2570–2580 *Nucleic Acids Research, 2008, Vol. 36, No. 8* *Published online 11 March 2008*
doi:10.1093/nar/gkn091

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*

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Received January 3, 2008; Revised February 11, 2008; Accepted February 12, 2008

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 mechansim (1,2). 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 (3). 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 (4).

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* (5). The primary *glmUS* transcripts are subject to processing by RNase E at the *glmU* stop codon (4,6). 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 (4). This sRNA is synthesized as a 210 nt long precursor and subsequently processed, presumably by RNase III, to

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yield a form of ∼155 nt (7,8). 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 (4). 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 (4). 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 (9). GlmY is encoded in the *purL–yfhK* intergenic region and evidence suggests that it is transcribed from a σ^{54}-dependent promoter (9). GlmY has been reported to exist in two different sizes of 184 and 148 nt, respectively. The shorter and more abundant form was suggested to result from 3′ end processing of the longer variant (9,10). 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 (4,6). 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 (11–13). 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 (14,15). These may be decay intermediates or primary transcripts carrying a *rho*-independent terminator at the 3′-end (16). 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 (17,18).

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°C 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 1. See Table 2 for the list of oligonucleotides used in this study. For DNA cloning, strain DH5α was used following standard procedures (19). 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 same sites on plasmid pBGG179. Plasmid pBGG179 carries the multiple cloning site of plasmid pBAD33 (20) 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 (21). 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 (22). 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 (23). 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

Table 1. Strains and plasmids used in this study

Strain/plasmid	Genotype or relevant structures	Reference, source or construction
A. strains		
IBPC903	as N3433, but Δ *pcnB::kan*	(6)
N3433	HfrH, *lacZ*43, λ, *relA*1, *spoT*1, *thi*1	(37)
R1279	CSH50 Δ(*pho-bgl*)201 Δ(*lac-pro*) *ara thi*	(38)
R2413	as R1279, but Δ[*ptsN*, Δ*yhbJ*, Δ*ptsO*]	(4)
Z8	as R1279, but *attB*::[*aadA glmS*-5'::*lacZ*] $strp^R$ $F'(pro^+)$	(4)
Z24	as R1279, but Δ*yhbJ*::*cat*	(4)
Z28	as R1279, but Δ*yhbJ*, *attB*::[*aadA glmS*-5'::*lacZ*] $strp^R$ $F'(pro^+)$	(4)
Z37	as R1279, but Δ*yhbJ*	(4)
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*	(4)
Z45	as R1279, but Δ*glmZ*	(4)
Z46	as R1279, but Δ[*ptsN*, Δ*yhbJ*, Δ*ptsO*], Δ*glmZ*::*cat*	(4)
Z47	as R1279, but Δ[*ptsN*, Δ*yhbJ*, Δ*ptsO*], 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*, Δ*yhbJ*, Δ*ptsO*], 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*	(20)
pBGG84	*glmZ* under P_{Ara}-control in pBAD30	(4)
pBGG149	as pBGG179, but *glmY* downstream of λ P_L	This work
pBGG179	ori pMB1, λP_L, MCS, *bla*	This work

ori, origin of replication; MCS, multiple cloning site.

Table 2. 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	GGGATGTTATTTCCCGATTCTCTGTGGCATAATAAACGAGTGT AGGCTGGAGCTGCTTCG		*glmZ* −39 to −1
BG185	CACCCGGAGGCAAGCACCTCCGGGGCCTTCCTGATACATCAT ATGAATATCCTCCTTAGTTCCTATTCC		*glmZ* + 248 to + 207
BG230	GTAGATGCTCATTCCATCTC		*glmZ* + 1 to + 20
BG231	CTAATACGACTCACTATAGGGagAAAACAGGTCTGTATGACAAC		*glmZ* + 172 to + 152
BG248	CAACAAAGCCGGGAATTACCCGGCTTTGTTATGGAAGTGTAGG CTGGAGCTGCTTCG		*glmY* + 185 to + 150
BG249	CTATTTTCTTTATTGGCACAGTTACTGCATAATAGTAACCCATAT GAATATCCTCCTTAGTTCCTATTCC		*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-AATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCA GGCATGCAAGCTTG	MCS[c] of pBAD33	
BG419	P-GATCCAAGCTTGCATGCCTGCAGGTCGACTCTAGA GGATCCCCGGGTACCGAGCTCG	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.

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 gel electrophoresis. 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 0.5 × 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). Here, 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°C. Total RNAs were extracted as described previously (24). Five micrograms were loaded on a high resolution 6% denaturing polyacrylamide gel, electrotransferred and hybridized with ^{32}P-labeled RNA probes as described previously (24). 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 (24) 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/µl BSA and 10% DMSO (25). After precipitation with ethanol, the pellet was re-suspended in 20 µl water. Five microlitres 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°C 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* (26) raised the possibility that YhbJ might have a global function and that it could also be a regulator of sRNAs

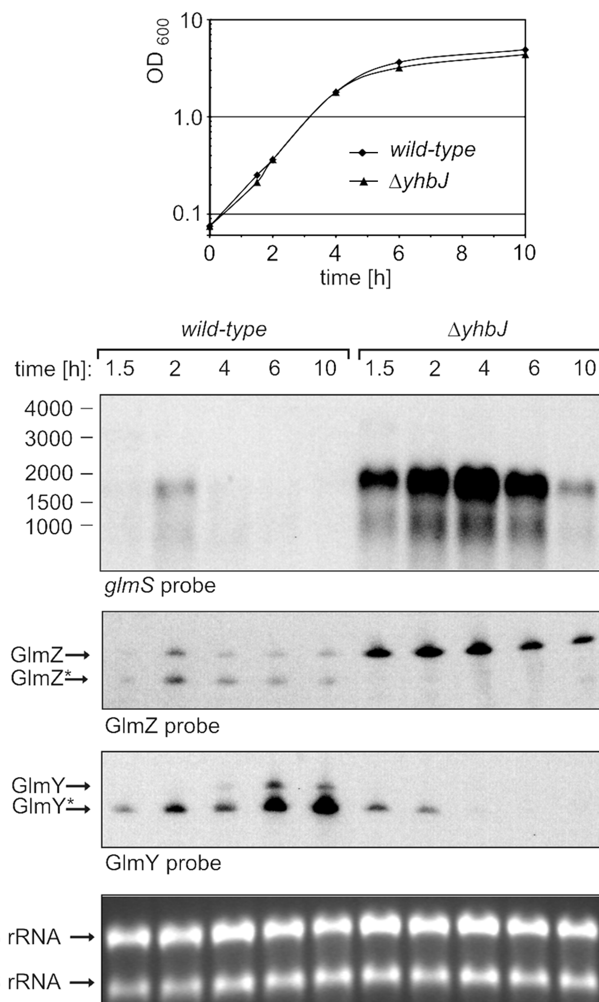

Figure 1. YhbJ has opposite effects on the amounts of GlmZ and GlmY. Northern blot analysis of RNA samples collected at various time points during growth of strains R1279 (wild-type) and Z37 (Δ*yhbJ*). The corresponding growth curves are shown at the top. The RNAs were hybridized with probes specific 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).

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* (7,8). 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 (Figure 1, first and second panels, 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 (4). 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 (10), whereas in the *yhbJ* mutant GlmY* was exclusively detectable in the early exponential growth phase (Figure 1, 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. 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 (Figure 2A). 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 (Figure 2A, lanes 1–3). As expected from previous data (4), the accumulation of the *glmS* transcript was abolished in the Δ*glmZ* Δ*yhbJ* double mutant (Figure 2A, 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 over-produced the *glmS* transcript whereas it was undetectable in the Δ*yhbJ* Δ*glmZ* Δ*glmY* triple mutant (Figure 2A, 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 (Figure 2A, 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 (Figure 2A, 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 (4). To see whether this is also the case in a Δ*glmY* mutant, we introduced a plasmid

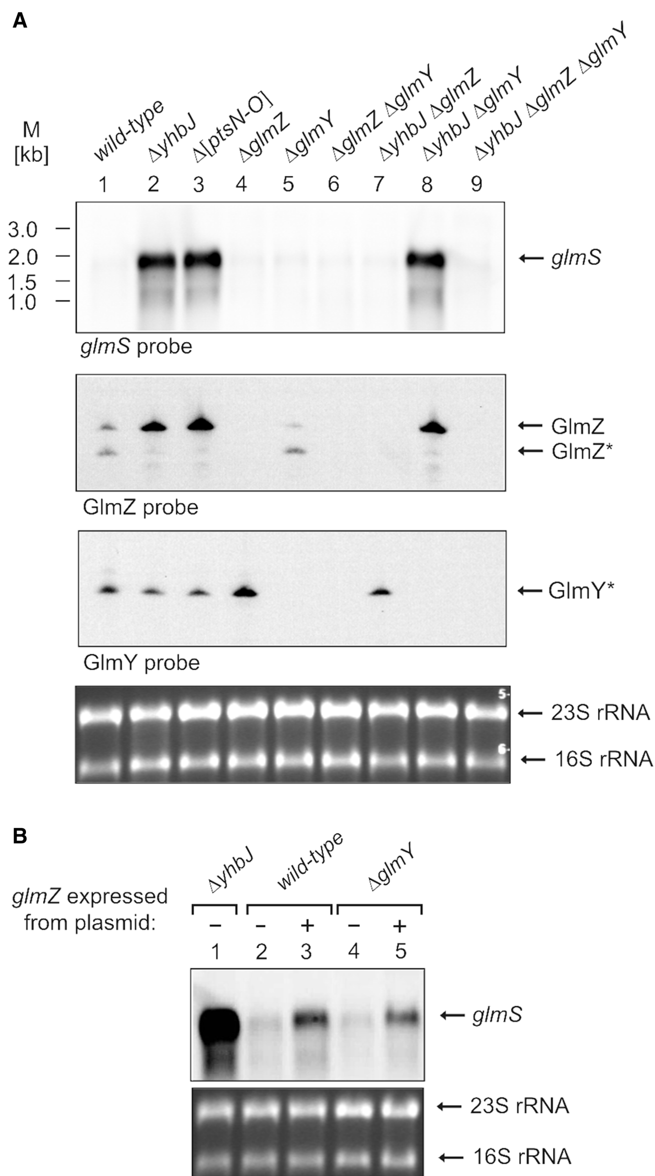

Figure 2. Activation of *glmS* expression by GlmZ is independent of GlmY. (**A**) Northern blot analyses to determine the effects of Δ*yhbJ*, Δ*glmZ* and Δ*glmY* mutations, alone or in various combinations, on the *glmS*, GlmY and GlmZ transcript levels. Total RNAs of strains R1279 (lane 1), Z37 (lane 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).

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

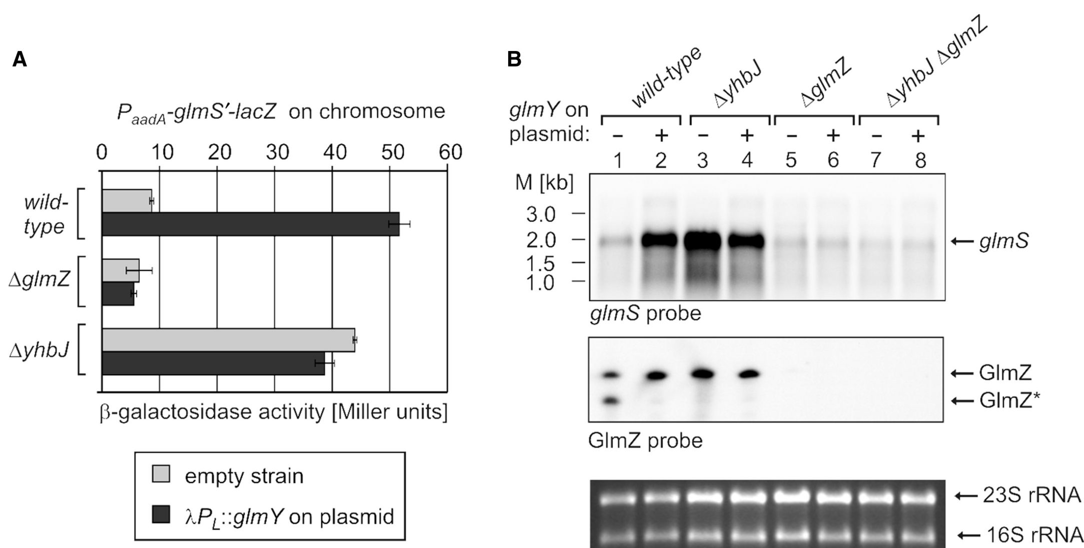

Figure 3. GlmY requires GlmZ for the activation of *glmS* expression. (**A**) Overexpression of *glmY* induces expression of the *glmS′-lacZ* reporter fusion in the wild-type but not in the Δ*glmZ* mutant. Strains Z8 (wild-type), Z38 (Δ*glmZ*) and Z28 (Δ*yhbJ*) were grown in the absence (grey bars) or 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).

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 (Figure 2B, lanes 2 and 3). The virtually same result was obtained when the Δ*glmY* mutant was tested (Figure 2B, 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 (4). This fusion is perfectly regulated by GlmZ and YhbJ. The presence of the *glmY* expression plasmid led to induction of *glmS′–lacZ* expression (Figure 3A), 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 (Figure 3B, 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 (9). 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 (Figure 3A) nor on the *glmS* transcript level as detected by northern analysis (Figure 3B, 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 (Figure 3A and B, lane 3). Additional overexpression of the *glmY* construct, however, had no additive effect on the GlmZ and *glmS* RNA levels (Figure 3B, lanes 3 and 4) and on the expression of the *glmS′–lacZ* fusion (Figure 3A).

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 (4). 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

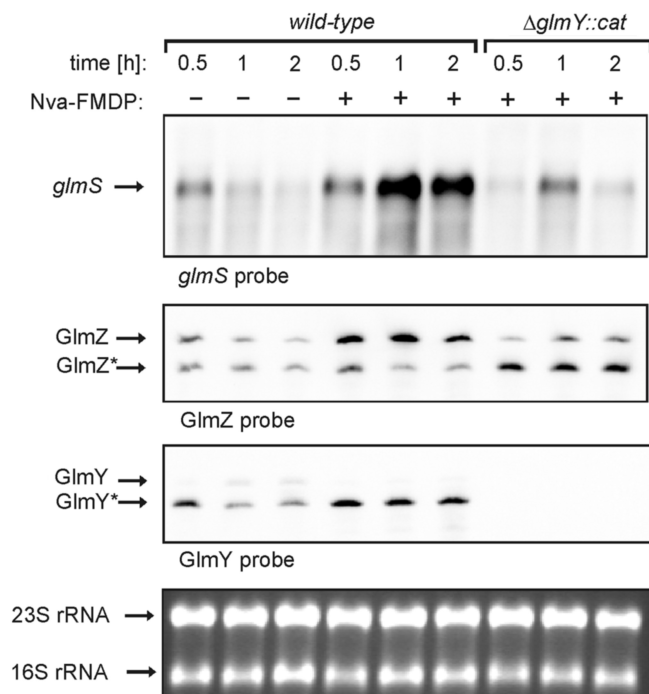

Figure 4. GlmY is essential for transduction of the GlcN-6-P signal to *glmS* and is itself regulated by GlcN-6-P. Northern blotting experiments to determine the effect of the inhibitor of GlmS enzymatic activity, Nva-FMDP, on the *glmS* transcript (*glmS* probe; top), on GlmZ (GlmZ probe; second panel) 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.

Nva-FMDP, a derivative of N3-(4-methoxyfumaroyl)-L-2,3-diaminopropanoic acid. This compound selectively inhibits GlmS enzymatic activity *in vivo* (27) 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 (4). 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 2 h) and total RNAs were isolated and subjected to northern analyses using probes directed against *glmS*, GlmZ and GlmY. As expected from previous data (4), Nva-FMDP caused the accumulation of *glmS* transcript and simultaneously of full-length GlmZ (Figure 4, first and second panels, compare lanes 1–3 with lanes 4–6). Intriguingly, presence of Nva-FMDP also caused the accumulation of GlmY* (Figure 4, 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 (Figure 4, 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 (Figure 4, 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 (6). In addition, full-length GlmZ accumulates in a *pcnB* mutant (4) 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. These experiments revealed that in the Δ*pcnB* mutant *glmS* mRNA and concomitantly full-length GlmZ strongly accumulate in the exponential growth phase (Figure 5, 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 (Figure 5, 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. 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 ± 0.1 min of full-length GlmZ in the wild-type strain (Figure 6A), which corresponds well with the previously reported half-life of ~2 min for GlmZ observed in wild-type cells during the exponential phase (10). In contrast, in the Δ*pcnB* mutant the half-life of GlmZ increased to of 20.2 ± 0.1 min (Figure 6A). 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 (Figure 6B). In this case, the half-life increased from 1.4 ± 0.1 min in the wild-type to 6.7 ± 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 (28). No such smear could be observed for GlmZ (Figure 6A). In sum, the data demonstrate that both GlmZ and GlmY* are stabilized by a *pcnB* mutation.

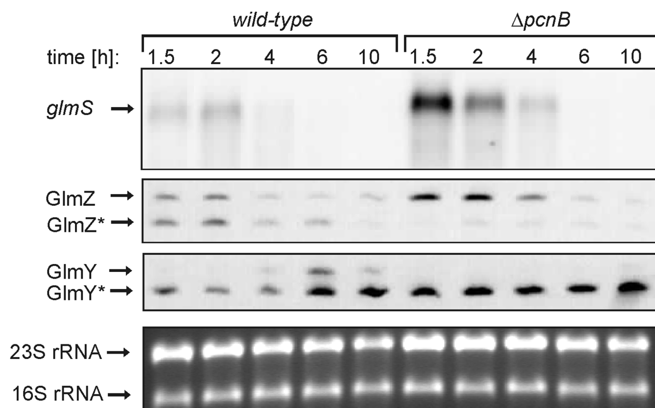

Figure 5. Mutation of *pcnB* results in accumulation of *glmS*, full-length GlmZ and GlmY* RNAs. Northern blot analysis of RNA samples collected at various times during growth of strains R1279 (wild-type) and Z129 (Δ*pcnB*). Specific RNAs were detected using probes directed against *glmS* (upper panel), GlmZ (second panel) and GlmY (third panel).

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 *glmS*. 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. These experiments showed that a *glmY* mutation prevents the accumulation of the *glmS* and full-length GlmZ RNAs in *pcnB* mutants (Figure 7A). 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 (Figure 5). 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 (29) 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 (Figure 7B). Nine clones harbored at the 3′ end short extensions of two or three A residues, which are added post-transcriptionally (Figure 7B). 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

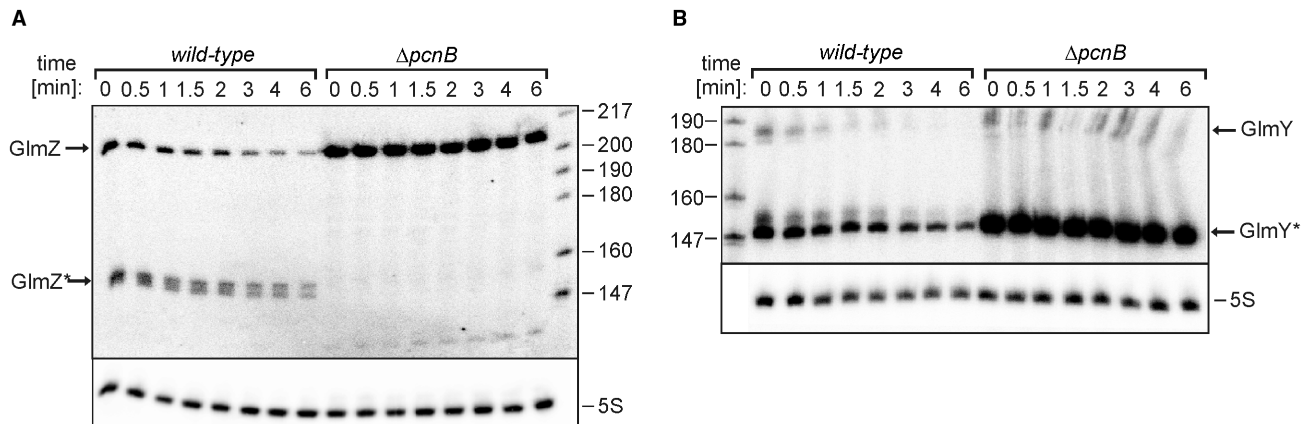

Figure 6. Mutation of *pcnB* strongly increases the half-lifes of full-length GlmZ and GlmY*. Strains N3433 (wild-type) and IBPC903 (Δ*pcnB*) were treated with rifampicin for the inhibition of transcription initiation and subsequently samples were harvested at the indicated times and the total RNAs were 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).

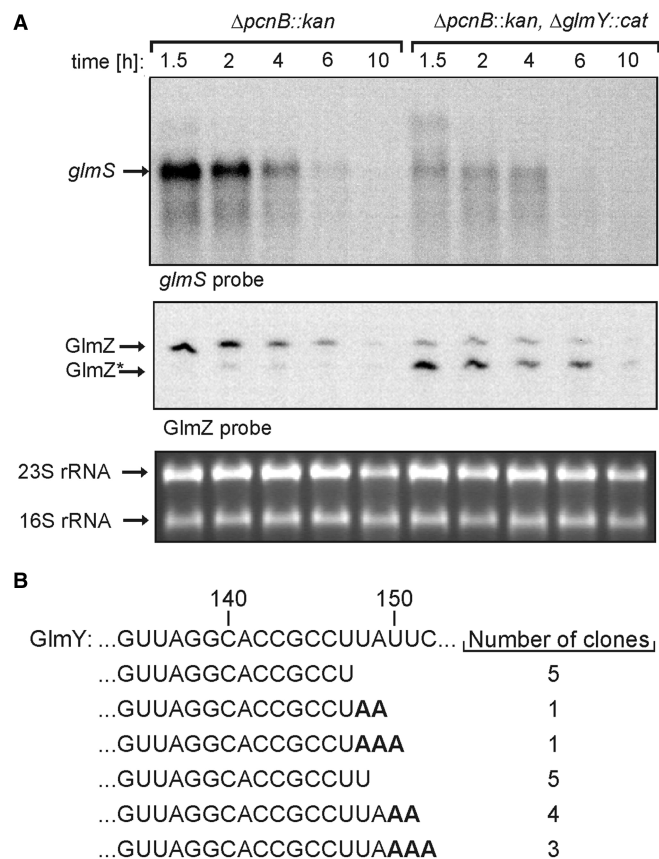

Figure 7. PAP I polyadenylates GlmY* and thereby indirectly destabilizes the *glmS* and GlmZ RNAs. (**A**) Northern blot analysis of total RNA samples collected at various times during growth of strains Z129 (Δ*pcnB*) and Z152 (Δ*pcnB*, Δ*glmY*). The *glmS* (top panel) and GlmZ (second panel) RNAs were 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.

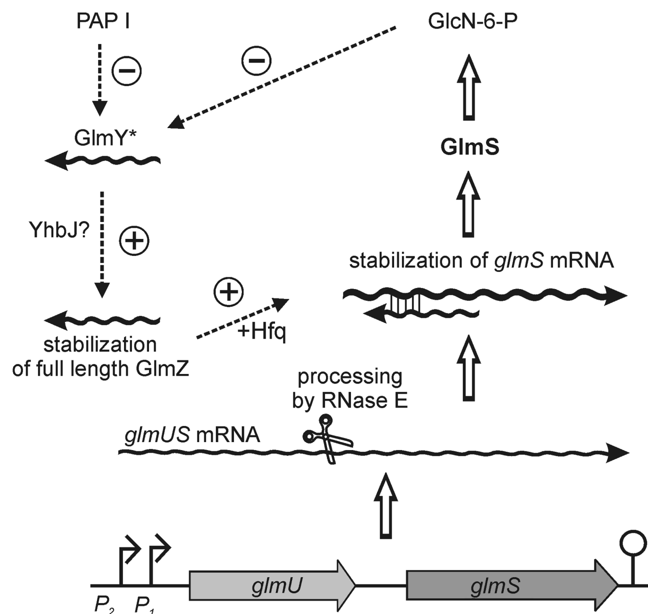

Figure 8. Model for the regulation of *glmS* expression by GlcN-6-P, PAP I, GlmY and GlmZ. PAP I polyadenylates and thereby destabilizes the sRNA GlmY. When the intracellular GlcN-6-P concentration drops, the short form of GlmY accumulates, which leads to stabilization of the full-length form of the 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 (4).

glmS monocistronic transcript and results in overproduction of GlmS protein [(4,9); this work]. 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. Third, 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 Figure 8). 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 (Figure 8). GlmY is conserved in the genomes of several *Enterobacteriaceae* (9). 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 (Figures 2 and 3). This suggests that GlmY acts upstream or in concert with YhbJ in the same pathway to regulate GlmZ (Figure 8). 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 (Figures 1 and 2). 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 (Figure S1 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 (Figure S1). 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 (6). 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 (Figure 8).

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 [(10); this work]. It is this shorter variant that accumulates in *pcnB* mutants (Figure 5) and that we detected as polyadenylated species in the 3′RACE experiments (Figure 7B). 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 (9).

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 (17,18). According to software analysis processed GlmY* carries an extensive secondary structure at the 3′ end, followed by only four or five unpaired nucleotides (9) (Figure 7B and Figure S1 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 (30). 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 (13,31). 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 (32–34). Recently, the turnover of SraL, a small RNA of unknown function, has been reported to be regulated by PAP I (28). 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 (35). Therefore, to function appropriately, it is necessary that trans-encoded regulatory RNAs are consumed upon action (36) 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.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We thank Karin Schnetz for plasmid pFDX1088 and Slawomir Milewski for the gift of Nva-FMDP. We are grateful to Kerstin Voigt for construction of plasmids and to Jörg Stülke for continuous support and lab space. This research was supported by grant GO 1355/2-1 of the Deutsche Forschungsgemeinschaft to B. Görke. B. Reichenbach is supported by a stipend of the ‘Studienstiftung des Deutschen Volkes’. E. Hajnsdorf is supported by the Centre National de la Recherche Scientifique (UPR9073), and the University of Paris VII.

Funding to pay the Open Access publication charge for this article was provided by the Deutsche Forschungsgemeinschaft (grant GO 1355/2-1).

Conflict of interest statement. None declared.

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