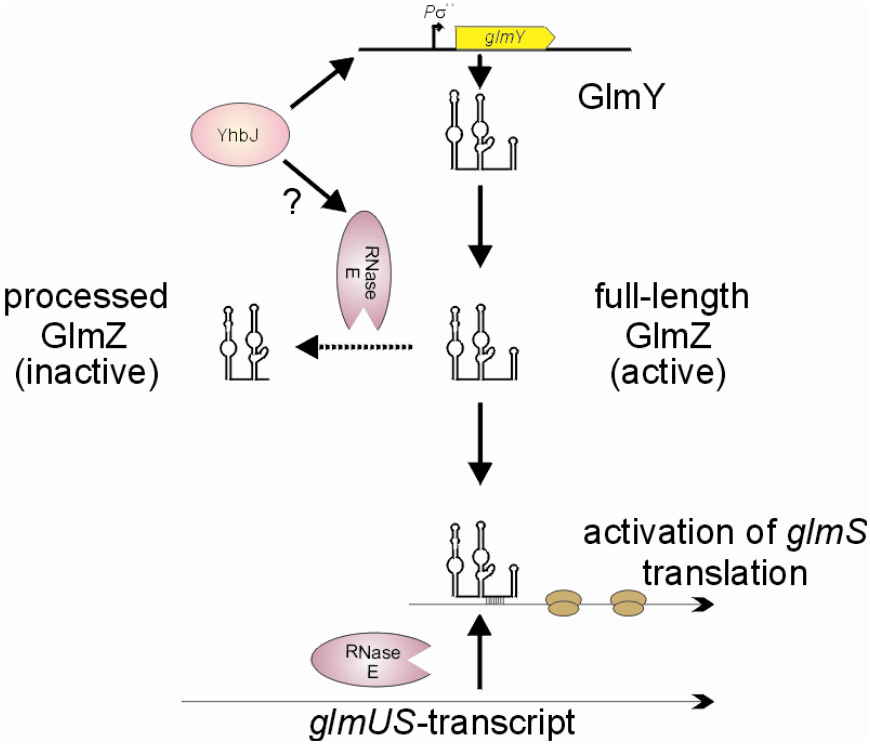

Figure 32: Model for the involvement of YhbJ in GlmY- and GlmZ-dependent regulation of *glmS* expression. When the intracellular concentration of GlcN6P is low, translation of the *glmS* mRNA is activated by a feed-back loop. Gene *glmS* is co-transcribed with *glmU*, and subsequently the *glmUS* co-transcript is processed by RNase E at the *glmU* stop-codon. Under none-inducing conditions both processing products are rapidly degraded. Under inducing conditions, the glmS mRNA is stabilized by increased translation thereof. The sRNA GlmY accumulates and by use of a currently unknown mechanism inhibits processing of GlmZ and thereby activates the accumulation of the stable unprocessed form of GlmZ. Unprocessed GlmZ then activates *glmS* translation by base-pairing with the *glmS* mRNA. The protein YhbJ has two functions in the regulation of this cascade. First, it is necessary for efficient transcription from the σ^{54}-dependent promoter of *glmY*. In addition, it is also needed for RNase E dependent processing of GlmZ. The mechanism of YhbJ action is unknown.

How is it possible that YhbJ regulates the processing of a sRNA and independently also regulates the transcription of another functionally related

sRNA? When considering our data, two possible explanations for YhbJ-dependent regulation of σ^{54}-promoter activity and RNase E dependent sRNA processing come to mind. One possibility is that YhbJ regulates a third sRNA, which then regulates σ^{54} amounts or the activity of σ^{54} RNA polymerase holoenzyme, as is the case for 6S RNA, which regulates the activity of σ^{70} RNA polymerase holoenzyme (Wassarman, 2007). Another possibility is that another factor is responsible for the direct regulation of RNase E dependent processing of GlmZ and that YhbJ regulates the expression of this factor possibly by regulation of another σ^{54}-dependent promoter.

Gene products of the *rpoN*-operon have been previously implicated in regulation of σ^{54}-dependent promoter activity in *Pseudomonas putida* and *Klebsiella pneumoniae* (Merrick and Coppard, 1989; Cases *et al.*, 1999; Merrick *et al.*, 1995). Could YhbJ be responsible for these regulations? For *K. pneumoniae* it was shown that disruption of *ptsN* made the σ^{54}-dependent *Pu* promoter unresponsive to C-source inhibition, which is normally exerted by glucose (Cases *et al.*, 1999). Since the effect of a *ptsN* deletion in *P. putida* could be fully complemented by ectopic expression of *ptsN*, it can be excluded that a polar effect on *yhbJ* expression, which might be caused by disruption of *ptsN,* is responsible for this regulation of *Pu* activity (Cases *et al.*, 1999). Proteome analyses showed that the regulation of *Pu* activity by IIA^{Ntr} is a special case of IIA^{Ntr}-dependent regulation. The C-source inhibition regulon and the σ^{54}-regulon are not significantly affected by deletion of *ptsN* in *P. putida* (Cases *et al.*, 2001). Until now, the mechanism of IIA^{Ntr}-dependent regulation of *Pu* could not be elucidated. In *K. pneumoniae*, the data are less clear. When the *rpoN*-operon of *K. pneumoniae* was first characterized, a mutational analysis was conducted to see if the function of the downstream encoded genes is connected to σ^{54} (Merrick and Coppard, 1989). Here it was observed that disruption of *hpf* or *ptsN* leads to strongly increased expression from the σ^{54}-dependent *pnifH* promoter and slightly increased expression from the σ^{54}-dependent *pnifL* and *glnAp2* promoters, while expression from non-σ^{54}-dependent promoters was unaffected. In this study, an ectopic complementation of the mutants failed and mutants were constructed in such

a way that polar effects on downstream encoded genes such as *yhbJ* are to be expected. In a later study, the analysis was repeated and here also a *npr* mutant was included into the analysis (Merrick *et al.*, 1995). This study produced results, which partially differed from those in the first study. Here, disruption of *ptsN* resulted in slightly increased transcription from the σ^{54}-dependent promoters *pnifL* and *pnifH* and strongly increased expression from *glnAp2*. At the same time, disruption of *npr* resulted in decreased transcription from all three inspected σ^{54}-dependent promoters. Complementation analyses were omitted in this study. Despite this omission and despite the polarity of the used *hpf* and *ptsN* mutants on *yhbJ*, the fact that inactivation of *npr* has the opposite effect on σ^{54}-dependent promoters as inactivation of *ptsN* is in support of the possibility that the observed modulations of σ^{54}-dependent promoter activities are indeed due to inactivation of the Ntr-PTS. Phosphorylated NPr could be the regulator of σ^{54}-dependent promoters in *K. pneumoniae*, since removal of NPr results in decreased promoter activities, while removal of IIA^{Ntr}, which should result in an increased phosphorylation status of NPr, activates expression from the σ^{54}-dependent promoters. Taking this into account it is feasible that the observed regulations of σ^{54}-depedent promoters are exerted by the Ntr-PTS. Nevertheless an involvement of YhbJ in regulation of the activities of σ^{54}-dependent promoters in *K. pneumonia* was not excluded in this study.

If YhbJ is a general modulator of σ^{54}-dependent promoter activity, this function might be conserved in other organisms. Gene *yhbJ* is highly conserved in bacteria and usually localizes to the *rpoN*-operon in Gram-negative bacteria (Comas *et al.*, 2008). Despite the frequent co-localization of *rpoN* and *yhbJ* and its high degree of conservation, some organisms exist, which possess a homolog of *yhbJ*, but do not encode σ^{54}, e.g. *Haemophilus influenzae* (Cases *et al.*, 1999). In such cases, *yhbJ* might be a relict, which has not yet been lost or YhbJ might have different functions in different organisms as is the case for the Ntr-PTS: potassium uptake is regulated by IIA^{Ntr} in *E. coli* (Lee *et al.*, 2007; Lüttmann *et al.*, 2009), but not in *Ralstonia eutropha* (Katja Karstens, Denise Lüttmann and Boris Görke, unpublished observation). Obviously, further experiments are necessary to substantiate the hypothesis that YhbJ is

a modulator of σ^{54}-dependent promoter activity. To this end, it will be necessary to analyze a Δ*rpoN* Δ*yhbJ* mutant for GlmZ processing. If RNase E dependent GlmZ processing is again possible in such a mutant, this would indicate that the abrogation of RNase E dependent GlmZ processing in Δ*yhbJ* mutants is due to altered expression of a σ^{54}-dependently expressed factor and YhbJ is indeed a modulator of σ^{54}-dependent promoters. The next step for identification of the GlmZ-processing regulating factor would be of interest to analyze double mutants of *yhbJ* and all σ^{54}-activator encoding genes for GlmZ processing. For definition of the scope of YhbJ-dependent modulation of σ^{54}-dependent promoters, it will be necessary to analyze transcription from all σ^{54}-dependent promoters in a Δ*yhbJ* mutant under conditions, which activate these promoters. These analyses are beyond the scope of this study and will be addressed in future works.

6. Discussion

It has become an accepted fact that regulation of gene expression by RNA molecules is a very important mechanism of gene regulation in eukaryotes. Meanwhile, regulation of gene expression by *trans*-encoded RNAs is also emerging as a widespread principle in prokaryotes. In bacteria, sRNA-dependent regulation is usually involved in stress response or in regulation of essential metabolic pathways. Most sRNAs downregulate their targets by inhibition of translation through direct binding to the Shine-Dalgarno region. In a few rare cases, expression of the target is upregulated by base-pairing of the sRNA with the mRNA target. This is usually achieved by resolving stem-loop structures, which inhibit the binding of ribosomes to the target mRNA. The present work revealed one of the rare examples where sRNAs activate gene expression. The two sRNAs, GlmZ and GlmY, activate synthesis of GlmS, an essential enzyme of the amino sugar biosynthesis pathway in response to low intracellular GlcN6P concentrations.

6.1 Feedback regulation of *glmS* expression by the sRNA GlmZ

Feedback regulation of GlmS activity is known in Gram-positive bacteria as well as in eukaryotes. In *B. subtilis*, *glmS* expression is downregulated post-transcriptionally by self-cleaving of the *cis*-encoded *glmS* ribozyme in response to an increasing intracellular GlcN6P concentration (Winkler *et al.*, 2004). In eukaryotes activity of the GlmS enzyme is inhibited in a feedback mechanism by binding of UDP-GlcNAc, the end-product of the amino sugar pathway (Milewski, 2002). The results presented in this work show that in the Gram-negative bacterium *E. coli* expression of *glmS* is feedback regulated at the post-transcriptional level by two *trans*-encoded sRNAs in response to GlcN6P. Intracellular depletion of GlcN6P, which is the product of the reaction catalyzed by GlmS, strongly activates *glmS* expression (Fig. 8 D; (Kalamorz *et al.*, 2007)). Gene *glmS* is co-transcribed with *glmU*, yielding a *glmUS* co-transcript (Plumbridge, 1995). Although both GlmU and GlmS are part of the pathway that leads to biosynthesis of UDP-GlcNAc, only GlmU is needed all the time. GlmS is only required, when no external sources for amino sugars are available (Durand *et al.*, 2008). It was a mystery, how differential

expression of *glmU* and *glmS* could be achieved. We found that the *glmUS* transcript is cleaved by RNase E within the *glmU*-stop codon (Fig. 4 A and Fig. S1; (Kalamorz *et al.*, 2007)). The cleavage site possesses properties, which are typical for RNase E cleavage sites (Kennell, 2002): it is located in an AU-rich region that is flanked by two stem-loop structures. The cleavage gives rise to a monocistronic *glmS* transcript, which is then regulated in response to GlcN6P depletion by the sRNA GlmZ. Both, *glmS* transcript levels and GlmS protein levels are strongly increased upon activation of this GlmZ-dependent feedback mechanism (Fig. 8 C, 8 D and 8 E; (Kalamorz *et al.*, 2007)). When sufficient GlcN6P is available, translation of *glmS* is inhibited by a secondary structure within the 5' UTR of *glmS*. This stem-loop structure inhibits access of ribosomes to the Shine-Dalgarno sequence. GlmZ activates translation of *glmS* by base-pairing with the left half-site of this secondary structure. This interaction liberates the Shine-Dalgarno sequence and makes it accessible to ribosomes (Fig. S3 and Fig. 33; (Kalamorz *et al.*, 2007; Urban and Vogel, 2008)).

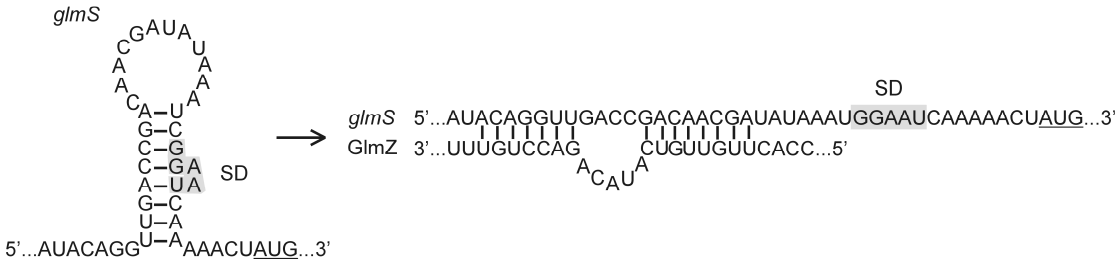

Figure 33: Mechanism of activation of *glmS* translation by GlmZ (according to (Görke and Vogel, 2008)). Upon base-pairing with the *glmS* mRNA GlmZ activates *glmS* translation by unfolding the stem-loop structure that masks the *glmS* SD.

This mechanism of activation of gene expression by a sRNA is similar to the mechanism employed by DsrA in activation of *rpoS* expression (Majdalani *et al.*, 1998). It has previously been shown that the half-life of mRNAs is strongly increased by bound ribosomes (Deana and Belasco, 2005; Iost and Dreyfus, 1995), i.e. ongoing translation stabilizes transcripts. Therefore, stabilization of the *glmS* transcript by base-pairing with GlmZ might be the indirect consequence of the increased translation rate of the *glmS* transcript. However, it cannot be excluded that the altered secondary structure of the GlmZ-bound *glmS* mRNA has a direct effect on the stability of the transcript. It is possible that binding of GlmZ prevents access of RNases to the *glmS*

transcript. A similar case is already known: in the case of the *ptsG* mRNA, both translation initiation and stability of the *ptsG* mRNA are regulated by binding of SgrS. SgrS masks the Shine-Dalgarno region of the *ptsG* transcript by binding to this region (Vanderpool and Gottesman, 2004; Kawamoto *et al.*, 2006; Kawamoto *et al.*, 2005). This leads to strongly reduced translation of *ptsG*. At the same time, binding of SgrS is assisted by Hfq, which recruits RNase E to the sRNA:mRNA complex, initiating its subsequent degradation (Morita *et al.*, 2005).

6.2 A second sRNA, GlmY, also regulates *glmS* expression

Expression of *glmS* is in addition regulated by a second sRNA, GlmY (Urban *et al.*, 2007). GlmY is homologous to GlmZ, both in sequence and in secondary structure (Fig. S3; (Reichenbach *et al.*, 2008; Urban and Vogel, 2008)). It appeared likely that GlmY and GlmZ activate *glmS* expression independently. Such a concerted action on the same target has been described for all other known homologous sRNAs and can be either additive, i.e. the homologous sRNAs act additively to give the full strength of regulation, or redundant, i.e. the action of one of the homologous sRNAs is sufficient to achieve full control of gene expression. Examples are the CsrB/CsrC-sRNAs, which additively regulate the carbon storage protein CsrA in *E. coli* (Weilbacher *et al.*, 2003; Liu *et al.*, 1997), the OmrA/OmrB-sRNAs, which also additively control expression of outer membrane proteins (Guillier and Gottesman, 2006), or the four Qrr1-4 sRNAs of *Vibrio cholerae* that redundantly control quorum sensing (Svenningsen *et al.*, 2009). Surprisingly, our analyses showed that GlmY is unable to activate *glmS* expression in the absence of GlmZ (Fig. 12 B; (Reichenbach *et al.*, 2008)). In addition, GlcN6P depletion does not activate *glmS* expression in the absence of GlmY (Fig. 13; (Reichenbach *et al.*, 2008)). These results show that GlmY and GlmZ do not activate *glmS* expression redundantly or additively as it is the case for all other known homologous sRNAs and their respective targets. Instead, GlmY and GlmZ act in a cascade with GlmY at the top of this cascade. Depletion of GlcN6P leads to accumulation of GlmY. GlmY in turn counteracts processing of GlmZ. The unprocessed form of GlmZ then activates *glmS* expression. In support of this model, GlmY does not possess the region for interaction with

the *glmS* target, which is present in GlmZ (Fig. S3; (Reichenbach *et al.*, 2008)). The GlmY/Z-system is the first report of two sRNAs that act in a cascade, reminiscient of similar cascades, which consist of protein regulators. Therefore, the GlmY/GlmZ sRNA cascade represents a novel mechanism for activation of gene expression by sRNAs.

6.3 On the role of Hfq in the GlmY/Z-system

Several additional factors are involved in regulation of *glmS* expression by GlmY and GlmZ: Hfq, RNase E, PAP I and YhbJ. As described above (section 1.1.3.2), *trans*-acting sRNAs such as GlmZ act on their targets by base-pairing through a short stretch of not perfectly matching nucleotides. In most cases, the RNA chaperone Hfq is needed for efficient formation of the sRNA:mRNA complex. Therefore, it is not surprising that Hfq is also necessary for activation of *glmS* expression by GlmZ (Fig. 4; (Kalamorz *et al.*, 2007)). It probably facilitates complex formation between GlmZ and *glmS*. In agreement, it was shown that Hfq binds to the *glmUS* intergenic region and that GlmZ is enriched 9.8-fold upon co-immunoprecipitation with Hfq in *S. typhimurium* (Sittka *et al.*, 2008). These data are strongly in support of the model that Hfq is necessary for base-pairing between the *glmS* transcript and GlmZ. Accordingly, GlmZ amounts are reduced in an *hfq* mutant (this work, data not shown). Interestingly, GlmY is enriched 4.6-fold co-immunoprecipitation with Hfq in *S. typhimurium* (Sittka *et al.*, 2008). This indicates that also GlmY might be involved in regulation of gene expression by base-pairing.

6.4 On the role of RNase E in the GlmY/Z-system

RNase E is involved in regulation of *glmS* expression at two independent steps. First, it is responsible for the processing of the *glmUS* transcript at the *glmU* stop codon into the *glmS* transcript and a *glmU* encompassing part of the transcript, which is rapidly degraded (Fig. 4A and Fig. S1; (Kalamorz *et al.*, 2007; Joanny *et al.*, 2007)). Because the stop codon is removed from *glmU* upon processing, all functional GlmU proteins probably are translated from the *glmUS* co-transcript. Translation from mRNAs lacking a stop codon cannot be terminated and as a result such mRNAs are rapidly degraded (Keiler, 2008).

Second, usually GlmZ is present in the cell in two forms: a processed form and an unprocessed form. The presemt work shows that processing of GlmZ is conducted by RNase E (Fig. 30). This is in conflict with a previous work, which reported that GlmZ is processed by RNase III (Argaman *et al.*, 2001). Full-length GlmZ possesses the site for base-pairing with the *glmS* transcript at its 3'end. This site is removed upon processing by RNase E. Therefore, only the unprocessed form of GlmZ has the ability to activate *glmS* expression in response to the GlcN6P signal. Upon decrease of the intracellular GlcN6P concentration, GlmZ accumulates in its unprocessed form in a GlmY-dependent manner and *glmS* expression is activated.

This shows that RNase E dependent processing of GlmZ is central to the regulation of *glmS* expression. On the one hand, processing of the active form of GlmZ into its inactive form constantly removes active GlmZ species from the cell. This avoids an unwanted activation of *glmS* expression. Therefore, processing of GlmZ by RNase E must be regulated very accurately: upon perception of the decreasing GlcN6P concentration, processing of GlmZ must be stopped or significantly slowed down. How can regulation of RNase E dependent processing of GlmZ be achieved by GlmY? Processing of GlmZ is not only decreased upon inactivation of RNase E and overexpression of GlmY, but also abrogated upon deletion of *yhbJ*. In a Δ*yhbJ* mutant, GlmZ is no longer processed by RNase E and accumulates to high amounts in its active unprocessed form. This leads to a very strong activation of *glmS* expression in the Δ*yhbJ* mutant. Therefore, it seems likely that YhbJ is involved in regulation of RNase E dependent processing of GlmZ in a GlmY-dependent manner. The involvement of YhbJ could either be direct or indirect via regulation of another unknown factor. GlmY and GlmZ are homologous sRNAs, both in sequence and in secondary structure (Fig. S4;(Reichenbach *et al.*, 2008; Urban and Vogel, 2008)). Therefore, it seems feasible that GlmY regulates processing of GlmZ by a mimicry mechanism: RNase E dependent processing might be dependent on binding of GlmZ to an auxiliary protein, which enables RNase E to recognize GlmZ as a substrate. One could imagine that such a protein would bind to both GlmY and GlmZ because of their homologous secondary structures. Upon accumulation of GlmY, a this protein

would bind to GlmY, which would outcompete GlmZ. As a result, GlmZ would accumulate in the active unprocessed form. This would in turn activate *glmS* expression. The fact that also overexpression of GlmY from *E. carotovora* is able to induce expression of *glmS* in *E. coli* (Urban *et al.*, 2007), is in support of such a mimicry mechanism. It is conceivable that YhbJ is the protein, which binds GlmY and GlmZ. Alternatively, YhbJ might regulate the expression or activity of another protein that in turn regulates RNase E activity (see section 6.7 for the function of YhbJ). A good candidate for such a protein is RraB. RraB is an inhibitor of RNase E activity (Gao *et al.*, 2006) and it was recently shown that that expression of *rraB* is activated in response to reduced GlmS activity (Zhou *et al.*, 2009). RraB is discussed in more detail in section 6.7.

6.5 Turning off the signal: Control of GlmY halflife by polyadenylation

Both GlmY and GlmZ are expressed at high levels (Fig. 21B; (Reichenbach *et al.*, 2009) and unpublished results of Denise Lüttmann and Boris Görke). To prevent an unwanted activation of *glmS* expression by uncontrolled accumulation of GlmY or GlmZ, active GlmY and GlmZ species must be continuously removed from the cell. As discussed above, active GlmZ species are constantly removed from the cell by RNase E dependent processing and subsequent degradation. For GlmY, the situation is different. Interestingly, inactivation of either *pcnB* encoding PAP I or *pnp* encoding PNPase results in accumulation of GlmY and activation of the GlmY/Z cascade (Fig. 30 and Fig. 14; (Reichenbach *et al.*, 2008)). A previous report showed that inactivation of the PAP I encoding gene *pcnB* leads to activation of *glmS* expression. Since the GlmY/Z cascade was unknown at the time of the report, it was suggested that PAP I acts directly on the monocistronic *glmS* transcript (Joanny *et al.*, 2007). In contrast, our study showed that this is not the case and that PAP I acts at the top of the GlmY/Z cascade (Reichenbach *et al.*, 2008). This finding was confirmed by a second study, which was conducted in parallel (Urban and Vogel, 2008). PAP I adds poly(A) tails to the 3'ends of RNA species, which possess stable secondary structures at their 3'ends. The exoribonuclease PNPase is unable to overcome these structures. Polyadenylation by PAP I enables PNPase to degrade these RNAs. We showed that GlmY is polyadenylated at the 3'end (Reichenbach *et al.*, 2008).

Since polyadenylation is the first step for RNA degradation, it seems likely that the function of PAP I in the GlmY/Z cascade is to allow constant degradation of superfluous GlmY species. This constant removal of GlmY keeps the system sensitive for GlcN6P signal perception. It has been reported that also sRNA SraL is subject to polyadenylation. SraL is stabilized in a mutant lacking PAP I (Argaman *et al.*, 2001; Viegas *et al.*, 2007). In many cases, sRNAs are removed from the cell by co-degradation with their targets following base-pairing to the target mRNA, e.g. SgrS is rapidly degraded along with its target mRNA *ptsG* (Morita *et al.*, 2005). GlmY does not function by base-pairing to a target mRNA. Therefore, GlmY must be removed from the cell by a different mechanism, namely by constant degradation following polyadenylation by PAP I. Therefore, the function of sRNA polyadenylation might be to remove those sRNAs from the cell that are not automatically removed by degradation as a consequence of their base-pairing function. Since the physiological function of SraL is unknown, GlmY is the first known case, where polyadenylation of a sRNA serves a function in regulation of gene expression by avoiding an unwanted activation of *glmS* expression.

Why are so many factors involved in the regulation of *glmS* expression? In theory, one could imagine that direct regulation of GlmZ in response to the GlcN6P signal should be sufficient for this regulation. Instead, two sRNAs and multiple protein factors are involved in this cascade. Therefore, in the case of regulation of *glmS* expression the GlmY/Z cascade must have an advantage over “classical” regulation by just one sRNA. One possibility is that the cascade allows multiple entry points for different signals. Another possibility is that the two sRNAs allow amplification of the activating signal or a faster response time in comparison to “classical” systems. Whether this is the case could be addressed by mathematical modeling of this system.

Another possible reason for the complexity of the GlmY/Z cascade is that GlmY and GlmZ might have additional regulatory functions. It is conceivable that the main function of the cascade is regulation of *glmS* expression during the exponential growth phase. In stationary phase, feedback regulation of *glmS* expression is no longer necessary, since synthesis of cell wall components and cell growth has stopped. In agreement, the σ^{70}-dependent

promoters of *glmZ* and *glmUS* are turned off in stationary phase (Fig. 10; (Reichenbach *et al.*, 2008)). However, expression of *glmY* increases in stationary phase (Fig. S6; (Reichenbach *et al.*, 2009)). Hence, it appears likely that GlmY has additional functions of GlmY during stationary phase, which could be absolutely unrelated to its function in regulation of *glmS* expression.

6.6 Regulation of GlmY expression by a dual promoter and the two-component system GlrK/R

In addition to the post-transcriptional regulation of GlmY by PAP I, *glmY* expression is also extensively regulated. GlmY is transcribed from two promoters: one σ^{70}-dependent promoter and one σ^{54}-dependent promoter (Fig. 20 A; (Reichenbach *et al.*, 2009)). These two promoters overlap in a manner that in both cases *glmY* transcription is started at the same position (Fig. 20; (Reichenbach *et al.*, 2009)). This is important because GlmY species with a different 5'end might have different physiological functions as it is the case for the IstR-1 and IstR-2 sRNAs. These sRNAs are generated from the same DNA template, but they have different 5'ends and also different functions (Vogel *et al.*, 2004). In the case of GlmY, both transcripts possess identical 5'ends and therefore should have the same physiological function. Why is it necessary to express *glmY* from two promoters? The two promoters could regulate the GlmY amount in response to different environmental signals. The σ^{70}-dependent promoter seems to be active mainly during exponential growth (Fig. 19; (Reichenbach *et al.*, 2009)), while the σ^{54}-dependent promoter is active all the time and activity increases 2-3-fold during transition to stationary phase (Fig. 19 and Fig. S6; (Reichenbach *et al.*, 2009)). GlmY might have an additional function, which is distinct from regulation of *glmS* expression during the exponential growth phase. Since the induction of *glmY* expression during transition to stationary phase is dependent on the σ^{54}-dependent promoter of *glmY*, this potential function should be connected to activation of the σ^{54}-dependent promoter.

All σ^{54}-dependent promoters require activation by a specific activator protein, which bind upstream of the promoter region (Reitzer and Schneider, 2001). This work identified the σ^{54}-activator protein GlrR as the specific activator of

the *glmY* σ^{54}-dependent promoter (Reichenbach *et al.*, 2009). GlrR is the response regulator in the GlrR/GlrK TCS, which is encoded just downstream of *glmY*. In TCS, the autophosphorylation rate of the histidine kinase, in this case GlrK, is altered –usually activated- upon sensing of the TCS-specific signal. The phosphoryl group is subsequently transferred to the response regulator, which in most cases is activated by phosphorylation. Indeed, first evidence suggests that phosphorylation of GlrR enhaces its DNA-binding acitivity (Sabine Zeides, Birte Reichenbach and Boris Görke, unpublished data). What is the signal that is sensed by GlrK and subsequently leads to GlrR-dependent activation of the σ^{54}-dependent *glmY* promoter? Since the σ^{54}-dependent promoter is active under all tested conditions, the signal that activates it should be present in exponentially growing cells in LB medium, although the signal intensity should increase upon entry of stationary growth phase. Interestingly, promoter activity of the σ^{54}-dependent promoter is two-fold increased in M9 minimal medium as compared to LB, but not in M9 minimal medium that is supplemented with a mixture of all amino acids (unpublished observation, Birte Reichenbach and Boris Görke). This indicates that the σ^{54}-activating signal might be connected to growth rate, media composition, diminished presence of a certain molecule or production of a signaling molecule by the cell.

Why is the σ^{54}-dependent promoter activated upon entry into stationary phase? During stationary growth phase, expression from the *glmZ* and *glmUS* σ^{70}-dependent promoters is strongly reduced (Fig. 10). This makes sense, since peptidoglycan and LPS biosynthesis is not necessary during stationary phase where cells do not grow. Therefore, also feedback activation of *glmS* expression is not necessary during these conditions. In this respect, the observed activation of GlmY expression upon entry of stationary phase does not make sense. For this reason it seems probable that, as proposed above, GlmY fulfills a function, which is distinct from regulation of *glmS* expression, during stationary growth phase. Microarray analyses of GlmY and GlmZ overexpressers and a Δ*yhbJ* strain show that the regulatory overlap between deletion of *yhbJ* and overexpression of GlmY or GlmZ is very small: only four genes are similarly regulated under these conditions (Kalamorz, 2009). In

contrast, the regulatory overlap between GlmY and GlmZ overexpression is much larger (Kai Papenfort, Tilmann Künzl, Jörg Vogel and Boris Görke, unpublished data). These observations hint at a proposed second function for GlmY during stationary growth phase. The nature of such a function remains to be defined.

6.7 What is the function of YhbJ?

The data in this work indicate two possible functions for YhbJ. First, YhbJ could be an RNA-binding protein that sequesters GlmY and/or GlmZ and may relay the signal from GlmY to GlmZ. In this scenario, YhbJ could regulate GlmZ activity by binding of RNase E facilitating processing of GlmZ. YhbJ-facilitated processing of GlmZ might be counteracted by binding of GlmY to YhbJ. Second, YhbJ could be a modulator of the activity of σ^{54}-dependent promoters. The activities of at least two σ^{54}-dependent promoters are affected by YhbJ, i.e. the σ^{54}-dependent *rtcB* promoter and the σ^{54}-dependent *glmY* promoter. In the latter case, promoter activity is strongly reduced in *yhbJ* mutants (Fig. 26 A). Overexpression of *yhbJ* has the opposite effect: in this case the activity of the σ^{54}-dependent *glmY* promoter is approximately two-fold higher as compared to the wild type (Fig. 26 C). In both cases, control of GlmZ processing as well as control of *glmY* promoter activity, the effects of the deletion of *yhbJ* can be complemented by expression of *yhbJ* from a plasmid. Moreover, the effects of the Δ*yhbJ* mutation on GlmZ processing and on GlmY expression are independent from each other. Modulation of σ^{54}-dependent promoter activity of P_{glmY} is independent of the presence of GlmY or GlmZ (Fig. 27) and abrogation of GlmZ processing in the Δ*yhbJ* strain cannot be rescued by deletion or overexpression of *glmY* (Fig. 11 A and Fig. 12 B; (Reichenbach *et al.*, 2008)). Therefore, it is evident that the effects of the *yhbJ* mutation on GlmY and GlmZ are independent of each other. Several possible explanations come into question: First, YhbJ might be both an RNA-binding protein and a modulator of the activity of σ^{54}-dependent promoters. Second, YhbJ might be an RNA-binding protein or alternatively a modulator of σ^{54}-dependent promoter activity. In these latter cases, either the effect on GlmZ or on the σ^{54}-promoters must be indirect. The modulation of σ^{54}-

promoters by YhbJ might occur through a so far unknown RNA-target of YhbJ that is not identical with GlmY or GlmZ. One possibility is that this RNA molecule affects σ^{54}-dependent promoter activity by interaction with the σ^{54} RNA polymerase holoenzyme. Regulation of an RNA polymerase holoenzyme by a sRNA is not unprecedented: transcription from σ^{70}-dependent promoters by σ^{70} RNA polymerase holoenzyme is downregulated by the sRNA 6S RNA at the onset of stationary phase (Wassarman, 2007). Another possibility is that the expression of σ^{54} is regulated by YhbJ either by direct binding or through a regulatory RNA. In addition, it is conceivable that YhbJ controls expression of a factor that regulates RNase E dependent processing of GlmZ in a GlmY-dependent manner. Such a factor should fulfill several prerequisites: it should be expressed from a σ^{54}-dependent promoter and it should control activity of RNase E or another factor involved in RNA metabolism. Several factors, which fulfill at least one of these prerequisites, are listed in table 8. Of these candidates, the most promising are encoded by the *rtcBA* operon and *rraB*.

The *rtcBA* operon is transcribed from a σ^{54}-dependent promoter, which is regulated by the divergently encoded σ^{54}-activator protein RtcR. Both *rtcB* and *rtcA* are highly conserved in prokaryotes and eukaryotes (Genschik *et al.*, 1997; Okada *et al.*, 2006) and RtcA might have a function in RNA metabolism as a RNA 3'-terminal phosphate cyclase (Genschik *et al.*, 1997; Genschik *et al.*, 1998). The actual physiological function of RtcA is unknown. Due to the high conservation of both genes and their co-localization in one operon, it is feasible that also RtcB might have a role in RNA metabolism. Therefore, expression from the *rtcB* σ^{54}-dependent promoter was analyzed in the wild type, in a Δ*rpoN* strain and in a Δ*yhbJ* strain. The data show that deletion of *yhbJ* increases expression from the σ^{54}-dependent promoter upstream of *rtcB*. It will be interesting to test, whether the effect of a *yhbJ* mutation on GlmZ processing is abolished in Δ*yhbJ* Δ*rtcA* or Δ*yhbJ* Δ*rtcB* double mutants.

Gene *rraB* is transcribed from a σ^{70}-dependent promoter. Expression of *rraB* is activated in response to reduced GlmS enzymatic activity (Zhou *et al.*, 2009). The mechanism of regulation of *rraB* expression by depletion of amino sugars is still unknown. Reduced GlmS activity results in low intracellular levels of GlcN6P and of the metabolites synthesized downstream of the

GlmS-catalyzed reaction. Reduced enzymatic activity of GlmS also activates the GlmY/GlmZ/*glmS* cascade, which ultimately activates *glmS* expression (Fig. 8 D, 8 E and Fig. 13; (Reichenbach *et al.*, 2008; Kalamorz *et al.*, 2007)). Therefore, RraB could have a function in regulation of the GlmY/GlmZ/*glmS* cascade. RraB is an inhibitor of RNase E activity (Gao *et al.*, 2006). Binding of RraB alters the composition of the degradosome. Both overexpression and deletion of *rraB* result in altered processing and abundance of many transcripts (Gao *et al.*, 2006; Yeom *et al.*, 2008). While processing of the *glmUS* transcript appears not to be regulated by GlcN6P, the situation is different for GlmZ processing. Expression of *glmS* is triggered by the RNase E dependent processing of GlmZ. RraB might be involved in regulation of *glmS* expression by regulation of GlmZ processing, since it is a regulator of RNase E activity. Two possibilities how RraB might be involved in regulation of GlmZ processing are depicted in Fig. 34.

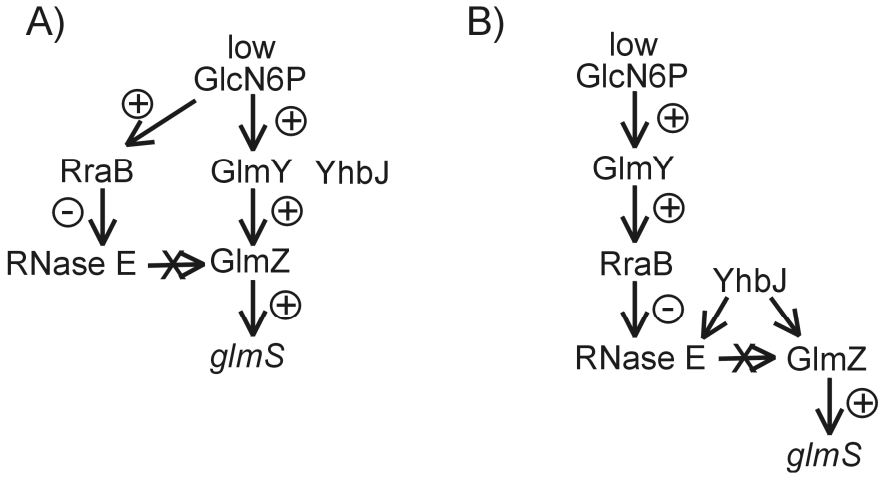

Figure 34: Two possibilities for an involvement of RraB in regulation of GlmZ processing. **A.** Transcription of *rraB* might be activated in response to a low GlcN6P concentration. RraB could function by specifically inhibiting RNase E dependent processing of GlmZ. **B.** Expression of *rraB* could be activated by GlmY in response to limiting GlcN6P levels. RraB might then, in turn inhibit RNase E dependent processing of GlmZ.

While YhbJ might be a direct activator of GlmZ processing, it is possible that RraB is the direct antagonist of YhbJ by acting as an inhibitor of GlmZ processing. If this is the case, GlmZ processing could be regulated by the cell in two different ways. In the first scenario (Fig. 34 A), YhbJ would function as an RNA binding protein that is titrated away from GlmZ by increased abundance of GlmY. This would in turn lead to reduced processing of GlmZ by RNase E, resulting in the accumulation of full-length GlmZ. Due to the high homology of GlmY and GlmZ, titration of YhbJ from GlmZ would probably not

be complete. To sufficiently reduce RNase E dependent processing it might therefore be necessary to inhibit YhbJ/RNase E dependent processing of GlmZ by specifically inhibiting this processing event. This might be achieved by increasing the expression of the RNase E inhibitor RraB in a GlcN6P concentration dependent manner. RraB might then specifically displace YhbJ from GlmZ, e.g. by binding to GlmZ. This would necessitate that RraB has a higher affinity for GlmZ than YhbJ. Displacement of YhbJ from GlmZ by RraB should be faster and more complete as compared to a strain lacking RraB. This scenario could also explain why a slight activation of *glmS* expression seems to be possible in response to GlcN6P limitation in a Δ*glmY* mutant strain (Fig. 13; (Reichenbach *et al.*, 2008)). This slight activation of *glmS* expression could be explained by displacement of YhbJ from GlmZ by RraB due to activation of *rraB* expression upon GlcN6P limitation. In the second scenario (Fig. 34 B), RraB might function as a specific inhibitor of RNase E dependent processing of GlmZ. Here, only GlmY abundance is directly upregulated by the GlcN6P limitation signal. GlmY could then induce expression of *rraB* by basepairing to and subsequent stabilization of the *rraB* messanger or increased translation of *rraB*. This would in turn inhibit RNase E dependent processing of GlmZ. To exclude one of these two possibilities, one could analyze expression of a *rraB::lacZ* fusion upon limitation of GlcN6P and upon overexpression of GlmY. If expression of *rraB* is only activated by limitation of GlcN6P, but not by overexpression of GlmY, this would exclude the second scenario.

6.8 How is the GlcN6P signal sensed by the cell?

The mechanism by which the cell senses the intracellular GlcN6P concentration is currently unknown. However, several facts are known that should be considered in subsequent experiments searching for the GlcN6P sensing mechanism. First, GlmY is required for sensing and/or transducing the signal (Fig. 13; (Reichenbach *et al.*, 2008)). Second, abundance of GlmY is increased upon decreasing intracellular GlcN6P levels (Fig. 13; (Reichenbach *et al.*, 2008)). Third, expression of *glmY* is not affected upon limitation of GlcN6P (Fig. S9; (Reichenbach *et al.*, 2009)). Therefore, the

signal should be sensed post-transcription of GlmY by a factor that governs the abundance of GlmY or by GlmY itself. Fig. 35 presents a schematic overview over the possible entry points for sensing of the GlcN6P signal.

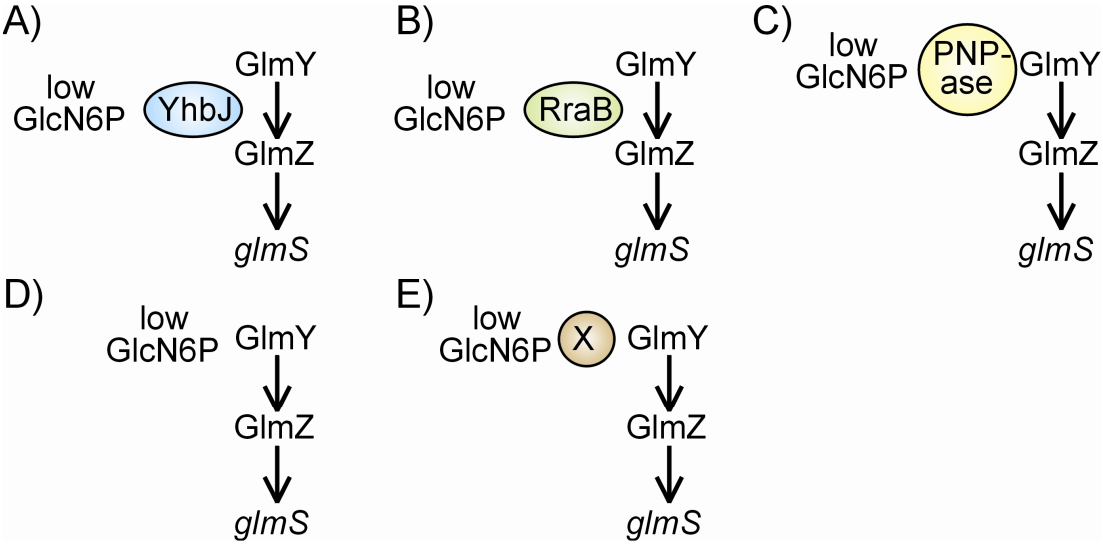

Figure 35: Overview over possible entry points for sensing of the GlcN6P signal. A. The signal is sensed by YhbJ or a protein upstream of YhbJ. **B.** The signal is sensed by or by a factor upstream of RraB. **C.** The signal is sensed by or by a factor upstream of PNPase. **D.** The signal is sensed directly by GlmY. **E.** The signal is sensed by an unknown factor.

One possibility is that the GlcN6P concentration would be directly sensed through binding of GlcN6P to GlmY (Fig. 35 D). In this scenario, a low GlcN6P concentration would result in unoccupied GlmY molecules, which should become stabilized and should therefore accumulate. This would then lead to activation of *glmS* expression. Such a mechanism would be reminiscient of the feedback regulation of *glmS* expression in *B. subtilis*. In *B. subtilis*, *glmS* expression is regulated through binding of GlcN6P to the 5'UTR of the *glmS* transcript (Winkler *et al.*, 2004). One factor that governs the abundance of GlmY is PAP I. However, a mutant lacking PAP I still responds to limitation of GlcN6P by activation of *glmS* expression (this work, data not shown). This excludes that GlcN6P sensing involves PAPI. Other factors that could serve as the sensor of the GlcN6P concentration are YhbJ, PNPase and RraB. PNPase could act as a GlcN6P sensor and function by stopping the degradation of GlmY, when the GlcN6P concentration is reduced (Fig. 35 C). Moreover, GlcN6P coiuld modulate the ability of YhbJ to bind GlmY. Upon binding, GlmY could be protected from degradation explaining explaining the increasing GlmY amounts upon GlcN6P limitation (Fig. 35 A). Finally, the

actor that regulates expression of *rraB* could represent the factor that senses GlcN6P. If this is the case, activation of *rraB* expression in response to a low GlcN6P concentration could result in displacement of YhbJ from GlmZ and increased binding of YhbJ to GlmY. This would result in RraB dependent inhibition of RNase E dependent processing of GlmZ (Fig. 35 B). It cannot be excluded that a so far unidentified factor is responsible for sensing of the GlcN6P signal (Fig. 35 E). To find the mechanism by which the intracellular GlcN6P concentration is sensed, further analyses are necessary. In a first step, it will be interesting to see, whether activation of *glmS* expression by GlcN6P limitation is possible in a Δ*rraB* mutant. If activation of *glmS* expression is not possible under this condition, it will be interesting to find the factor that activates expression of *rraB*. To this end, one might employ a plasmid bank of all *E. coli* genes to screen for the activator of *rraB* expression.

6.9 Evolution of GlmY and GlmZ

GlmY and GlmZ are homologous both in sequence and in secondary structure (Fig. S4; (Reichenbach *et al.*, 2008)). Although the advantage of regulating *glmS* expression by a cascade consisting of two sRNAs remains to be elucidated, it is possible to draw conclusions concerning the evolutionary development of the GlmY/GlmZ system. It is unlikely that GlmY and GlmZ have developed independently from one another, because GlmY and GlmZ are highly homologous. In contrast, it seems that GlmY and GlmZ evolved by duplication of an ancestral sRNA species, resulting in the two homologous RNAs. All genomes that possess a copy of GlmY also possess a copy of GlmZ and vice versa. However, there must have been an ancestor that contained only one copy of GlmY/Z. It is possible that a bacterium that retained this ancestral organization might be discovered in the future. It will be interesting to see, how *glmS* expression is regulated in such an organism.

Alignments of the control regions that are located upstream of *glmY* and *glmZ* from different organisms, showed that in addition to GlmY also GlmZ is putatively transcribed from a σ^{54}-dependent promoter in most species (Fig. S7 and Fig. S10; (Reichenbach *et al.*, 2009)). The *glmZ* gene apparently possesses a σ^{54}-dependent promoter in all analyzed species, except for *E.*

coli, *S. flexneri* and *Klebsiella pneumoniae* (Fig. S10; (Reichenbach *et al.*, 2009)). In the latter cases, *glmZ* should be transcribed from a σ^{70}-dependent promoter, at least in *E. coli* K12 (Denise Lüttmann and Boris Görke, unpublished observation). It is tempting to speculate that in the ancestral bacterium, in which the duplication of GlmY/Z occurred, both sRNAs were expressed the from σ^{54}-dependent promoters. Subsequently, the σ^{54}-dependent *glmZ* promoter was substituted by a σ^{70}-dependent promoter in some species such as *E. coli*, *Shigella flexneri* and *Klebsiella pneumoniae*. Interestingly, *Salmonella* species, which are closely related to *E. coli*, retained the σ^{54}-dependent promoter. It is reasonable to assume that the substitution of the *glmZ* σ^{54}-dependent promoter by a σ^{70}-dependent promoter occurred only in the ancestor of either *E. coli* and *Shigella*, which are closely related, or of *Klebsiella*, which is not as closely related as e.g. *Salmonella*. Hence, either *E. coli* and *Shigella* or *Klebsiella* might have acquired the σ^{70}-dependent promoter by horizontal gene transfer. It is also interesting to note that some, but not all analyzed organisms possess both a rather perfect σ^{54}-promoter consensus sequence and a potential σ^{70}-promoter sequence in front of the *glmY* gene (Fig. S7; (Reichenbach *et al.*, 2009)). This could indicate that the GlmY/GlmZ system is in the process of evolving from a σ^{54}-dependently expressed system to a σ^{70}-dependently expressed system. In all cases where GlmZ is transcribed from a σ^{54}-dependent promoter, the GlrR activator binding sites, which were identified upstream of the *E. coli glmY* gene, are also present. Therefore, also GlmZ expression should be regulated by the GlrR/GlrK two component system in response to the unknown signal in these organisms.

It is interesting to note that while GlmY and GlmZ are conserved in most *Enterobacteriacea*, homologs of YhbJ can be found in most bacterial species. Hence, YhbJ cannot be involved in *glmS* regulation in these organisms. In agreement, no effect on *glmS* expression could be observed in a *B. subtilis* mutant lacking the YhbJ homolog YvcJ (Luciano *et al.*, 2009). This raises the question whether YhbJ is a specific regulator of *glmS* expression in *E. coli*, or whether the effect on processing of GlmZ in a Δ*yhbJ* mutant is an indirect consequence of a yet unknown function of YhbJ. The fact that also at least

two σ^{54}-dependent promoters are affected in their activities by YhbJ indicates that YhbJ might have a function in regulation of promoter activity. In this respect, it is also interesting to note that microarray analyses of a Δ*yhbJ* mutant, a GlmY overexpression strain and a GlmZ overexpressing strain showed that the regulatory overlap between GlmY/Z and YhbJ appears to be very small. In contrast, several genes are equally regulated in the GlmY and GlmZ overexpressing strains (Tilmann Künzl, Kai Papenfort, Falk Kalamorz, Jörg Vogel and Boris Görke, unpublished observation). Therefore, it would not be surprising, if it turned out that the effect of the *yhbJ* deletion on GlmZ is indirect.

6.10 Function of the GlrR/GlrK two component system

While the signal that is sensed by GlrK in *E. coli* K12 is unknown, two previous studies have implicated an involvement of the GlrR/GlrK two component system in regulation of $espF_U$ expression in the pathogenic enterohemorrhagic *E. coli* strain O157:H7 (EHEC) (Reading *et al.*, 2007; Reading *et al.*, 2009). Gene $espF_U$ is encoded within the bi-cistronic *espJ-*$espF_U$ operon and it encodes a proline rich homolog of *espF* (Garmendia and Frankel, 2005). This operon is not present in *E. coli* K12 and it encodes functions, which are associated with virulence: $espF_U$ (also known as *tccP*) encodes the type III effector protein Tir-cytoskeleton coupling protein, which is involved in recruiting factors important for adhesion to the host cell (Garmendia *et al.*, 2004) and *espJ* encodes a type III effector protein that modulates infection dynamics (Dahan *et al.*, 2005). Pathogenicity of EHEC is dependent on the type III secretion system that is encoded within the Locus of Enterocyte Effacement (LEE) pathogenicity island (Elliott *et al.*, 1998; Ritchie and Waldor, 2005). The LEE island encodes not only the type III secretion system, but also effectors and translocators of this system. Additional effectors of type III secretion, such as $espF_U$ and *espF*, are encoded outside of the LEE island in prophages (Gruenheid *et al.*, 2004; Tobe *et al.*, 2005). In contrast to a report claiming that *espJ* and $espF_U$ form an operon (Garmendia and Frankel, 2005), a later study showed that both $espF_U$ and *espJ* constitute individual transcripts, which are transcribed from independent promoters

(Reading *et al.*, 2007). This study also showed that transcription of *espF*$_U$ is slightly reduced in mutants lacking *glrK* and strongly reduced in mutants lacking *glrR*. Since *espF*$_U$ does not possess a σ^{54}-dependent promoter (Reading *et al.*, 2007), it appears likely that the GlrR σ^{54}-activator protein does not directly regulate *espF*$_U$ expression. In contrast, a so far unknown factor should exist, which is regulated by the GlrK/GlrR two component system in a σ^{54}-dependent manner and in turn regulates *espF*$_U$ expression. This factor could be GlmY, because it is transcribed from a σ^{54}-dependent promoter and was identified as a target of the GlrK/GlrR two component system. Furthermore, preliminary software analyses indicate that *glmY* is the only target of GlrR (unpublished observation). Therefore, it was analyzed whether expression of an *espF*$_U$*::lacZ* fusion is also affected in *E. coli* K12 in a Δ*glrR* strain as compared to the wild type, but no alteration in *espF*$_U$*::lacZ* expression could be observed upon deletion of *glrR* (this work, data not shown). This indicates that the factor, which is regulated by GlrR and which then regulates *espF*$_U$ expression is not present in *E. coli* K12. Although this result does not exclude the possibility that GlmY is involved in regulation of *espF*$_U$ expression, the direct effector of *espF*$_U$ expression should be encoded within one of the EHEC-specific pathogenicity islands. Such a factor could then be regulated by GlmY in a GlrR-dependent manner. A recent analysis compared transcript amounts of LEE encoded genes and type III secretion system effectors, which are encoded outside of the LEE island, in an EHEC wild type strain and an EHEC Δ*hfq* strain (Shakhnovich *et al.*, 2009). The study showed that all LEE encoded genes and approximately half of the non-LEE encoded effectors of the type III secretion system are differentially regulated in a Δ*hfq* mutant as compared to the wild type, including the non-LEE encoded *espF*$_U$ gene. Expression of *espF*$_U$ is upregulated 2.4-fold in the Δ*hfq* mutant as compared to the wild type. Hfq is often involved in sRNA dependent regulations (Sittka *et al.*, 2008). Therefore it is possible that a sRNA might be responsible for the observed Hfq-dependent regulation of the type III secretion system and its effectors. Due to the observed indirect regulation of *espF*$_U$ expression by GlrR ((Reading *et al.*, 2007); this work) and the observed regulation of *espF*$_U$ amounts by Hfq (Shakhnovich *et al.*, 2009),

it is possible that these regulations are carried out by GlmY via at least one additional pathogenicity island encoded factor.

It is interesting to note that *E. coli* EHEC is not the only organism where deletion of *glrR* results in a phenotype of reduced pathogenicity. For *Yersinia pseudotuberculosis* 32777 it was found that deletion of *glrR* reduces virulence in a mouse model (Flamez *et al.*, 2008). It is tempting to speculate that GlrR and maybe also GlmY might have a role in regulation of pathogenicity in some pathogenic relatives of *E. coli* K12.

7. Conclusion and perspectives

This work presents the discovery of a novel mechanism for the regulation by bacterial small non-coding RNAs. It has not only discovered one of a few rare cases, where a sRNA acts as an activator of its target, but it has also found the first example of two (homologous) sRNAs that act in a cascade to regulate gene expression. In addition, the present work reports the discovery of the first example for transcripts that are started at the same position from overlapping σ^{70}-dependent and σ^{54}-dependent promoters. Nevertheless, several open questions remain.

The exact mechanism of how the activating signal is sensed and subsequently transduced to activate *glmS* expression is unknown. To gain a better insight into this mechanism, the next steps should be the analysis of a Δ*rraB* mutant strain. Is signal transduction of the GlcN6P signal possible in this strain? Is the abundance of GlmY, GlmZ or *glmS* altered? In addition it will be interesting to see, how expression of *rraB* is regulated. Is *rraB* expression activated by GlmY overexpression? Or is it only induced upon induction of GlcN6P limitation?

Another open question is, how YhbJ can at the same time regulate the processing of GlmZ and also regulate the activity of the σ^{54}-dependent *glmY* promoter. In a first step, one could analyze, whether YhbJ specifically binds to GlmY and/or GlmZ. To this end, one could see whether GlmY or GlmZ specifically co-purify with YhbJ and vice versa. In addition one could conduct gel shift experiments employing purified YhbJ protein and *in vitro* transcribed GlmY and GlmZ RNAs. If these experiments show that YhbJ is indeed an RNA binding protein, one could screen a library of sRNA mutants in a Δ*yhbJ* strain for such a sRNA that is responsible for reducing *glmY* σ^{54}-dependent expression in a Δ*yhbJ* strain.

Bioinformatical analyses show that *glmZ* appears to be transcribed from a σ^{54}-dependent promoter in many species, but not in *E. coli*. It will be interesting to see, if transcription of *glmZ* depends on *rpoN* and *glrR* in e.g. *Y. pseudotuberculosis*. To see if this is the case, one could perform gene expression analyses of *glmZ* in *rpoN* and *glrR* mutant strains of *Y. pseudotuberculosis*. In addition, employing *in vitro* gel shift experiments one

could see whether *Y. pseudotuberculosis* GlrR binds to the *glmZ* promoter region of *Y. pseudotuberculosis*, but not to that of *E. coli glmZ*.

Since expression of GlmY increases in stationary phase, it is conceivable that GlmY might have additional target that are regulated by GlmY during stationary phase. In this respect, it will be necessary to identify more targets of GlmY. Preliminary analyses of expression of genes, which were differentially regulated upon GlmY overexpression in a microarray analysis, indicate that indeed more targets of GlmY might exist (Tilmann Künzl, Birte Reichenbach and Boris Görke, unpublished).

8. References

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