Common and divergent features in transcriptional control of the homologous small RNAs GlmY and GlmZ in *Enterobacteriaceae*

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ABSTRACT

Small RNAs GlmY and GlmZ compose a cascade that feedback-regulates synthesis of enzyme GlmS in *Enterobacteriaceae*. Here, we analyzed the transcriptional regulation of *glmY/glmZ* from *Yersinia pseudotuberculosis*, *Salmonella typhimurium* and *Escherichia coli*, as representatives for other enterobacterial species, which exhibit similar promoter architectures. The GlmY and GlmZ sRNAs of *Y. pseudotuberculosis* are transcribed from σ^{54}-promoters that require activation by the response regulator GlrR through binding to three conserved sites located upstream of the promoters. This also applies to *glmY/glmZ* of *S. typhimurium* and *glmY* of *E. coli*, but as a difference additional σ^{70}-promoters overlap the σ^{54}-promoters and initiate transcription at the same site. In contrast, *E. coli glmZ* is transcribed from a single σ^{70}-promoter. Thus, transcription of *glmY* and *glmZ* is controlled by σ^{54} and the two-component system GlrR/GlrK (QseF/QseE) in *Y. pseudotuberculosis* and presumably in many other Enterobacteria. However, in a subset of species such as *E. coli* this relationship is partially lost in favor of σ^{70}-dependent transcription. In addition, we show that activity of the σ^{54}-promoter of *E. coli glmY* requires binding of the integration host factor to sites upstream of the promoter. Finally, evidence is provided that phosphorylation of GlrR increases its activity and thereby sRNA expression.

INTRODUCTION

Post-transcriptional gene regulation involving regulatory RNAs has emerged as a widespread principle occurring in all three domains of life. In bacteria, one important mode of riboregulation involves *trans*-encoded small RNAs (sRNAs), which appear to be involved in regulation of almost every important physiological function (1–5). The majority of sRNAs acts by base-pairing with target mRNAs usually in the vicinity of the ribosome binding site (4,6). Most often, this interaction represses translation and/or stimulates mRNA degradation, although a few cases are known where sRNA–mRNA interaction increases gene expression (7). One example is provided by the sRNA GlmZ in *Escherichia coli*. Binding of GlmZ to its target mRNA *glmS* destroys an inhibitory stem loop that sequesters the Shine–Dalgarno sequence of *glmS*. GlmZ is also an unusual case, because it works in concert with a second homologous sRNA, GlmY (1,8,9). However, while other homologous sRNAs regulate their targets redundantly or additively (6), GlmY/GlmZ act hierarchically to activate expression of the *glmS* gene, which encodes glucosamine-6-phosphate (GlcN6P) synthase GlmS. GlmS catalyzes formation of GlcN6P, which initiates the pathway that generates precursors of cell wall synthesis. Of both sRNAs, only GlmZ is able to base-pair with *glmS* mRNA. However, ongoing processing removes most of the base-pairing residues and thereby inactivates GlmZ. Upon depletion of GlcN6P, the second sRNA GlmY accumulates and counteracts processing of GlmZ. This activates synthesis of GlmS, which re-synthesizes GlcN6P. Hence, both sRNAs work in a cascade to mediate feedback control of GlmS (8–11).

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To understand the impact of sRNAs on bacterial physiology, it is important to identify the signals and mechanisms that control expression of a particular sRNA. sRNA transcription is often controlled by transcriptional regulatory proteins similar to that of protein-coding genes [for an overview, see (4)]. Some sRNA genes are controlled by two-component systems (TCS) and/or alternative sigma factors, which are the key devices for perception of environmental signals and their conversion into gene expression changes (3,12,13). Evidence is accumulating that sRNAs are also members of the modulon controlled by σ^{54} involving genes important for nitrogen and carbon-utilization, uptake of metal ions, stress responses and other apparently unrelated functions. Transcription of sRNA genes from σ^{54}-dependent promoters has been demonstrated in *Pseudomonas aeruginosa* and *Vibrio harveyi* (14,15). It is estimated that there are ∼70 σ^{54}-dependent promoters in *E. coli* (16,17). σ^{54} is unique among σ factors since it is not related to other σ factors and recognizes a different sequence composed of −24/−12 motifs (18). The σ^{54}-RNAP holo-enzyme is unable to catalyze formation of the open promoter complex. This reaction requires interaction with an activator protein that usually binds to activating binding sites (ABS) located far upstream of the promoter.

Despite the parallels in the transcriptional control of protein-coding and sRNA genes, there appears to be at least one difference: many protein-coding genes are transcribed from multiple promoters that can be activated by different σ factors and use different transcriptional start sites (19,20). While differing 5′ sequences of mRNAs are without consequences for the nature of the encoded protein, they have functional consequences for sRNAs as shown for the IstR-1 and IstR-2 sRNAs, which are transcribed from consecutive promoters (21). To allow transcription of identical sRNA species from alternative promoters, these promoters must overlap to allow transcription initiation at the same nucleotide. Such an unorthodox arrangement has recently been identified for the *E. coli glmY* gene, where overlapping σ^{70}- and σ^{54}-promoters start transcription at the same site (22). The σ^{54}-promoter requires activation by the TCS GlrR/GlrK (alternative names: QseF/QseE or YfhA/YfhK), which is encoded downstream of *glmY* and transcribed independently (22). The activator protein GlrR consists of an N-terminal response regulatory domain, a central σ^{54}-interaction module and a C-terminal helix-turn-helix DNA-binding motif. GlrR binds three TGTCN$_{10}$GACA motifs located more than 100 bp upstream of *glmY* and thereby activates the σ^{54}-promoter, while activity of the σ^{70}-promoter is unaffected. Both promoters are moderately active during the exponential growth phase. Their activities interfere since binding of σ^{54} represses activity of the overlapping σ^{70}-promoter to some extent (22).

In this work, we analyzed the transcriptional regulation of *glmY* and *glmZ*. The TCS GlrR/GlrK as well as *glmY* and *glmZ* are conserved in *Enterobacteriaceae*. *In silico* analyses of the *glmY* and *glmZ* promoter sequences identified three groups within the enterobacterial species. *Yersinia pseudotuberculosis*, *Salmonella enterica* subsp.

enterica serovar Typhimurium str. LT2 and *E. coli* K12 are representatives of each group and were analyzed. We show that both, *glmY* and *glmZ*, are controlled by GlrR and σ^{54} in *Y. pseudotuberculosis*, *S. typhimurium* and presumably in many other species. In these species, both sRNAs are expressed from σ^{54}-dependent promoters that require activation by GlrR. However, overlapping σ^{70}-promoters additionally contribute to expression in *S. typhimurium*. In *E. coli*, *glmY* is transcribed from overlapping σ^{54}- and σ^{70}-promoters, while *glmZ* is expressed from a single σ^{70}-promoter that is constitutively active. In conclusion, *glmY* and *glmZ* appear to be strictly σ^{54}-dependent genes in one subgroup of *Enterobacteriaceae*, while σ^{54}-dependency is lost in favor of unregulated σ^{70}-promoters in a second subgroup. Furthermore, we show for *E. coli glmY* that activity of the σ^{54}-promoter requires the integration host factor IHF, which presumably binds to two conserved sites flanking the proximal GlrR binding site. Finally, our data indicate that phosphorylation of GlrR increases its affinity for its target sites on the DNA.

MATERIALS AND METHODS

Growth conditions and strains

LB was used as standard medium for cultivation of bacteria. *Escherichia coli* and *S. typhimurium* LT2 were grown routinely under agitation (200 r.p.m.) at 37°C and *Y. pseudotuberculosis* YPIII was cultivated at 25°C. When necessary, antibiotics were added to the medium (ampicillin 100 μg/ml, kanamycin 30 μg/ml, chloramphenicol 15 μg/ml, spectinomycin 50 μg/ml). For induction of the P_{Ara} promoter on pBAD plasmids, 0.2% L-arabinose was added. The *E. coli* strains are listed in Table 1, including a description of their relevant genotypes. The *ΔihfA::kan* and *ΔihfB::kan* alleles were transduced to strains Z190 and Z197 using bacteriophage T4GT7 (23). Most of the *lacZ* reporter fusions used in this study were first established on plasmids and subsequently integrated into the *λattB*-site on the *E. coli* chromosome by site-specific recombination yielding the strains as indicated in Table 1. Recombination was achieved using helper plasmid pLDR8 as described (24). Briefly, origin-less DNA-fragments encompassing the respective *lacZ* fusion, the *aadA* spectinomycin resistance gene and the *λattP*-site were isolated by BamHI digestion and agarose gel-electrophoresis. The DNA-fragments were self-ligated and subsequently introduced into target strains carrying the temperature-sensitive λ-integrase expression plasmid pLDR8. Recombinants were obtained by selection on spectinomycin-plates at 42°C. Correct integration was verified by PCR using appropriate primers and loss of plasmid pLDR8 was confirmed by sensitivity to kanamycin.

Construction and site-directed mutagenesis of plasmids

DNA cloning was carried out in *E. coli* strain DH5α following standard procedures. The plasmids and oligonucleotides used in this study are listed in Supplementary Tables S1 and S2, respectively (see 'Supplementary data').

Table 1. *E. coli* strains used in this study

Name	Genotype	Reference or construction
DH5α	φ80d *lacZ*ΔM15, *recA1, endA1, gyrA96, thi-1, hsdR17* (r_K^-, m_K^+), *supE44, relA1, deoR,* Δ(*lacZYA-argF*) U169	Laboratory collection
JW0895	F-, Δ(*araD-araB*)567, Δ*lacZ4787*(::*rrnB-3*), Δ*ihfB735*::*kan, LAM-, rph-1,* Δ(*rhaD-rhaB*)568, *hsdR514*	(39)
JW1702	Δ(*araD-araB*)567, Δ*lacZ4787*(::*rrnB-3*), λ-, Δ*ihfA786*::*kan, rph-1,* Δ(*rhaD-rhaB*)568, *hsdR514*	(39)
R1279	CSH50 Δ(*pho-bgl*)201 Δ(*lac-pro*) *ara thi*	(40)
Z179	As R1279, but Δ*glr-R*	(22)
Z184	As R1279, but Δ*rpoN*	(22)
Z190	As R1279, but *attB*::[*E.c. glmY*(−238 to +22)-*lacZ,* −10 mutated]	(22)
Z196	As R1279, but Δ*glr-R, attB*::[*E.c. glmY*(−238 to +22)-*lacZ,* −10 mutated]	(22)
Z197	As R1279, but *attB*::[*E.c. glmY*(−238 to +22)-*lacZ*]	(22)
Z206	As R1279, but Δ*glr-R, attB*::[*E.c. glmY*(−238 to +22)-*lacZ*]	(22)
Z227	As R1279, but Δ*rpoN, attB*::[*E.c. glmY*(−238 to +22)-*lacZ*]	(22)
Z360	As R1279, but *attB*::[*E.c. glmZ*(−424 to +32)-*lacZ*]	pBGG59/BamHI→R1279, this work
Z361	As R1279, but Δ*glr-R, attB*::[*E.c. glmZ*(−424 to +32)-*lacZ*]	pBGG59/BamHI→Z179, this work
Z362	As R1279, but *attB*::[*Y.p. glmY*(−257 to +22)-*lacZ*]	pYG1/BamHI→R1279, this work
Z363	As R1279, but Δ*glr-R, attB*::[*Y.p. glmY*(−257 to +22)-*lacZ*]	pYG1/BamHI→Z179, this work
Z364	As R1279, but *attB*::[*Y.p. glmZ*(−303 to +22)-*lacZ*]	pYG2/BamHI→R1279, this work
Z365	As R1279, but Δ*glr-R, attB*::[*Y.p. glmZ*(−303 to +22)-*lacZ*]	pYG2/BamHI→Z179, this work
Z370	As R1279, but *attB*::[*E.c. glmY*(−238 to +22)-*lacZ;* IHF1 mutated]	pBGG390/BamHI→R1279, this work
Z371	As R1279, but *attB*::[*E.c. glmY*(−238 to +22)-*lacZ;* IHF1 mutated; −10 mutated]	pBGG391/BamHI→R1279, this work
Z372	As R1279, but Δ*glr-R, attB*::[*E.c. glmY*(−238 to +22)-*lacZ;* IHF1 mutated]	pBGG390/BamHI→Z179, this work
Z373	As R1279, but Δ*glr-R, attB*::[*E.c. glmY*(−238 to +22)-*lacZ;* IHF1 mutated; −10 mutated]	pBGG391/BamHI→Z179, this work
Z388	As R1279, but *attB*::[*S.t. glmY*(−242 to +22)-*lacZ*]	pYG7/BamHI→R1279, this work
Z389	As R1279, but Δ*glr-R, attB*::[*S.t. glmY*(−242 to +22)-*lacZ*]	pYG7/BamHI→Z179, this work
Z390	As R1279, but *attB*::[*S.t. glmZ*(−242 to +22)-*lacZ*]	pYG8/BamHI→R1279, this work
Z391	As R1279, but Δ*glr-R, attB*::[*S.t. glmZ*(−242 to +22)-*lacZ*]	pYG8/BamHI→Z179, this work
Z392	As R1279, but *attB*::[*E.c. glmY*(−238 to +22)-*lacZ,* −10 mutated], *ihfB*::*kan*	T4GT7 (JW0895)→Z190, this work
Z393	As R1279, but *attB*::[*E.c. glmY*(−238 to +22)-*lacZ*], *ihfB*::*kan*	T4GT7 (JW0895)→Z197, this work
Z394	As R1279, but *attB*::[*E.c. glmY*(−238 to +22)-*lacZ,* −10 mutated], *ihfA*::*kan*	T4GT7 (JW1702)→Z190, this work
Z395	As R1279, but *attB*::[*E.c. glmY*(−238 to +22)-*lacZ*], *ihfA*::*kan*	T4GT7 (JW1702)→Z197, this work
Z397	As R1279, but Δ*glr-R, attB*::[*Y.p. glmZ*(−292 to +22)-*lacZ;* ABS1 mutated]	pYG9/BamHI→Z179, this work
Z398	As R1279, but Δ*glr-R, attB*::[*Y.p. glmZ*(−303 to +22)-*lacZ;* ABS2 mutated]	pYG10/BamHI→Z179, this work
Z399	As R1279, but Δ*glr-R, attB*::[*Y.p. glmZ*(−303 to +22)-*lacZ;* ABS3 mutated]	pYG11/BamHI→Z179, this work
Z400	As R1279, but Δ*glr-R, attB*::[*Y.p. glmZ*(−292 to +22)-*lacZ;* ABS1,2,3 mutated]	pYG12/BamHI→Z179, this work
Z443	As R1279, but Δ*rpoN, attB*::[*E.c. glmZ*(−424 to +32)-*lacZ*]	pBGG59/BamHI→Z184, this work
Z444	As R1279, but Δ*rpoN, attB*::[*Y.p. glmY*(−257 to +22)-*lacZ*]	pYG1/BamHI→Z184, this work
Z445	As R1279, but Δ*rpoN, attB*::[*Y.p. glmZ*(−303 to +22)-*lacZ*]	pYG2/BamHI→Z184, this work
Z446	As R1279, but Δ*rpoN, attB*::[*S.t. glmY*(−242 to +22)-*lacZ*]	pYG7/BamHI→Z184, this work
Z447	As R1279, but Δ*rpoN, attB*::[*S.t. glmZ*(−242 to +22)-*lacZ*]	pYG8/BamHI→Z184, this work

E.c., Escherichia coli-K12; *Y.p., Yersinia pseudotuberculosis* YPIII, *S.t., Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2

Plasmid constructions are also described under 'Supplementary data'.

Analysis of *glmY* and *glmZ* transcription (β-galactosidase assays)

Overnight cultures of *E. coli* were inoculated into fresh LB medium to an OD_{600} of 0.1 and grown to an OD_{600} of 0.5–0.7. Subsequently, the cells were harvested and the β-galactosidase activities were determined as previously described (25). β-Galactosidase activities were determined from *Y. pseudotuberculosis* cells as described recently (26). The presented values are the average of at least three measurements using independent cultures.

Protein purification

C-terminally His-tagged *E. coli* and *Y. pseudotuberculosis* GlrR proteins were overproduced in *E. coli* DH5α carrying plasmid pBGG219 or pBGG397, respectively. Cells were grown in 11 LB-ampicillin to an $OD_{600} = 0.5$–0.8. After addition of 1 mM IPTG for the induction of $GlrR::His_{10}$ synthesis, growth was continued for one additional hour. 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 at 2600 Ψ (Constant systems Ltd.) and subsequently cleared by low speed centrifugation followed by ultracentrifugation. The cleared lysates were loaded onto pre-equilibrated Ni-NTA Superflow columns (Qiagen) and proteins were eluted with a gradient of imidazol solved in ZAP buffer. Samples of the different purification steps and elution fractions were analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie blue staining. The 250 mM imidazol fractions contained the pure $GlrR-His_{10}$ proteins. These fractions were dialysed two times for 24 h against buffer (20 mM Tris–HCl pH 7.5, 100 mM KCl, 2 mM DTT). In the second dialysis step, the buffer additionally contained 25% (v/v) glycerol. The purified proteins were aliquoted and stored at $-20°C$ until their use.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assays (EMSAs) were carried out as described previously (22,27). The DNA fragments tested in the EMSAs were amplified by PCR using the same oligonucleotides that were used for construction of the corresponding *glmY'*- and *glmZ'-lacZ* gene fusions (Supplementary Table S1). The 200 and 400 bp *lacZ* promoter fragments, which were used as internal controls, were generated by PCR using primer pairs BG580/BG581 and BG578/BG579, respectively. DNA concentrations were determined with the NanoDrop Spectrometer ND-1000 (Peqlab). Binding assays were carried out in 10 µl volume containing binding buffer (20 mM Tris–HCl, pH 7.5, 100 mM KCl, 2 mM DTT, 10% glycerol), 30 ng of each DNA-fragment and the protein concentrations as indicated in the Figures. The reactions were incubated at 30°C for 20 min and subsequently 6 µl of the samples were separated at 4°C alongside with a DNA size marker on non-denaturing 8% acrylamide gels prepared in 0.5 × TBE. Gels were stained with ethidium bromide for visualization of the DNA. For testing the effect of acetyl phosphate, GlrR protein was incubated for 1 h at 37°C in binding buffer containing 50 mM acetyl phosphate and then used for the binding assays.

RESULTS

Conservation and gene synteny of *glmY and glmZ* in *Enterobacteriaceae*

In *E. coli*, transcription of *glmY* is controlled by overlapping σ^{70}- and σ^{54}-dependent promoters. Activity of the σ^{54}-promoter is governed by the TCS GlrR/GlrK, which is encoded downstream of *glmY* (22). To investigate, whether this unusual promoter architecture is conserved in other bacteria and to increase our understanding of regulation of *glmZ* transcription, we compared the promoter sequences of *glmY* and *glmZ* from a comprehensive number of genomes. To retrieve these sequences, we used the sRNA sequences of *Escherichia coli* K12 (strain MG1655) as queries in NCBI Blast analyses. This search generated a list of species, all belonging to the *Enterobacteriaceae* family, which coincidently contained both sRNA genes. Inspection of gene synteny using the MicrobesOnline tool (28) and the KEGG database (29) revealed conserved localization of *glmZ* downstream of the divergently orientated *hemCDXY* operon encoding enzymes involved in tetrapyrrole synthesis, whereas the region upstream of *glmZ* is variable and may carry insertion elements (Figure 1A and Supplementary Figure S1). Gene *glmY* is always located upstream of the gene cluster *glrK-yfhG-glrR* (Figure 1A and Supplementary Figure S2). Collectively, these observations suggest that sRNA genes *glmY* and *glmZ* are elements of the core genome conserved in *Enterobacteriaceae*. The conserved co-localization of *glmY* with the genes encoding the sensor kinase GlrK and the response regulator GlrR suggests that regulation of *glmY* expression by this TCS might be likewise conserved.

Sequences for a σ^{54}-promoter and for binding sites of the response regulator GlrR as well as of IHF are shared features of the *glmY* and *glmZ* promoter regions of many, but not all *Enterobacteriaceae*

We performed sequence alignments of the promoter regions of the *glmY* as well as *glmZ* genes retrieved from 39 genome sequences representing the most important genera of *Enterobacteriaceae*. The σ^{54}-dependent promoter of *E. coli glmY* is conserved in all species (Supplementary Figure S3). The GlrR binding sites are likewise conserved although sequence deviations from the consensus $TGTCN_{10}GACA$ occur in a few cases, in particular in ABS 1 and 3. Two additional regions flanking ABS3 exhibit a higher degree of conservation and show similarity to binding sites of IHF, which are represented by the consensus WATCARXXXXTTR (30). The previously characterized -10 sequence (CATA AT) of the σ^{70}-promoter, which overlaps with the -12 sequence of the σ^{54}-promoter of *glmY* in *E. coli*, is conserved only in a subset of genera, i.e. in *Escherichia* (which includes *Shigella* strains), *Klebsiella*, *Salmonella*,
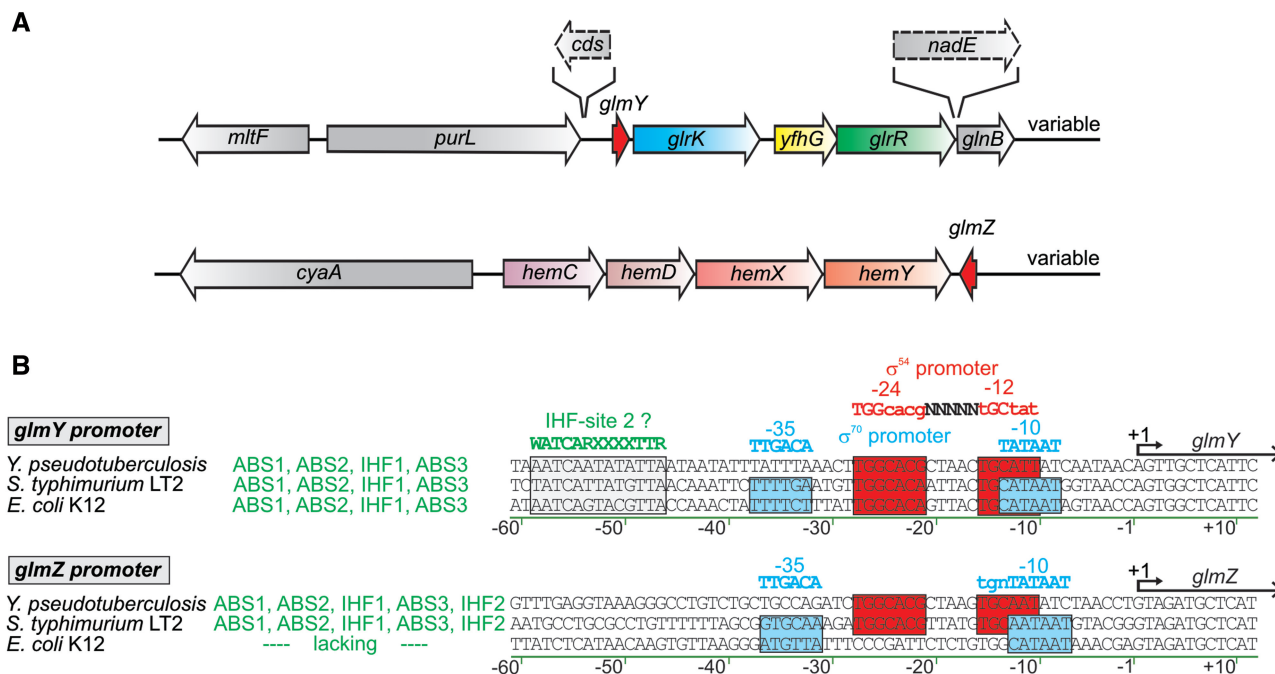
Figure 1. Organization of the *glmY* and *glmZ* genes in *Enterobacteriaceae*. (**A**) Diagram illustrating gene synteny of the *glmY* and *glmZ* regions in *Enterobacteriaceae*. The gene cluster *glmY-glrK-yfhG-glrR-glnB* is conserved in *Enterobacteriacea*, but in some species e.g. *Yersinia* and *Photorhabdus*, gene *nadE* is inserted between *glrR* and *glnB*. Upstream of *glmY*, genes *mltF* and *purL* are present except for *Providencia sp.* Small *orfs* of unknown function are interspersed between *purL* and *glmY* in *Yersinia*, *Photorhabdus* and other species. Gene *glmZ* clusters with the downstream located and divergently orientated *hemCDXY* cluster, while the region upstream is variable. (**B**) Organization of enterobacterial *glmY* and *glmZ* promoters. Sequence alignments of the *glmY* and *glmZ* promoter regions from 39 enterobacterial genomes classified the species into three groups, for which *Y. pseudotuberculosis*, *S. typhimurium* and *E. coli* are representatively shown (for details, see Supplementary Figures S3 and S4). *Yersinia* possesses the sequences for a σ^{54}-promoter (labeled in red) and three GlrR binding sites upstream of both sRNA genes, while overlapping σ^{70}-promoters appear to be absent. GlrR binding sites and σ^{54}-promoters are also detectable upstream of both sRNA genes in *Salmonella*, but in addition putative σ^{70}-promoters (labeled in blue) that overlap the σ^{54}-promoters, are detectable. This arrangement is also found upstream of *E. coli glmY*. However, *E. coli glmZ* appears to be transcribed from a single σ^{70}-promoter. The sequence alignment also detected two putative IHF binding sites that coincide with the occurrence of σ^{54}-promoters.

Enterobacter, *Citrobacter* and *Cronobacter*. Putative −35 sequences are also detectable at the appropriate positions. In contrast, in other genera, such as *Erwinia, Photorhabdus, Serratia* and *Yersinia*, overlapping potential σ^{70}-promoter sequences are not detectable (Supplementary Figure S3).

The analysis of the promoter of the second sRNA gene *glmZ* revealed two groups of sequences, which exhibit no similarity and could not be aligned with each other (Supplementary Figure S4). In the group comprising the majority of sequences, the *glmZ* promoter region is strongly reminiscent of the organization of the *glmY* promoter. Sequence motifs of a σ^{54}-promoter, three GlrR binding sites and two IHF binding sites are detectable. The putative ABS1 and IHF-sites are less conserved in comparison to the *glmY* promoters (compare Supplementary Figures S3 and S4). In a subset of genera, i.e. *Cronobacter, Citrobacter, Enterobacter* and *Salmonella*, putative σ^{70}-promoters overlapping with the σ^{54}-promoters are also detectable upstream of *glmZ* (Supplementary Figure S4). Interestingly, these species also possess overlapping σ^{70}- and σ^{54}-promoter sequences upstream of *glmY* (Supplementary Figure S3). The second group comprised the genera *Klebsiella* and *Escherichia*. In these cases, sequence motifs for σ^{54}-promoters and for

GlrR- and IHF-binding sites are lacking. Instead of that, putative σ^{70}-promoter sequences (ATGTTA-N$_{15}$-tggCATAAT in *Escherichia* sp. and *Shigella* strains and ATGCAA-N$_{15}$-tgcGATAAT in *Klebsiella pneumoniae*) are present at the appropriate positions.

From these analyses we hypothesized that enterobacterial species can be classified into three groups in respect to control of *glmY* and *glmZ* expression (Figure 1B): (i) Species of the genera *Pantoea, Erwinia, Pectobacterium, Arsenophonus, Photorhabdus, Serratia, Proteus, Yersinia* and *Dickeya* may transcribe both, *glmY* and *glmZ*, from σ^{54}-dependent promoters, which might be controlled by GlrR/GlrK. (ii) This may also apply to species of the genera *Cronobacter, Citrobacter, Enterobacter* and *Salmonella*, but as a difference, additional overlapping σ^{70}-promoters are present, which may start transcription at the same site. (iii) Overlapping σ^{54}- and σ^{70}-promoters also control expression of *glmY* in *Klebsiella* and *Escherichia* species. In contrast, transcription of *glmZ* is driven exclusively from σ^{70}-promoters.

Finally, IHF might be important for the activities of the σ^{54}-dependent *glmY* and *glmZ* promoters. To address these hypotheses, we selected one species per group to experimentally analyse the *glmY* and *glmZ* promoters (Figure 1B). These were *Y. pseudotuberculosis* YPIII

(group I), *S. enterica subsp. enterica* serovar *typhimurium* str. LT2 (group II) and *E. coli* K12 (group III).

Response regulator GlrR binds to the *glmY* promoter regions of *S. typhimurium* and *Y. pseudotuberculosis*

First, we wanted to verify if the putative σ^{54}-dependent *glmY* promoters of *S. typhimurium* and *Y. pseudotuberculosis* are controlled by the response regulator GlrR. Therefore, we tested whether purified GlrR protein is able to bind to these promoters. EMSAs were carried out using purified GlrR protein from *E. coli* and DNA fragments covering the *glmY* promoter regions of these species. For comparison, binding of GlrR to the corresponding DNA fragment of *E. coli* was tested. Different concentrations of purified His-tagged GlrR protein were incubated with the various *glmY* promoter fragments,

respectively. In order to verify binding specificity, an additional DNA fragment, which covered the *lacZ* promoter and had a size of either 400 or 200 bp was simultaneously present in these assays. Protein/DNA-complexes and unbound DNA were separated by polyacrylamide gel electrophoresis (Figure 2A). The *glmY* promoter fragments of all three species were shifted to distinct slower migrating bands indicating DNA/GlrR complexes, while the *lacZ* control fragments were not bound. Comparable protein concentrations were required to achieve binding, indicating that GlrR binds with similar affinities to all these *glmY* fragments. GlrR of *E. coli* shares 95% and 87% amino acid sequence identity with its homologs from *S. typhimurium* and *Y. pseudotuberculosis*, respectively. To confirm that the results obtained with the heterologous GlrR protein are valid, we additionally performed
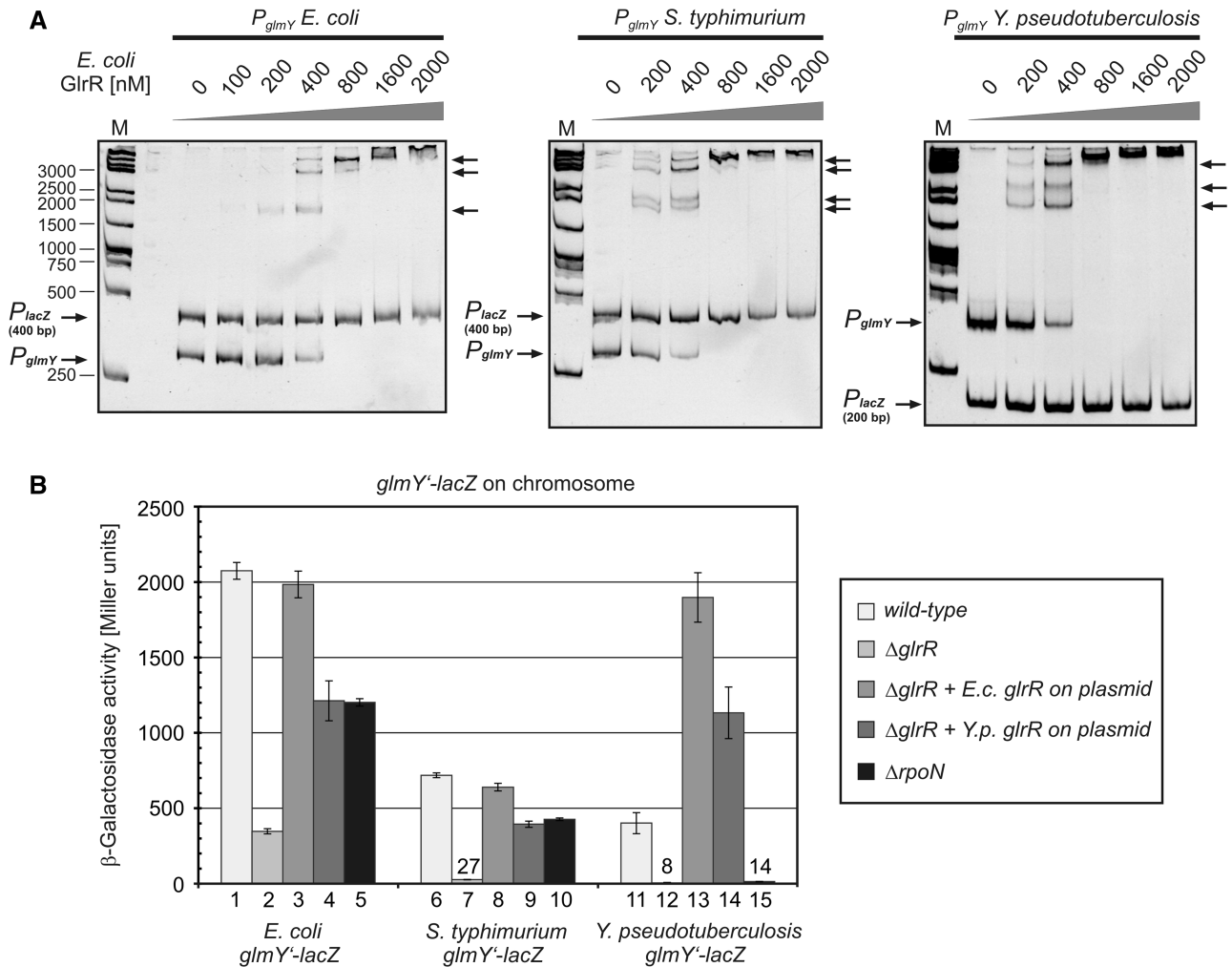

Figure 2. Comparison of the roles of GlrR and σ^{54} for expression of *glmY* from *E. coli*, *S. typhimurium* and *Y. pseudotuberculosis*. (**A**) EMSAs to test binding of *E. coli* GlrR protein to the *glmY* promoter regions of *E. coli* (−238 to +22), *S. typhimurium* (−242 to +22) and *Y. pseudotuberculosis* (−257 to +22). In addition to the *glmY* promoter fragments, 400 bp (panels 1 and 2) or 200 bp DNA fragments (panel 3) covering the *lacZ* promoter were present as internal controls. The sizes of the DNA size standard are given at the left. The apparent K_D values are 360 nM for the *E. coli glmY* promoter, 230 nM for the *Salmonella glmY* promoter and 290 nM for the *Y. pseudotuberculosis glmY* promoter. (**B**) β-Galactosidase activities of *E. coli* strains carrying fusions of *glmY'* from *E. coli*, *S. typhimurium* and *Y. pseudotuberculosis* to the *lacZ* reporter gene. In addition, these strains had the genotypes indicated in the legend. The following strains and transformants were tested (corresponding to the columns from left to right): Z197, Z206, Z206+pBGG223, Z206+pYG6, Z227, Z388, Z389, Z389+pBGG223, Z389+pYG6, Z446, Z362, Z363, Z363+pBGG223, Z363+pYG6 and Z444.

EMSAs using purified *Y. pseudotuberculosis* GlrR. This protein also bound the *glmY* promoter DNA fragments of both, *Y. pseudotuberculosis* and *E. coli*, with comparable affinities (Supplementary Figure S5). However, in comparison to GlrR from *E. coli* higher protein concentrations were required to achieve binding.

Analysis of *S. typhimurium* and *Y. pseudotuberculosis* *glmY* expression

The EMSAs suggested that *glmY* expression is regulated by GlrR in all three species. To validate this conclusion and to determine whether single or overlapping σ^{70}- and σ^{54}-promoters control expression of *glmY*, we constructed fusions of the *glmY* genes of all three species to the *lacZ* reporter gene. The fusions were integrated into the chromosome of *E. coli* wild-type and isogenic *ΔglrR* and *ΔrpoN* mutants (*rpoN* encodes σ^{54}). The resulting strains were grown to exponential phase and the β-galactosidase activities were determined. The *E. coli glmY'-lacZ* fusion was readily expressed in the wild-type, while its expression was 6-fold lower in the *ΔglrR* mutant reflecting the lack of activity of the σ^{54}-promoter (Figure 2B, columns 1 and 2). However, a certain level of expression was retained in the *ΔglrR* mutant, which is due to the activity of the overlapping σ^{70}-promoter (22). Complementation of the *ΔglrR* mutant with a plasmid carrying *E. coli glrR* under P_{Ara} promoter control restored expression of *glmY'-lacZ* to wild-type levels (Figure 2B, columns 1 and 3), while a somewhat lower activity was obtained when a plasmid carrying *glrR* from *Y. pseudotuberculosis* was used (Figure 2B, column 4). This effect was also seen in all subsequent complementation experiments suggesting that GlrR from *Y. pseudotuberculosis* is less active than the *E. coli* GlrR protein. In agreement with previous data (22), the *E. coli glmY'-lacZ* fusion was expressed at higher levels in the *ΔrpoN* mutant in comparison to the *ΔglrR* mutant (Figure 2B, columns 2 and 5). This difference results from repression of the σ^{70}-dependent promoter by binding of σ^{54}-RNAP to the overlapping σ^{54}-promoter in the *ΔglrR* mutant (22).

Similar results were obtained using the *S. typhimurium glmY'-lacZ* fusion (Figure 2B, columns 6–10). However, expression of this fusion was almost completely abolished in the *ΔglrR* mutant (Figure 2B, columns 2 and 7). A considerable level of expression was detectable in the *ΔrpoN* mutant as it was also observed for the *E. coli glmY'-lacZ* fusion (Figure 2B, columns 5 and 10). Hence, the data are compatible with overlapping σ^{70}- and σ^{54}-promoters, as predicted by the sequence alignment (Figure 1B, Supplementary Figure S3). The σ^{70}-promoter of *S. typhimurium glmY* appears to be completely repressed by binding of σ^{54} to the overlapping σ^{54}-promoter. The *Y. pseudotuberculosis glmY'-lacZ* fusion exhibited a different pattern of expression (Figure 2B, columns 11–15). This fusion was neither expressed in the *ΔglrR* nor in the *ΔrpoN* mutant. Complementation of the *ΔglrR* mutant with plasmids encoding *glrR* either from *E. coli* or *Y. pseudotuberculosis* restored expression to higher levels than in the wild-type strain (Figure 2B, columns 11, 13, 14). Collectively, the

data support the conclusions drawn from the sequence alignments: The *glmY* genes of all three species are transcribed from σ^{54}-dependent promoters that require activation by GlrR. An additional σ^{70}-promoter overlapping the σ^{54}-promoter exists in *E. coli* and *S. typhimurium*, but not in *Y. pseudotuberculosis*.

The response regulator GlrR binds the *glmZ* promoter region of *S. typhimurium* and *Y. pseudotuberculosis*, while the *E. coli glmZ* promoter is not bound

The sequence alignment analysis of the *glmZ* promoter regions had revealed putative σ^{54}-promoters and GlrR binding sites in *S. typhimurium* and *Y. pseudotuberculosis*, while these elements are missing upstream of *E. coli glmZ* (Figure 1B, Supplementary Figure S4). To determine whether GlrR is able to bind to these promoter regions, EMSAs were carried out using *E. coli* GlrR protein and DNA fragments encompassing the respective *glmZ* promoter regions. These experiments showed that GlrR binds the *glmZ* promoters of *S. typhimurium* and *Y. pseudotuberculosis* with comparable affinities, whereas the *E. coli glmZ* promoter is not bound (Figure 3A). In addition, EMSAs were carried out using GlrR from *Y. pseudotuberculosis* (Supplementary Figure S6). Binding of the *glmZ* promoter fragment from *Y. pseudotuberculosis* was detectable, but four times higher protein concentrations were required in comparison to GlrR from *E. coli*, as already observed in the EMSAs using the *glmY* promoter fragments (Supplementary Figure S5). In contrast, the *E. coli glmZ* promoter fragment was not bound (Supplementary Figure S6). In conclusion, GlrR binds the *glmZ* promoters of *Y. pseudotuberculosis* and *S. typhimurium*, but not of *E. coli*.

Analysis of *E. coli*, *S. typhimurium* and *Y. pseudotuberculosis glmZ* expression

To obtain further evidence that σ^{54} and GlrR regulate the *glmZ* genes of *S. typhimurium* and *Y. pseudotuberculosis* and are not involved in *E. coli glmZ* regulation, *lacZ* fusions of the *glmZ* genes were constructed and integrated into the chromosome of *E. coli* wild-type, *ΔglrR* and *ΔrpoN* strains. Expression of the *E. coli glmZ'-lacZ* fusion was neither affected by the *ΔglrR* nor by the *ΔrpoN* mutation and expression of *glrR* from a plasmid had also no stimulatory effect (Figure 3B, columns 1–5). Hence, expression of *E. coli glmZ* is not controlled by GlrR or σ^{54}. Expression of the *S. typhimurium glmZ'-lacZ* fusion was also not decreased in the *ΔglrR* mutant. In contrast to the *E. coli glmZ'-lacZ* fusion, expression was significantly increased when *glrR* was expressed from a plasmid (Figure 3B, compare columns 6–9 and 1–4). Interestingly, expression of this fusion was also strongly increased in the *ΔrpoN* mutant (Figure 3B, columns 6 and 10). These results can be explained by the existence of overlapping σ^{70}- and σ^{54}-promoters. The high levels of *glmZ* transcription detected in the *ΔglrR* and *ΔrpoN* mutants (Figure 3B, columns 7 and 10) suggest that this σ^{70}-promoter is stronger than the σ^{70}-promoter preceding the *glmY* gene in *S. typhimurium*.
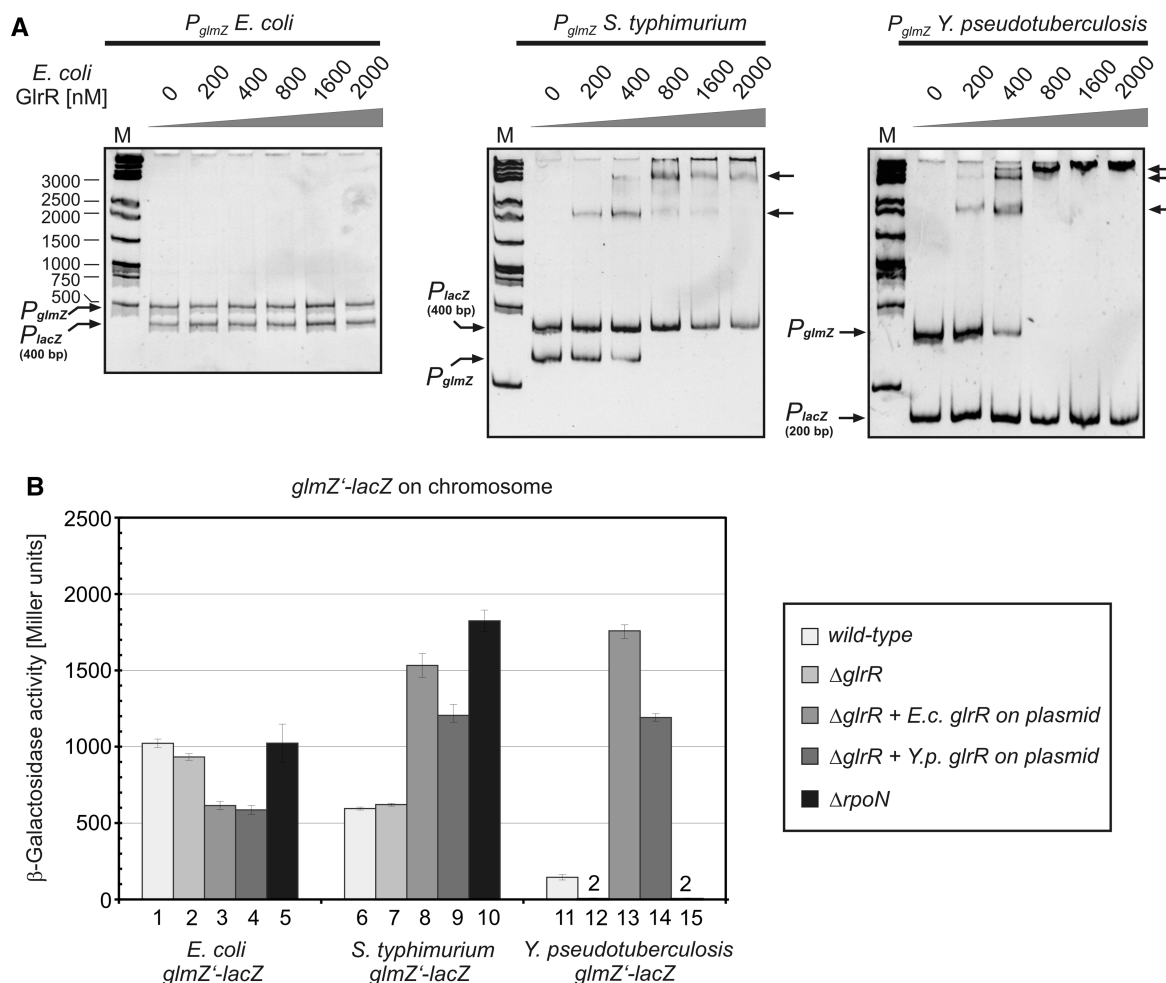
Figure 3. Comparison of the roles of GlrR and σ^{54} for expression of *glmZ* from *E. coli*, *S. typhimurium* and *Y. pseudotuberculosis*. (**A**) EMSAs to test binding of *E. coli* GlrR protein to the *glmZ* promoter regions of *E. coli* (−424 to +32), *S. typhimurium* (−242 to + 22) and *Y. pseudotuberculosis* (−303 to +22). The apparent K_D values for binding of GlrR to the *S. typhimurium* and *Y. pseudotuberculosis glmY* promoter fragments are 370 nM in both cases. (**B**) β-Galactosidase activities of *E. coli* strains carrying fusions of *glmZ'* from *E. coli*, *S. typhimurium* and *Y. pseudotuberculosis* to the *lacZ* reporter gene. In addition, these strains had the genotypes indicated in the legend. The following strains and transformants were tested (corresponding to the columns from left to right): Z360, Z361, Z361+pBGG223, Z361+pYG6, Z443, Z390, Z391, Z391+pBGG223, Z391+pYG6, Z447, Z364, Z365, Z365+pBGG223, Z365+pYG6 and Z445.

The *Y. pseudotuberculosis glmZ'-lacZ* fusion showed an expression pattern that was reminiscent of the results obtained with the cognate *glmY'-lacZ* fusion. Expression of both fusions was abolished in *ΔglrR* as well as *ΔrpoN* mutants (columns 12 and 15 in Figures 2B and 3B, respectively). Complementation of the *ΔglrR* mutant with plasmids carrying *glrR* either from *E. coli* or *Y. pseudotuberculosis* restored expression to levels that were even higher than in the wild-type strain (columns 11, 13 and 14 in Figures 2B and 3B). In conclusion, *Y. pseudotuberculosis glmY* as well as *glmZ* appear to be expressed exclusively from σ^{54}-dependent promoters that require activation by GlrR. Apparently, overlapping σ^{70}-promoters do not exist in these cases.

Expression of *glmY* and *glmZ* in *Y. pseudotuberculosis*

Among *Enterobacteriaceae*, *E. coli* and *Y. pseudotuberculosis* are distantly related (31). Although the transcriptional machinery and all elements involved in regulation of *glmY* and *glmZ* expression are conserved in both species, one might argue that the patterns of *Y. pseudotuberculosis glmY* and *glmZ* expression, as observed here in *E. coli*, do not appropriately reflect expression of these sRNAs in the authentic host. To address this possibility, we transformed *Y. pseudotuberculosis* with plasmids carrying either the *Y. pseudotuberculosis glmY'-lacZ* or the *glmZ'-lacZ* fusion or with the empty fusion vector. The cells carrying the *glmY'-lacZ* or the *glmZ'-lacZ* fusion displayed significantly higher β-galactosidase activities than the transformant carrying the empty *lacZ* fusion plasmid (Supplementary Figure S7A). Thus, both fusions are expressed in *Y. pseudotuberculosis*. The *glmY'-lacZ* fusion was approximately two-fold higher expressed than the *glmZ'-lacZ* fusion. The same difference was observed in *E. coli* (compare columns 11 in Figures 2B and 3B). Next, a second compatible plasmid carrying either *glrR* from *E. coli* or *Y. pseudotuberculosis* or no gene (empty vector)

under control of the P_{Ara} promoter was introduced. Presence of the *glrR* expression plasmids strongly increased expression of the *lacZ* fusions (Supplementary Figure S7B). Expression of *E. coli glrR* resulted in higher expression levels of the *lacZ* fusions in comparison to *Y. pseudotuberculosis glrR*. These differences were also detected in *E. coli* (Figures 2B and 3B). Taken together, it appears justified to conclude that the data obtained with these *lacZ* fusions in *E. coli* reflect their expression in *Y. pseudotuberculosis*.

E. coli glmZ is exclusively transcribed from a σ^{70}-promoter, while Y. pseudotuberculosis glmZ transcription depends on σ^{54} and GlrR

Our data suggested that *glmZ* of *Y. pseudotuberculosis* is transcribed from a single promoter that requires activation by σ^{54} and GlrR, whereas expression of *E. coli glmZ* is not affected by these factors. To confirm this conclusion, we mutated the left half-site of each of the three putative ABS of GlrR individually or in combination (Figure 4A, left). Fusions of *Y. pseudotuberculosis glmZ'* to *lacZ* carrying these mutations were integrated into the chromosome of the *E. coli ΔglrR* mutant. These strains were subsequently complemented with the plasmid carrying *Y. pseudotuberculosis glrR* under P_{Ara} promoter

control and the β-galactosidase activities were determined (Figure 4A, right). Mutation of ABS 1 had no negative impact on *Y. pseudotuberculosis glmZ'-lacZ* transcription, whereas mutation of ABS 2 or ABS 3 reduced expression more than two-fold. Expression was completely abolished, when all three ABS were simultaneously mutated. To corroborate these data, we performed EMSA experiments using *Y. pseudotuberculosis glmZ* promoter fragments carrying a mutation in ABS3 or simultaneously in all three ABS. These EMSAs were carried out using purified GlrR from *E. coli* (Figure 4B) or from *Y. pseudotuberculosis* (Supplementary Figure S8). In addition, a truncated *glmZ* promoter fragment lacking all three ABSs was tested in EMSA with *Y. pseudotuberculosis* GlrR (Supplementary Figure S8). The data show that mutation of ABS3 decreased binding efficiency significantly. Finally, binding of GlrR was completely prevented, when all three ABS were truncated or simultaneously mutated (Figure 4B, Supplementary Figure S8). These results show that *Y. pseudotuberculosis glmZ* is transcribed from a single σ^{54}-promoter, which requires activation by binding of GlrR to its upstream located ABS.

To further confirm that *E. coli glmZ* expression is independent of upstream activating sequences, a promoter deletion analysis was performed. For this purpose, DNA
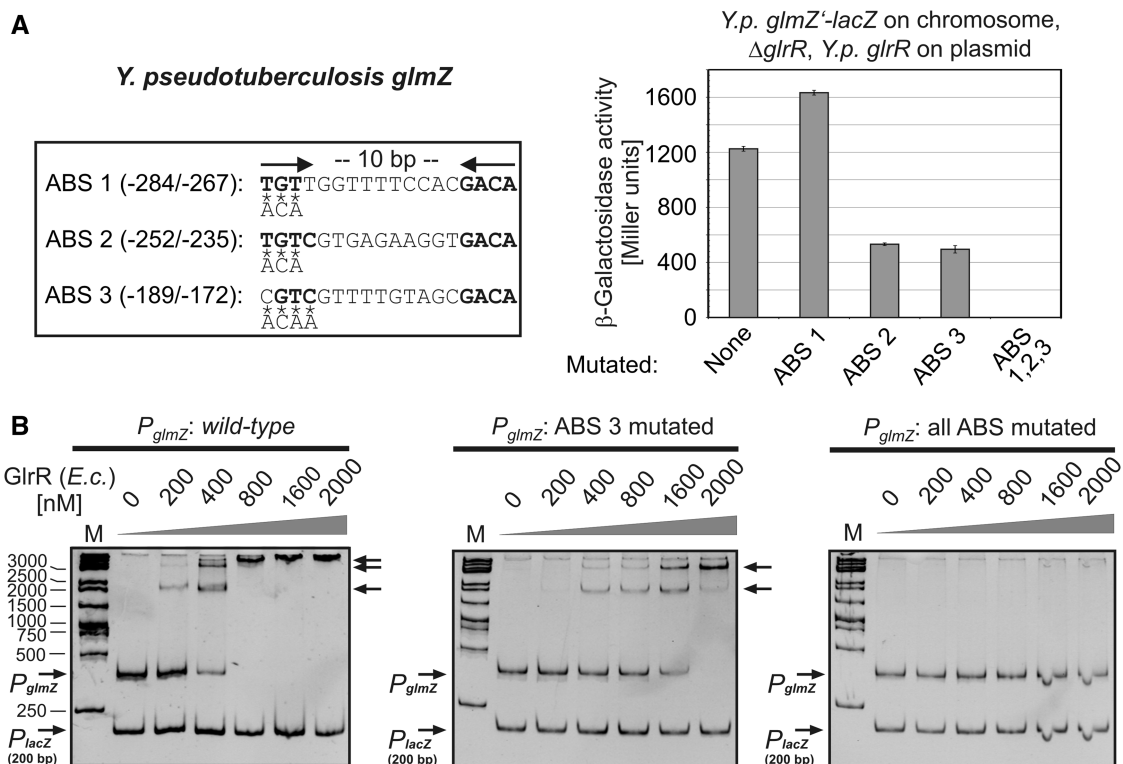

Figure 4. Transcription of *Y. pseudotuberculosis glmZ* depends on binding of GlrR to its three target sites upstream of the promoter. (**A**) β-Galactosidase activities of *E. coli* strains carrying mutated GlrR binding sites in the chromosomal *Y. pseudotuberculosis glmZ'-lacZ* fusion. In order to monitor activation by the cognate GlrR protein, *Y. pseudotuberculosis glrR* was expressed from plasmid pYG6, while the endogenous *glrR* gene was deleted. The nucleotide exchanges introduced into the ABS are depicted at the left. The following strains were employed (corresponding to the columns from left to right): Z365, Z397, Z398, Z399 and Z400. (**B**) EMSAs to monitor binding of *E. coli* GlrR to DNA fragments covering the *Y. pseudotuberculosis glmZ* promoter and carrying mutations in the ABS as depicted in the Figure.

fragments carrying gradually 5′-truncated versions of the *aslA-glmZ* intergenic region were fused to *lacZ* (Figure 5A). Plasmids carrying these various fusions were subsequently introduced into *E. coli* wild-type and the β-galactosidase activities were determined. The data show that the region upstream of position −40 relative to *glmZ* is dispensable for promoter activity (Figure 5B). Deletion of the sequences upstream position −20, which removes the −35 motif of the putative σ[70]-promoter (Figure 5A and Supplementary Figure S4), abrogates expression. To verify if the assumed −35 and −10 sequences are indeed elements of a functional σ[70]-promoter, these sequence elements were mutated. Mutation of the three bases matching the consensus sequence TTGACA within the putative −35 sequence (Figure 5A) reduced expression of the fusion drastically (Figure 5C). Mutation of the right half site of the putative −10 motif completely abolished expression (Figure 5C). These data confirm that *E. coli glmZ* is transcribed from a single σ[70]-promoter, which is constitutively active and apparently unregulated, at least under the tested conditions.

Activity of the σ[54]-dependent *glmY* promoter requires binding of IHF

The sequence alignment analyses detected two additional sequence motifs with similarity to the binding site of the global transcriptional regulator IHF. These sequence elements were detectable in all species, except for the *glmZ* promoters of *Escherichia*, *Shigella* and *Klebsiella* (Supplementary Figures S3 and S4), which according to all evidence are transcribed from single σ[70]-promoters. This suggested a role of these sites for activities of the σ[54]-promoters upstream of *glmY* and *glmZ* (Figure 6A). Therefore, we tested whether IHF is able to bind to the promoter fragments of *E. coli glmY* and *Y. pseudotuberculosis glmZ*. Both DNA fragments were bound by IHF protein (Figure 6B). The *lacZ* promoter fragments, which served as internal controls, were also
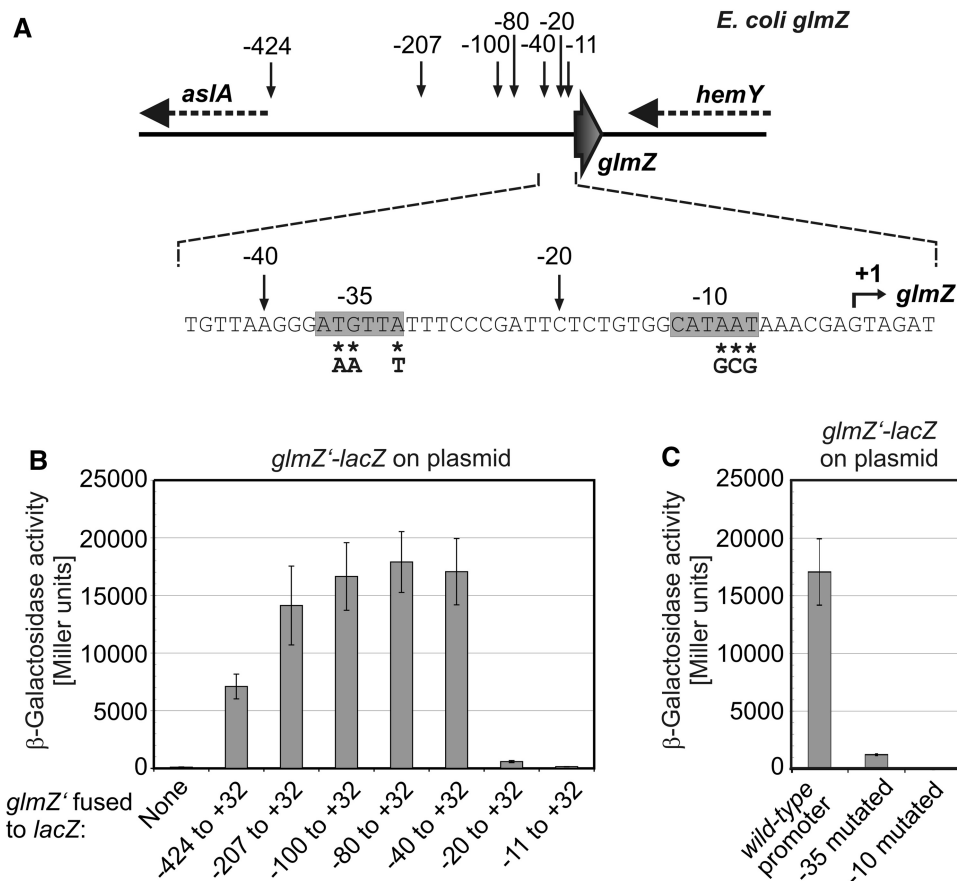

Figure 5. Analysis of the *E. coli glmZ* promoter (**A**) Schematic representation of the *aslA-hemY* intergenic region comprising the *E. coli glmZ* gene. DNA fragments extending until position +32 relative to the *glmZ* start site and with the 5′ ends indicated by arrows were fused to *lacZ*. The sequence of the *glmZ* promoter region with the putative −35/−10 motifs of a σ[70]-promoter is shown below. The nucleotide exchanges that were introduced into these motifs and tested in (C) are marked with asterisks. (**B**) 5′→3′ deletion analysis of the *E. coli glmZ* upstream region. β-Galactosidase activities of *E. coli* wild-type strain R1279 carrying the gradually 5′ truncated *glmZ'-lacZ* fusions on plasmids. The following plasmids were tested (corresponding to the columns from left to right): pKEM04, pBGG59, pBGG111, pBGG112, pBGG113, pBGG114, pBGG170 and pBGG135. (**C**) Mutational analysis of the *glmZ* promoter. The putative −35 and −10 sequences were mutated as indicated in (A) in the context of the *glmZ'* (−40 to +32)-*lacZ* fusion. Plasmids pBGG114, pBGG157 and pBGG171 (corresponding to the columns from left to right) were introduced into wild-type strain R1279 and the β-galactosidase activities were determined.
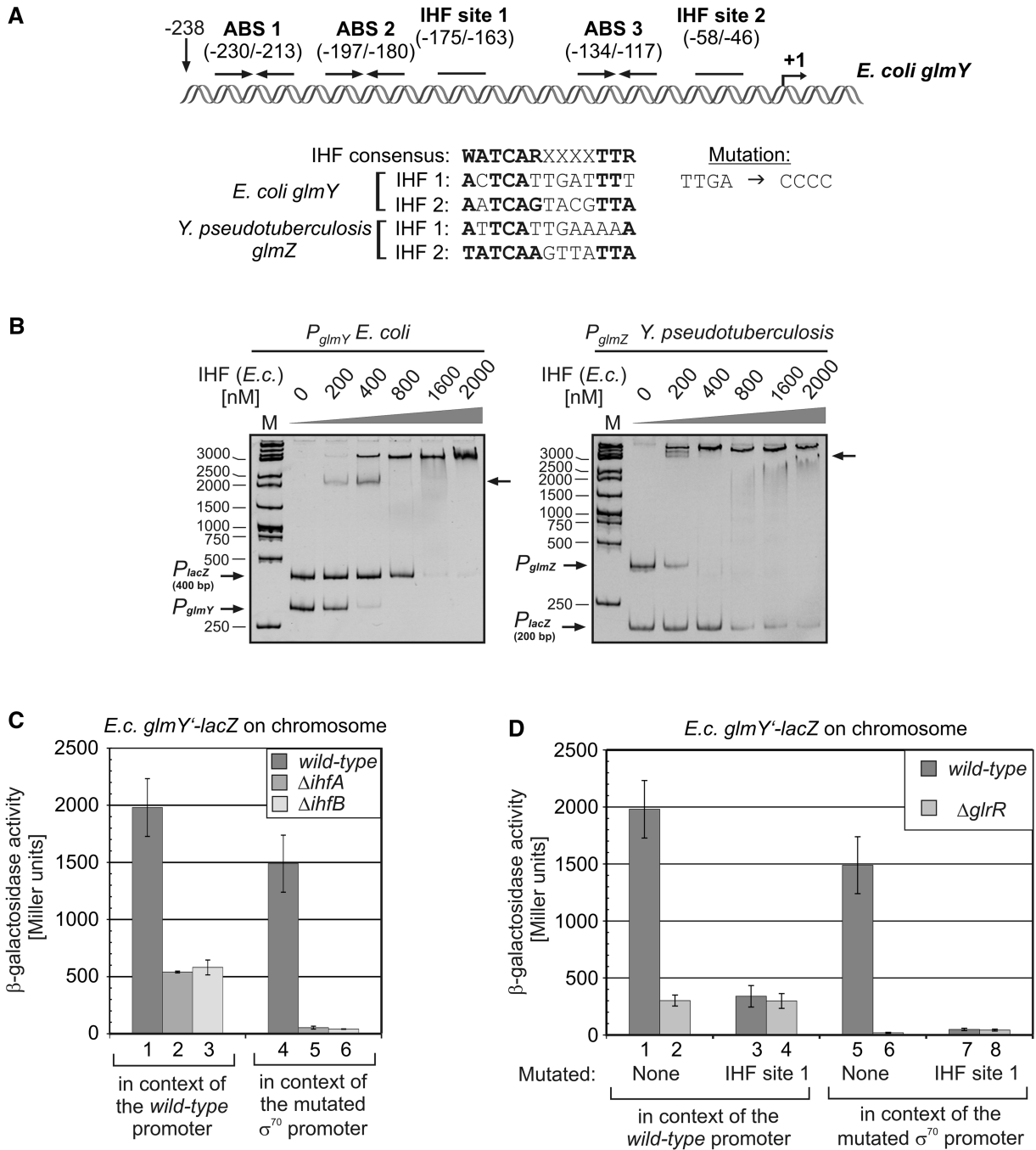
Figure 6. Role of IHF for expression of *glmY*. (**A**) Schematic representation of the *E. coli glmY* promoter region and location of GlrR and putative IHF binding sites. The sequences of the putative IHF binding sites upstream of *E. coli glmY* and *Y. pseudotuberculosis glmZ* are shown and the nucleotide exchanges introduced in IHF site 1 of the *E. coli glmY* promoter are indicated. (**B**) EMSAs to test binding of purified IHF to the *glmY* and *glmZ* promoter regions of *E. coli* and *Y. pseudotuberculosis*, respectively. The DNA fragments were obtained by PCR making use of the primer pairs BG377/BG456 and BG700/BG701, respectively. As controls, DNA fragments encompassing the *lac* promoter were additionally present. (**C**) Expression of *E. coli glmY* in *ΔihfA* and *ΔihfB* mutants. β-Galactosidase activities of strains carrying the chromosomal *E. coli glmY'-lacZ* fusion in the context of the *wild-type* promoter (columns 1–3) or in the context of the mutated σ^{70}-promoter leaving the σ^{54}-promoter as single active promoter (columns 4–6). Genes *ihfA* or *ihfB* were deleted as indicated in the legend. The following strains were tested (corresponding to the columns from left to right): Z197, Z395, Z393, Z190, Z394 and Z392. (**D**) Mutational analysis of the putative IHF site 1 in the *E. coli glmY* promoter region. β-Galactosidase activities of *wild-type* and *ΔglrR E. coli* strains carrying the wild-type or mutated alleles of the *E. coli glmY'-lacZ* fusion. Mutations were either in the putative IHF-site 1 (columns 3, 4, 7, 8) as indicated in (A) or in the −10 sequence of the *glmY* promoter (columns 5–8) rendering *glmY'-lacZ* expression fully dependent on σ^{54}. The following strains were employed (corresponding to the columns from left to right): Z197, Z206, Z370, Z372, Z190, Z196, Z371 and Z373.

bound, but at higher protein concentrations. The *lacZ* promoter is not known to contain any IHF site indicating unspecific binding. To confirm this conclusion, we repeated the experiments using a DNA fragment covering the *ptsG* promoter from *Bacillus subtilis* as internal control. *B. subtilis* does not possess IHF. Once more, efficient binding of the *glmY* and *glmZ* promoters could be observed, while the *ptsG* promoter was only bound at higher protein concentrations (Supplementary Figure S9). Hence, binding of IHF to the *lacZ* and *ptsG* promoters is unspecific, which is in line with previous data reporting that IHF binds DNA with lower affinity also in sequence-independent manner (30).

Next, we determined whether IHF is important for the activities of the σ^{54}-promoters. Therefore, we examined the role of IHF for expression of *E. coli glmY*. Expression of the chromosomally encoded *E. coli glmY'-lacZ* fusion was determined in mutants lacking *ihfA* or *ihfB*, which encode the subunits of IHF (30). In both mutants, expression of the fusion was reduced ~four-fold (Figure 6C, columns 1–3). The remaining activities were comparable with the expression level of this fusion in the $\Delta glrR$ mutant (Figure 2B, column 2), suggesting that it is caused by activity of the overlapping σ^{70}-promoter (22). Therefore, we repeated the experiment using a *glmY'-lacZ* fusion in which the −10 sequence of the σ^{70}-promoter is mutated, while the σ^{54}-promoter is unaffected (22). Expression of this fusion was abolished in the $\Delta ihfA$ and $\Delta ihfB$ mutants (Figure 6C, columns 4–6). This demonstrates that IHF is essential for activity of the σ^{54}-promoter of *glmY*.

To assess whether the two sequence elements resembling IHF binding sites are important for σ^{54}-promoter activity, we mutated the putative IHF-site 1 in the *E. coli glmY'-lacZ* fusion. Four highly conserved nucleotides (Supplementary Figure S3) were exchanged within the putative IHF site 1 (Figure 6A). This mutation yielded the same effects as the $\Delta ihfA$ and $\Delta ihfB$ mutations. Expression of the *glmY'-lacZ* fusion dropped five-fold and the remaining expression was comparable with the expression obtained in the $\Delta glrR$ mutant, in which solely the σ^{70}-promoter is active (Figure 6D, columns 1–3). Mutation of the putative IHF-1 site had no further negative impact on the residual expression of the fusion in the $\Delta glrR$ mutant (Figure 6D, columns 2 and 4) suggesting that activity of the σ^{70}-promoter is unaffected by this mutation. To verify the role of site 1 for activity of the σ^{54}-promoter, the experiments were repeated using the *glmY'-lacZ* fusion in which the σ^{70}-promoter had been mutated. Mutation of IHF site 1 abolished expression of this fusion and therefore had the same effect as a $\Delta glrR$ or the Δihf mutations (Figure 6D, columns 5–8; Figure 6C, columns 4–6). Hence, site 1 is essential for activity of the σ^{54}-promoter. Collectively, these data show that activity of the σ^{54}-promotor of *E. coli glmY* requires binding of IHF to the promoter region. The two sites identified by sequence alignment are likely candidates for these IHF binding sites. In contrast, activity of the overlapping σ^{70}-promoter appears to be unaffected by IHF.

Phosphorylated GlrR is active and stimulates sRNA expression

GlrR contains a response regulatory domain including the conserved putative phosphorylation site aspartate 56 at its N-terminus. Phosphorylation of GlrR by its cognate kinase GlrK has been previously demonstrated *in vitro* (32). Furthermore, a $\Delta glrK$ mutation was shown to abolish activity of the σ^{54}-promoter of *glmY* in *E. coli*, suggesting that GlrK controls activity of this promoter through modulation of the phosphorylation state of GlrR (22). In many TCS, the histidine kinase is capable of phosphorylating as well as dephosphorylating the response regulator. Phosphorylation of the response regulator results in structural changes, which in most cases activate the protein and stimulate interaction with the target DNA (33). In a few cases the dephosphorylated protein was shown to be active (34). We wanted to discriminate, whether phosphorylated or dephosphorylated GlrR is active. Therefore, we exploited the fact that many response regulators can autophosphorylate *in vitro* using small molecules such as acetyl phosphate as phosphoryl group donors (35). Therefore, EMSAs were carried out using the *E. coli glmY* promoter fragment and the *E. coli* GlrR protein that was pre-incubated with 50 mM acetyl phosphate for 1 h at 37°C prior to EMSA. Since ongoing incubation of GlrR at 37°C resulted in increasing inactivation of the protein (compare left panels in Figures 2A and 7A), a control experiment was performed in which GlrR was treated the same way but acetyl phosphate was omitted. These experiments revealed that binding affinity of GlrR was somewhat increased by the acetyl phosphate treatment relative to the control (compare panels in Figure 7A).

To obtain *in vivo* evidence that phosphorylated rather than dephosphorylated GlrR is active, we replaced the phosphorylation site Asp 56 in GlrR with an alanine and a glutamate residue, respectively. An Ala replacement is reported to mimic the dephosphorylated form of a response regulator, while a Glu replacement is able to mimic the phosphorylated Asp in some response regulators resulting in kinase-independent activation (35). Plasmids carrying the various *glrR* variants or no gene (empty vector control) under P_{Ara} promoter control were used to complement the $\Delta glrR$ mutant that carries the *E. coli glmY'-lacZ* fusion on the chromosome. Subsequently the β-galactosidase activities were determined from these transformants. Expression of the *glrR*-D56A allele resulted in ~two-fold lower activity when compared with wild-type *glrR* (Figure 7B, columns 2 and 3). In contrast, expression of *glrR*-D56E enhanced *glmY'-lacZ* expression five-fold. Taken together, the data indicate that phosphorylation of GlrR increases its DNA binding activity and thereby expression of the sRNA.

DISCUSSION

In this study we addressed the transcriptional regulation of two sRNA genes, *glmY* and *glmZ*, which are conserved in *Enterobacteriaceae*. Our analysis reveals three different scenarios of control of *glmY* and *glmZ* expression
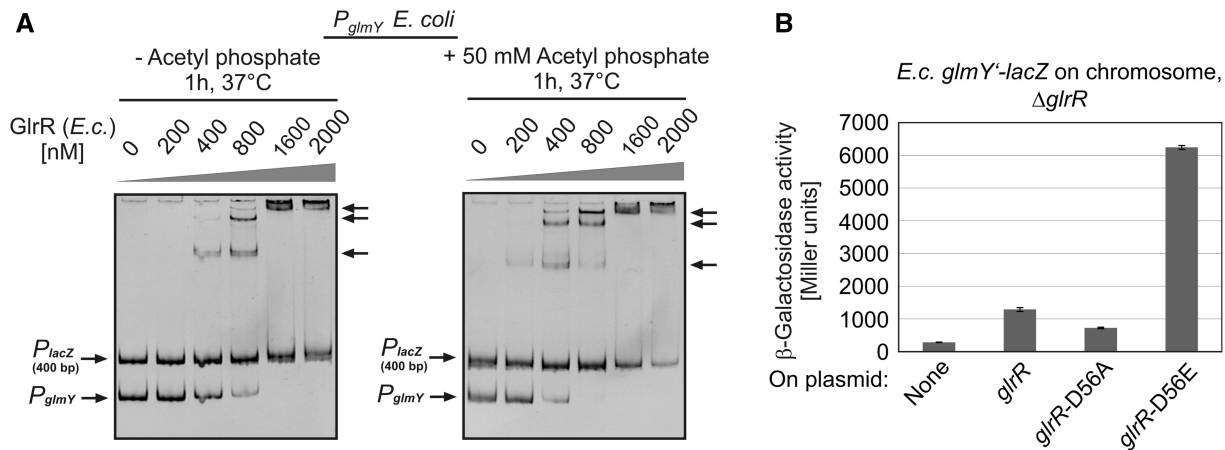
Figure 7. Phosphorylation increases activity of response regulator GlrR. (**A**) Effect of acetyl phosphate on the DNA binding activity of GlrR as revealed by EMSA. EMSAs were performed using purified *E. coli* GlrR and the *E. coli glmY* promoter fragment. To test the possible effect of phosphorylation on GlrR activity, the protein was pre-incubated at 37°C for 1 h in the absence (left panel) or presence (right panel) of 50 mM acetyl phosphate before continuing with the EMSA protocol. (**B**) A glutamate replacement of the phosphorylation site Asp56 in GlrR strongly up-regulates *glmY* expression. *E. coli* strain Z206 carrying a *ΔglrR* mutation and the *E. coli glmY'-lacZ* fusion on the chromosome was complemented with plasmids carrying *E. coli* wild-type *glrR* (pBGG389, column 2), *glrR*-D56A (pBGG398, column 3), *glrR*-D56E (pBGG399, column 4) or no gene (pBAD33, column 1) under P_{Ara} promoter control. Subsequently, the β-galactosidase activities were determined from these transformants.

operative in enterobacterial species as described for *Y. pseudotuberculosis*, *S. typhimurium* and *E. coli*. Sequence alignment analyses (Supplementary Figures S3 and S4) suggest that these species are representatives for other species showing similar *glmY* and *glmZ* promoter architectures, respectively (Figure 8). Most importantly, our results suggest that in most species expression of both sRNAs is controlled by σ^{54} and the response regulator GlrR (Figure 8). This adds two sRNA genes to the regulon governed by σ^{54} in *Enterobacteriaceae*. The *glmY* and *glmZ* genes of *Y. pseudotuberculosis* exhibit all features of canonical σ^{54}-dependent genes. Their expression depends on σ^{54} (Figures 2 and 3) and on binding of the activator protein GlrR to ABS present upstream of the σ^{54}-promoter, as demonstrated for *Y. pseudotuberculosis glmZ* (Figure 4 and Supplementary Figure S8). In conclusion, transcription is initiated from single σ^{54}-promoters that require activation by GlrR and the same may also hold true for species of the genera *Arsenophonus*, *Dickeya*, *Erwinia*, *Pectobacterium*, *Photorhabdus*, *Proteus* and *Serratia* (Figure 8). A somewhat different scenario is operative in the case of *S. typhimurium glmY* and *glmZ*. The corresponding promoter regions also contain three ABS and a σ^{54}-promoter. Accordingly, GlrR specifically binds to these regions and stimulates transcription (Figures 2 and 3). However, both genes are still expressed in mutants lacking σ^{54}, which is at first glance incompatible with the properties of genuine σ^{54}-dependent genes. The expression in the absence of σ^{54} is explained by additional σ^{70}-promoters that overlap the σ^{54}-promoters and can potentially start transcription at the same site. According to the sequence alignment, such overlapping σ^{70}- and σ^{54}-promoters may also exist in *Citrobacter*, *Cronobacter* and *Enterobacter* species (Figure 8). We have recently shown that in *E. coli* transcription of *glmY* is controlled by a similar mechanism (22). In contrast, *E. coli glmZ* is not controlled by GlrR or σ^{54} and

accordingly GlrR does not bind the *E. coli glmZ* promoter (Figure 3). A single constitutively active σ^{70}-promoter directs expression of *glmZ* in *E. coli* (Figure 5) and presumably also in *Klebsiella* and other *Escherichia* species (including *Shigella*) (Figure 8). In sum, our work suggests that *glmY* and *glmZ* transcription is controlled by σ^{54} and the TCS GlrR/GlrK in most Enterobacteria, but in a subset of species this relation is gradually lost in favor of unregulated σ^{70}-dependent transcription.

How did these different scenarios evolve? GlmY and GlmZ are homologous sRNAs (8,9). A sequence alignment of the *glmY/glmZ* genes of several species reveals sequence elements that are conserved in both sRNAs, while the *glmS* binding site is exclusively present in GlmZ species (Supplementary Figure S10). A phylogenetic tree built from this sequence alignment clusters *glmZ* genes together, while the *glmY* genes form a distinct group (Supplementary Figure S11). A similar clustering can be observed when the sequences of the corresponding promoter regions are used for tree construction (Supplementary Figure S12). Accordingly, *glmY* and *glmZ* most likely originated from duplication of a single sRNA locus in an ancestor of *Enterobacteriaceae* and transcription of this ancient sRNA was presumably already controlled by σ^{54} and GlrR. Following duplication, divergence of the promoter regions by mutation might have generated the different promoter architectures detectable in recent bacteria.

What is the physiological meaning of regulation of *glmY/glmZ* transcription by GlrR/GlrK? In *E. coli*, GlmYZ feedback-regulate synthesis of the enzyme GlmS and are therefore crucial for maintaining the intracellular GlcN6P concentration required for undisturbed synthesis of the cell wall and the outer membrane (8,10). This important role of GlmYZ may also apply to other *Enterobacteriacea*, since the GlmZ/*glmS* base-pairing appears to be conserved (7). In *E. coli*, a decrease in the
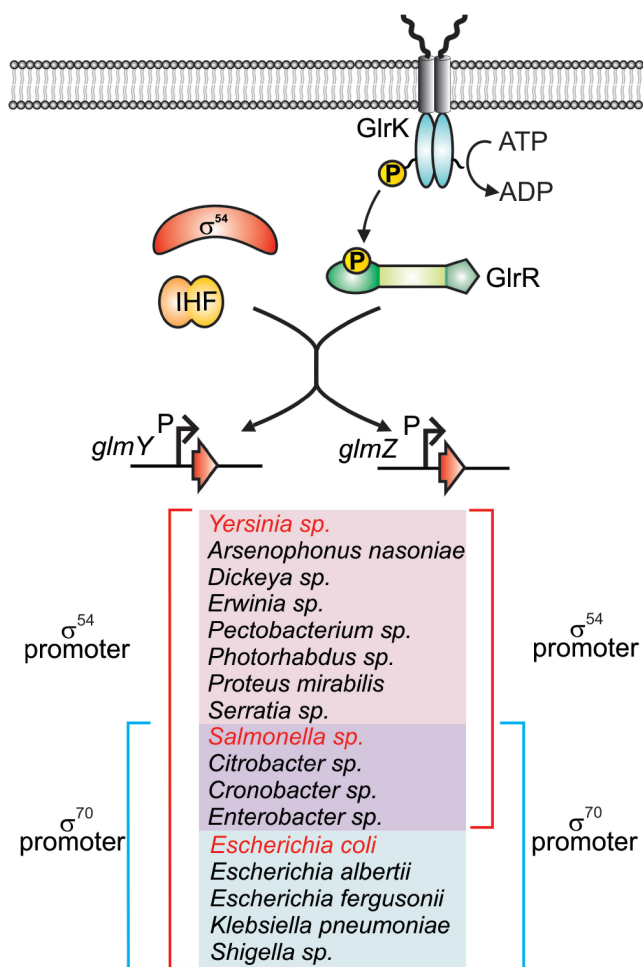
Figure 8. Model illustrating the roles of the TCS GlrR/GlrK, σ^{54} and IHF for transcription of sRNA genes *glmY* and *glmZ* in *Enterobacteriaceae*. Histidine kinase GlrK phosphorylates response regulator GlrR, which stimulates binding of GlrR to its target sites on the DNA. GlrR binds to three activator binding sites present upstream of σ^{54}-dependent promoters that control the expression of sRNA genes *glmY* in all species and *glmZ* in a subset of species. GlrR, which contains a σ^{54} interaction domain, is absolutely required for activity of these σ^{54}-promoters. In addition, promoter activity depends on IHF, which might facilitate interaction of GlrR with the σ^{54}-RNA polymerase by binding-induced bending of the promoter DNA. In *Y. pseudotuberculosis*, transcription of *glmY* and *glmZ* is directed by single σ^{54}-promoters that require activation by GlrR. Hence, *glmY* and *glmZ* compose a regulon controlled by GlrR and σ^{54}. A similar arrangement is found in *S. typhimurium*, but σ^{70}-promoters that overlap the σ^{54}-promoters additionally contribute to *glmY* and *glmZ* expression. Overlapping σ^{54}- and σ^{70}-promoters also direct expression of the *E. coli glmY* gene, while expression of *glmZ* is achieved from a single constitutively active σ^{70}-promoter. Sequence alignment analyses suggest that these three different arrangements might also apply to other enterobacterial species as shown in the Figure.

intracellular GlcN6P concentration induces accumulation of GlmY, which in turn increases concentration of the full-length form of GlmZ that is competent in *glmS* base-pairing (8,10). Most likely, GlmY acts on GlmZ through sequestration of a protein that targets GlmZ to processing (8,9), but it is unknown whether this mechanism is also operative in other species.

In conclusion, up-regulation of the GlmYZ cascade in response to GlcN6P depletion occurs at the post-transcriptional level and involves stabilization of the sRNAs rather than activation of their transcription in *E. coli* (22). Accordingly, the basal level of transcription of the sRNAs, as observed in the exponential growth phase, is sufficient for this function. However, GlrR/GlrK strongly up-regulate *glmY* expression through activation of the σ^{54}-promoter, when cells enter the stationary growth phase (22). In contrast, GlmZ levels decrease, i.e. stabilization of GlmZ as a consequence of accumulation of GlmY does not occur in this growth phase (8). Hence, GlmY accumulates in *E. coli* when growth ceases and ongoing cell wall synthesis and up-regulation of *glmS* are not required. This indicates a second function of GlmY, which requires a higher concentration of the sRNA and becomes relevant during transition to the stationary growth phase. We speculate that GlmY may have multiple functions and this may also hold for GlmZ in those species, which control expression of both sRNA through GlrR/GlrK: GlmYZ regulate *glmS* and thereby GlcN6P synthesis during the exponential growth phase and basal expression levels are sufficient for this purpose. In addition, they might have another function that requires further up-regulation of the sRNAs through the TCS GlrR/GlrK. What is this additional function? Interestingly, GlrR/GlrK have been implicated to play a role for virulence: Mutants of *Y. pseudotuberculosis* lacking GlrR exhibited reduced pathogenicity in mice (36). In enterohemorrhagic *E. coli* (EHEC) GlrR/GlrK (QseF/QseE) are required for transcription of *espFU*, which is an EHEC-specific gene and encodes an effector protein translocated to the host cell. Consequently, loss of GlrR/GlrK results in the inability to form attaching and effacing lesions that are required for destruction of microvilli, pedestal formation and rearrangement of the cytoskeleton of host cells (37,38). In conclusion, GlrR/GlrK controls functions important for interaction with eukaryotic cells in at least two different bacteria. Whether this also holds for other *Enterobacteriaceae* and involves GlmY(Z) remains to be determined.

What is the reason for the existence of additional σ^{70}-promoters overlapping with the σ^{54}-dependent *glmY*/ *glmZ* promoters in a subgroup of *Enterobacteriaceae*? They may allow better fine-tuning of the expression to meet the requirements of the multiple functions of these sRNAs, e.g. the σ^{70}-promoters ensure sRNA expression when the activating signal for GlrR/GlrK is absent and the σ^{54}-promoter is inactive. Alternatively, the σ^{70}-promoters could also be regulated and may allow regulation of the sRNAs in response to another yet unknown process. It is also possible, that the functional overlap of σ^{54}- and σ^{70}-dependent promoters is a more global phenomenon in certain species such as *E. coli*. Extensive functional overlap with σ^{70}-promoters has been observed for σ^{24}- and σ^{32}-dependent genes in *E. coli* (20). Both, σ^{24} and σ^{32} recognize distinct promoter sequences. However, many of these promoters also contain matches to overlapping σ^{70}-promoters. Thus, the majority of the σ^{32}-promoters and about half of the σ^{24}-promoters are

also recognized by σ^{70}-RNAP and transcription initiation at the same start site was demonstrated for some of these promoters (20). This was interpreted to means that the primary function of alternative σ factors is to increase transcription of σ^{70}-dependent genes. A recent study reported that 14% of the σ^{54}-dependent genes in *E. coli* can also be transcribed by σ^{70}-RNAP *in vitro* (17). Whether this occurs from overlapping or consecutive promoters is not known. However, our studies prove that arrangements of overlapping σ^{70}- and σ^{54}-promoters exist [(22); the present study]. It remains to be elucidated whether functional overlap between σ^{70} and σ^{54} is a peculiarity of *E. coli* and its closest relatives or may apply to a wider range of bacterial species.

Activation of the σ^{54}-dependent *glmY* and *glmZ* promoters requires binding of GlrR to ABS located upstream of the promoter. However, the impact of each of the three ABS on the promoter activity appears to vary from case to case, e.g. ABS2 and ABS3 were shown to be essential for activity of the σ^{54}-promoter of *E. coli glmY* (22), while mutation of one of these sites upstream of *Y. pseudotuberculosis glmZ* reduced promoter activity only two-fold (Figure 4A). ABS1 appears to be dispensable for promoter activity in both cases, as reflected by its lower degree of conservation. Interaction of activator proteins with σ^{54}-RNAP requires bending of the DNA, which is usually induced by IHF (18,30). IHF might also be required for the activities of the σ^{54}-dependent *glmY* and *glmZ* promoters as demonstrated for the σ^{54}-promoter of *E. coli glmY* (Figure 6). Two putative IHF binding sites were detected and we demonstrated an essential role for σ^{54}-promoter activity for the distal site (Figure 6D). We also provided evidence that phosphorylation of GlrR enhances *glmY* expression (Figure 7). Substitution of the phosphorylation site Asp 56 with Ala reduced *glmY* expression two-fold, whereas a Glu exchange mimicking phosphorylation led to much stronger expression (Figure 7B). In addition, pre-incubation of GlrR with acetyl phosphate increased its binding affinity for the *glmY* promoter (Figure 7A). Taken together this indicates that the DNA-binding activity of GlrR is activated by its phosphorylation although it cannot be excluded yet that the mutations in GlrR affected the stability rather than activity of the protein. Our data indicate that just a minor fraction of GlrR is phosphorylated by GlrK during exponential growth, which is in line with previous data suggesting that this TCS drastically increases *glmY* expression at the on-set of the stationary growth phase in *E. coli* (22). So far, *glmY* and *glmZ* are the only known direct targets of GlrR/GlrK suggesting that this TCS acts predominantly through these sRNAs.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

1. Görke,B. and Vogel,J. (2008) Noncoding RNA control of the making and breaking of sugars. *Genes Dev.*, **22**, 2914–2925.
2. Repoila,F. and Darfeuille,F. (2009) Small regulatory non-coding RNAs in bacteria: physiology and mechanistic aspects. *Biol. Cell*, **101**, 117–131.
3. Guillier,M., Gottesman,S. and Storz,G. (2006) Modulating the outer membrane with small RNAs. *Genes Dev.*, **20**, 2338–2348.
4. Brantl,S. (2009) Bacterial chromosome-encoded small regulatory RNAs. *Future Microbiol.*, **4**, 85–103.
5. Vanderpool,C.K. (2007) Physiological consequences of small RNA-mediated regulation of glucose-phosphate stress. *Curr. Opin. Microbiol.*, **10**, 146–151.
6. Waters,L.S. and Storz,G. (2009) Regulatory RNAs in bacteria. *Cell*, **136**, 615–628.
7. Fröhlich,K.S. and Vogel,J. (2009) Activation of gene expression by small RNA. *Curr. Opin. Microbiol.*, **12**, 674–682.
8. 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**, 2570–2580.
9. Urban,J.H. and Vogel,J. (2008) Two seemingly homologous noncoding RNAs act hierarchically to activate *glmS* mRNA translation. *PLoS Biol.*, **6**, e64.
10. Kalamorz,F., Reichenbach,B., März,W., Rak,B. and Görke,B. (2007) Feedback control of glucosamine-6-phosphate synthase GlmS expression depends on the small RNA GlmZ and involves the novel protein YhbJ in *Escherichia coli*. *Mol. Microbiol.*, **65**, 1518–1533.
11. Urban,J.H., Papenfort,K., Thomsen,J., Schmitz,R.A. and Vogel,J. (2007) A conserved small RNA promotes discoordinate expression of the *glmUS* operon mRNA to activate GlmS synthesis. *J. Mol. Biol.*, **373**, 521–528.
12. Valverde,C. and Haas,D. (2008) Small RNAs controlled by two-component systems. *Adv. Exp. Med. Biol.*, **631**, 54–79.
13. Vogel,J. (2009) A rough guide to the non-coding RNA world of *Salmonella*. *Mol. Microbiol.*, **71**, 1–11.
14. Sonnleitner,E., Abdou,L. and Haas,D. (2009) Small RNA as global regulator of carbon catabolite repression in *Pseudomonas aeruginosa*. *Proc. Natl Acad. Sci. USA*, **106**, 21866–21871.
15. Tu,K.C. and Bassler,B.L. (2007) Multiple small RNAs act additively to integrate sensory information and control quorum sensing in *Vibrio harveyi*. *Genes Dev.*, **21**, 221–233.
16. Reitzer,L. and Schneider,B.L. (2001) Metabolic context and possible physiological themes of sigma(54)-dependent genes in *Escherichia coli*. *Microbiol. Mol. Biol. Rev.*, **65**, 422–444.
17. Zhao,K., Liu,M. and Burgess,R.R. (2010) Promoter and regulon analysis of nitrogen assimilation factor, sigma54, reveal alternative strategy for *E. coli* MG1655 flagellar biosynthesis. *Nucleic Acids Res.*, **38**, 1273–1283.
18. Wigneshweraraj,S., Bose,D., Burrows,P.C., Joly,N., Schumacher,J., Rappas,M., Pape,T., Zhang,X., Stockley,P.,

Severinov,K. *et al.* (2008) Modus operandi of the bacterial RNA polymerase containing the sigma54 promoter-specificity factor. *Mol. Microbiol.*, **68**, 538–546.

19. Mendoza-Vargas,A., Olvera,L., Olvera,M., Grande,R., Vega-Alvarado,L., Taboada,B., Jimenez-Jacinto,V., Salgado,H., Juarez,K., Contreras-Moreira,B. *et al.* (2009) Genome-wide identification of transcription start sites, promoters and transcription factor binding sites in *E. coli. PLoS ONE*, **4**, e7526.

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

21. Darfeuille,F., Unoson,C., Vogel,J. and Wagner,E.G. (2007) An antisense RNA inhibits translation by competing with standby ribosomes. *Mol. Cell.*, **26**, 381–392.

22. Reichenbach,B., Göpel,Y. and Görke,B. (2009) Dual control by perfectly overlapping sigma 54- and sigma 70- promoters adjusts small RNA GlmY expression to different environmental signals. *Mol. Microbiol.*, **74**, 1054–1070.

23. Wilson,G.G., Young,K.Y., Edlin,G.J. and Konigsberg,W. (1979) High-frequency generalised transduction by bacteriophage T4. *Nature*, **280**, 80–82.

24. Diederich,L., Rasmussen,L.J. and Messer,W. (1992) New cloning vectors for integration in the *lambda* attachment site *attB* of the *Escherichia coli* chromosome. *Plasmid*, **28**, 14–24.

25. Miller,J. (1972) *Experiments in Molecular Genetics.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

26. Heroven,A.K., Böhme,K., Rohde,M. and Dersch,P. (2008) A Csr-type regulatory system, including small non-coding RNAs, regulates the global virulence regulator RovA of *Yersinia pseudotuberculosis* through RovM. *Mol. Microbiol.*, **68**, 1179–1195.

27. Stratmann,T., Madhusudan,S. and Schnetz,K. (2008) Regulation of the *yjjQ-bglJ* operon, encoding LuxR-type transcription factors, and the divergent *yjjP* gene by H-NS and LeuO. *J. Bacteriol.*, **190**, 926–935.

28. Dehal,P.S., Joachimiak,M.P., Price,M.N., Bates,J.T., Baumohl,J.K., Chivian,D., Friedland,G.D., Huang,K.H., Keller,K., Novichkov,P.S. *et al.* (2009) MicrobesOnline: an integrated portal for comparative and functional genomics. *Nucleic Acids Res.*, **38**, D396–400.

29. Kanehisa,M., Araki,M., Goto,S., Hattori,M., Hirakawa,M., Itoh,M., Katayama,T., Kawashima,S., Okuda,S., Tokimatsu,T. *et al.* (2008) KEGG for linking genomes to life and the environment. *Nucleic Acids Res.*, **36**, D480–D484.

30. Swinger,K.K. and Rice,P.A. (2004) IHF and HU: flexible architects of bent DNA. *Curr. Opin. Struct. Biol.*, **14**, 28–35.

31. Paradis,S., Boissinot,M., Paquette,N., Belanger,S.D., Martel,E.A., Boudreau,D.K., Picard,F.J., Ouellette,M., Roy,P.H. and Bergeron,M.G. (2005) Phylogeny of the *Enterobacteriaceae* based on genes encoding elongation factor Tu and F-ATPase beta-subunit. *Int. J. Syst. Evol. Microbiol.*, **55**, 2013–2025.

32. Yamamoto,K., Hirao,K., Oshima,T., Aiba,H., Utsumi,R. and Ishihama,A. (2005) Functional characterization *in vitro* of all two-component signal transduction systems from *Escherichia coli. J. Biol. Chem.*, **280**, 1448–1456.

33. Stock,A.M., Robinson,V.L. and Goudreau,P.N. (2000) Two-component signal transduction. *Annu. Rev. Biochem.*, **69**, 183–215.

34. Zakikhany,K., Harrington,C.R., Nimtz,M., Hinton,J.C. and Römling,U. (2010) Unphosphorylated CsgD controls biofilm formation in *Salmonella enterica* serovar Typhimurium. *Mol. Microbiol.*, **77**, 771–786.

35. Scharf,B.E. (2010) Summary of useful methods for two-component system research. *Curr. Opin. Microbiol.*, **13**, 246–252.

36. Flamez,C., Ricard,I., Arafah,S., Simonet,M. and Marceau,M. (2008) Phenotypic analysis of *Yersinia pseudotuberculosis* 32777 response regulator mutants: new insights into two-component system regulon plasticity in bacteria. *Int. J. Med. Microbiol.*, **298**, 193–207.

37. Reading,N.C., Torres,A.G., Kendall,M.M., Hughes,D.T., Yamamoto,K. and Sperandio,V. (2007) A novel two-component signaling system that activates transcription of an enterohemorrhagic *Escherichia coli* effector involved in remodeling of host actin. *J. Bacteriol.*, **189**, 2468–2476.

38. Reading,N.C., Rasko,D.A., Torres,A.G. and Sperandio,V. (2009) The two-component system QseEF and the membrane protein QseG link adrenergic and stress sensing to bacterial pathogenesis. *Proc. Natl Acad. Sci. USA*, **106**, 5889–5894.

39. Baba,T., Ara,T., Hasegawa,M., Takai,Y., Okumura,Y., Baba,M., Datsenko,K.A., Tomita,M., Wanner,B.L. and Mori,H. (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol. Syst. Biol.*, **2**, 1–11.

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