Published in final edited form as:
Mol Microbiol. 2009 December ; 74(6): 1314–1330. doi:10.1111/j.1365-2958.2009.06944.x.

A PhoQ/P-Regulated small RNA Regulates Sensitivity of *Escherichia coli* to Antimicrobial Peptides

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Abstract

Non-coding small RNAs (sRNAs) play a major role in post-transcriptional regulation of gene expression. Of the 80 small RNAs that have been identified in *E. coli*, one-third bind to the RNA chaperone Hfq. Hfq both stabilizes these sRNAs *in vivo* and stimulates pairing to targets *in vitro*. A novel Hfq-dependent RNA, called here MgrR, was identified by its ability to bind Hfq. Expression of MgrR requires the PhoQ/PhoP two-component system; the PhoP response regulator is active under low Mg^{2+} concentrations and is an important virulence regulator in *Salmonella; mgrR* is also found in Salmonella species. Negatively regulated targets of MgrR identified using microarrays include *eptB*, involved in lipopolysaccharide (LPS) modification, and *ygdQ*, encoding a hypothetical protein. Cell sensitivity to the antimicrobial Polymyxin B is affected by LPS modifications, and cells carrying an *mgrR* deletion were approximately 10 times more resistant than wild type cells to Polymyxin B. Thus, lower Mg^{2+} concentrations, sensed by PhoQ/PhoP, lead to expression of MgrR, changing LPS. sRNAs have previously been shown to regulate many outer membrane proteins. This work demonstrates that LPS, a major contributor of bacterial interactions with mammalian cells, is also subject to regulation by sRNAs.

Keywords

extended -10; *yneM*

Introduction

Non-coding small RNAs (sRNAs) have been shown to provide a new level of gene regulation in cells across different kingdoms of life. In prokaryotes, many sRNAs have been found to be expressed specifically during adaptation to different environmental stresses, including low temperature, oxidative stress, and iron starvation (Gottesman *et al.*, 2006, Gottesman, 2004). To date, close to one hundred different sRNAs have been identified in *E. coli* by a variety of genome-wide approaches (Vogel & Sharma, 2005). In many cases, these sRNAs regulate target mRNAs at the post-transcriptional level via direct base pairing, and, as a consequence, modify translation and/or message stability.

The majority of these RNAs known to base pair with their target RNA require an RNA binding protein, Hfq, for proper function in gene regulation (Majdalani *et al.*, 2005, Gottesman, 2004, Storz *et al.*, 2005). Hfq stabilizes many regulatory sRNAs and facilitates the base pairing between sRNAs and their target mRNAs (Brennan & Link, 2007, Valentin-Hansen *et al.*, 2004). Both positive and negative regulation of translation have been demonstrated, although negative regulation is more common.

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A variety of evidence suggests that Hfq-dependent RNAs may have important roles during pathogenesis and in the interaction of bacteria and host. *hfq* null mutants were shown to attenuate the ability of *Salmonella* to invade epithelial cells, to secrete virulence factors, and to survive inside macrophages (Sittka *et al.*, 2007). Type IV pili-dependent twitching motility, important for *P. aeruginosa* virulence, is reduced in *hfq* mutants of *Pseudomonas aeruginosa* (Sonnleitner et al., 2003). The loss of Hfq increased the sensitivity to hydrogen peroxide and decreased survival in acidic conditions in *Brucella abortus*, which causes disease in cattle (Robertson & Roop, 1999). In uropathogenic *E. coli* (UPEC), Hfq is critical for colonization within the urinary tract as well as biofilm formation and motility (Kulesus *et al.*, 2008). One characteristic of the Hfq-dependent sRNAs that have been studied in *E. coli* and in *Salmonella typhimurium* is that many have been found to negatively regulate outer membrane proteins, and therefore would be expected to significantly alter the cell surface (Valentin-Hansen *et al.*, 2007, Guillier *et al.*, 2006, Vogel & Papenfort, 2006)

A large number of different transcriptional regulators have been found to regulate sRNAs. In particular, a number of two-component systems or phosphorelays have been shown to include sRNAs in their regulons. OmrA and OmrB are dependent on the EnvZ/OmpR two-component system (Guillier & Gottesman, 2006). RprA is dependent on the Rcs phosphorelay (Majdalani *et al.*, 2002).

Hfq co-immunoprecipitation followed by genome-wide detection on microarrays or deep sequencing has proven to be a sensitive method of identifying Hfq-dependent sRNAs. In *E. coli*, such experiments suggested the presence of a novel Hfq-bound small RNA in the intergenic region between *ydeH* and *ydeE* (Zhang *et al.*, 2003). In this study, we focus on the regulation and function of the sRNA found in this intergenic region, now named MgrR. We show that the expression of *mgrR* is dependent on the two-component system, PhoQ/PhoP. PhoQ, the sensor kinase, has been shown to become active in response to low Mg^{2+} concentrations in the environment and in response to treatment of cells with some antimicrobial peptides; when it is active, it phosphorylates PhoP, leading to increased synthesis of a large set of genes (Soncini *et al.*, 1996). These genes include Mg^{2+} transporters, and also include proteins that modify LPS, changing its charge and therefore changing cell sensitivity to antimicrobial peptides (Fields *et al.*, 1989). PhoQ/PhoP are known to be essential for Salmonella virulence in mouse infections (Miller *et al.*, 1989, Fields *et al.*, 1989, Galan & Curtiss, 1989). We identified two negatively regulated targets of MgrR, *eptB*, an LPS modifying enzyme, and *ygdQ*, a conserved protein of unknown function, which therefore are indirectly negatively regulated by PhoQ/PhoP. MgrR expression leads to changes in sensitivity of cells to antimicrobial peptides, probably due to changes in LPS modification, suggesting a possible role of MgrR in bacterial survival *in vivo*.

While the study reported here was being carried out, an additional report of an Hfq-bound sRNA in the same intergenic region was provided by Sittka *et al.*, using deep sequencing of Hfq-bound RNAs in *Salmonella* (Sittka *et al.*, 2008). Our results suggest that our findings with *E. coli* with respect to expression of this sRNA and with respect to its targets is likely to be true in *Salmonella* as well.

Results

Expression pattern for MgrR

MgrR was first observed as a strong signal during a microarray analysis of sRNAs that immunoprecipitated with Hfq (Zhang *et al.*, 2003), but was not at that time confirmed by Northern blot. The signal was located in a conserved intergenic region between the hypothetical protein genes *ydeH* and *ydeE* (Fig. 1A). This intergenic region had previously

been shown to encode a small open reading frame (orf), which is named *yneM* (Wassarman *et al*., 2001), on the strand opposite to that predicted for the Hfq-binding sRNA (Fig. 1A). Both *mgrR* and *yneM* contain conserved factor-independent terminators; these do not overlap (Fig. 1B). *yneM* and *mgrR* are conserved and adjacent to each other in the genomes of enterobacterial species closely related to *E. coli*, although in some species the flanking genes have changed (Fig. S1). A recent paper by Sittka *et al.* (2008) also reported a possible Hfq binding small RNA in *Salmonella* (STnc560) in the same intergenic region, as well as the expression of the *yneM* mRNA.

We analyzed the expression of MgrR by Northern blot at various stages of growth. It was highly expressed in LB at all ODs examined, but the level of expression of MgrR decreased at least five-fold in an *hfq* deletion mutant, consistent with it being an Hfq-binding sRNA, stabilized by Hfq (Fig. 1C). The *yneM* transcript showed no major change in abundance in an *hfq* mutant (Fig. 1C). We confirmed that MgrR was enriched by Hfq immunoprecipitation, consistent with the previous reports from microarray data and deep sequencing (data not shown).

The turnover rate of MgrR was measured, both from the chromosome after treatment with rifampicin and from a Plac-*mgrR* plasmid, in the absence of rifampicin, by washing out inducer to stop new synthesis. As has previously been seen with RyhB, DsrA, and OxyS (Massé et al, 2003), MgrR was quite stable (half-life of 15') in the presence of rifampicin, but quite unstable when measured by shutting down the inducible promoter (Fig. S2). This difference in stability has been interpreted as indicating that the sRNA is degraded as it is used, after pairing with a target mRNA; in rifampicin, no new target mRNA is made and therefore the sRNA is stable (Massé et al, 2003). The results with MgrR are consistent with this model.

Conservation and regulation of *mgrR*

The 5' end of MgrR was mapped using 5'-rapid amplification of cDNA ends (RACE) (Urban & Vogel, 2007). This analysis, combined with the predicted Rho-independent terminator at the 3' end of MgrR, suggested it to be 98nt in size (Fig. 2). This observation was consistent with the size observed by Northern blot analysis. MgrR contains an A-rich region between two conserved stem loops; this A-rich region may be an Hfq binding site. A similar sRNA sequence was found in all other sequenced *E. coli* strains, including pathogenic ones, as well as in *Salmonella*, *Citrobacter*, *Enterobacter*, and *Klebsiella* (Fig. 2). In particular, the region just upstream of the terminator (bracketed in Fig. 2) is highly conserved in all of these organisms. This conservation suggests that closely related organisms contain *mgrR* homologues that may be functionally similar to the *E. coli* K12 MgrR.

The promoter of *mgrR* is somewhat less conserved than the sRNA itself; the conserved regions may define the critical regulatory sites in this promoter. Assuming the same +1, conservation of an extended −10 sequence, TGcTACtGT, was found. Upstream, conservation was seen overlapping the −35 region, extending both downstream and upstream, with a GTTTA sequence upstream of the −35 region (Fig. 2). In *E. coli* and *Shigella*, a second GTTTA sequence was seen, 6 nt upstream of the conserved sequence; this suggested the possibility of a PhoP binding site in this region. PhoP, the response regulator of the PhoQ/PhoP two-component system, has been found to regulate a large number of genes in *E. coli* and *Salmonella* (Groisman, 2001,Ernst *et al.*, 2001). The sensor kinase PhoQ is active under low Mg^{2+}conditions, leading to phosphorylation and activation of PhoP, which generally acts as a transcriptional activator (Prost & Miller, 2008). Studies of PhoP binding sites have suggested a consensus sequence of (T/G)GTTTA-5nt-(T/G)GTTTA (Minagawa *et al.*, 2003,Kato *et al.*, 1999). As described below, regulation of

MgrR is consistent with this suggestion, and is the basis for naming this sRNA (Mg^{2+} responsive RNA).

To examine whether PhoP is an activator for MgrR, two *mgrR-lacZ* transcriptional fusions were constructed at the chromosomal *lac* site and assayed in wild-type and *phoP* or *phoQ* mutants. A full-length promoter fusion construct contained the *mgrR* upstream region from positions −60 to +10, (P$mgrR_{(-60)}$), strain KM112); a second construct contained the region from the −35 to +10 (P$mgrR_{(-35)}$), strain KM 113). The transcriptional activity of the P$mgrR_{(-60)}$ -*lacZ* fusion was 80-fold higher than that of the control without a promoter insert; the activity of the P$mgrR_{(-35)}$-*lacZ* fusion was very low, equivalent to the promoterless control (Fig. 3A). This data was consistent with the involvement of a positive regulator with essential binding site elements upstream of −35. A *phoP* deletion abolished expression of β-galactosidase in the P$mgrR_{(-60)}$ -*lacZ* fusion, as did a *phoQ* deletion (Fig. 3A). The dependence upon *phoP* was confirmed by Northern blot analysis; no *mgrR* transcript was observed in the *phoP* knockout strain (Fig. 3B). These data confirmed that PhoQ/PhoP are essential positive regulators for MgrR expression, and are consistent with the PhoP binding sites shown in Figure 2. No sequences upstream of −60 are necessary for this regulation.

Regulation of *yneM*

The 5’ end of *yneM* was mapped as well, and the gene was found to have a 58 nt leader, as shown in Fig. 1B. The expression of the mRNA for *yneM* was monitored in experiments in which *mgrR* was measured and also found to be dependent upon PhoP for accumulation (Fig. S3). An examination of the *yneM* promoter identified a possible site for PhoP binding (Fig. S3).

Mg^{2+} sensing and MgrR expression

In the experiments above, expression of MgrR was easily detected in LB broth, conditions at which other PhoQP-dependent RNAs were poorly expressed. We therefore carried out a series of experiments to examine the effect of changing Mg^{2+} concentrations on *mgrR* expression, and compared this to other PhoQ/PhoP-dependent promoters.

We compared the expression of *mgrR* to five different PhoQ/PhoP regulated promoters, *phoP* (Soncini *et al.*, 1995, Kato *et al.*, 1999), *mgrB* (Kato *et al.*, 1999), *mgtA* (Minagawa *et al.*, 2003), *iraM* (Bougdour *et al.*, 2008), and *yneM* (Fig. 3C) using Real-time PCR analysis after growth at seven different Mg^{2+} concentrations ranging from 0 to 100 mM (Fig. 4), as well as by Northern blot (data not shown). Cells were grown in minimal medium with 2mM Mg^{2+}, filtered and resuspended in various Mg^{2+} concentrations, and RNA samples were collected 50’ after filtration. The expression of all PhoP-regulated genes were highest without Mg^{2+} in the media and decreased with increasing Mg^{2+} concentration, as expected for PhoQ-dependent expression. However, the sensitivity of different promoters to levels of Mg^{2+} differed significantly, with *mgrR* the least sensitive to higher Mg^{2+} concentrations and *iraM*, *mgtA*, and *yneM* among the most sensitive to raised Mg^{2+} levels (Fig. 4).

One possible explanation for the higher activity of the *mgrR* promoter at moderate levels of Mg^{2+} would be that this promoter can be activated by relatively low levels of PhoP-P (phosphorylated PhoP). A prediction of this might be that while activity would be primarily dependent upon the histidine kinase PhoQ (as seen in Fig. 3A), alternative sources of phosphorylation might contribute to *mgrR* activity. Consistent with this idea, on lactose MacConkey plates a strain expressing an *mgrR-lacZ* fusion was inactive in a *phoP* mutant but partially active in a *phoQ* mutant, while the tightly regulated *yneM-lacZ* fusion was inactive in either mutant (Fig. 3C).

The *mgrR* promoter has a conserved extended −10 (Fig. 2: TGcTACTGT) that may contribute to its high activity. A derivative of the fusion was created in which TG was changed to AC (−10 region: ACcTACTGT). The level of expression was much reduced for cells carrying this mutant fusion; expression was decreased by 20–30 fold either in LB medium (data not shown) or in minimal media (Table 1). However, the degree of repression by Mg^{2+} was comparable (4.4× for the wild-type promoter, and 6 fold for the mutant promoter; Table 1), and as for the wild-type promoter, the expression level at 2 mM Mg^{2+} was only modestly decreased relative to the level after growth in low Mg^{2+}. This experiment confirms the Mg^{2+} responsive behavior of the *mgrR* promoter and shows that the −10 region affects level of expression but not response to Mg^{2+}.

We also asked if PhoQ/PhoP regulation of *mgrR* sRNA was conserved in *Salmonella*, since the PhoP site was not as close to consensus in that organism (Fig. 2). In this case, the *Salmonella* promoter sequences were substituted for those of *E. coli* in the fusion construct (sequence changed is underlined in Fig. 2). Expression levels were comparable to those found for *E. coli* sequences, and expression was still PhoP and PhoQ dependent (Fig. 3A).

Genes Regulated by MgrR

Hfq-binding sRNAs base pair with target RNAs, frequently leading to rapid degradation of target messages or, less frequently, to stabilization, both of which can be assayed by using microarrays (Masse *et al.*, 2005, Guillier & Gottesman, 2006, De Lay & Gottesman, 2009). In order to search for the target genes of MgrR, we therefore examined the consequences of MgrR expression on mRNA abundance under two conditions. In condition 1, the chromosomal copy of *mgrR* was deleted and MgrR was expressed for 15' from an induced plac-*mgrR* plasmid and compared to cells carrying a vector induced for the same period. We estimate that levels of MgrR under these conditions were about 5-fold higher than that made from the chromosome (Fig. S4). In condition 2, the expression of mRNAs was compared in wild-type cells ($mgrR^+$) and the *mgrR* deletion strain, both grown in LB to an OD_{600} of 0.5. Because MgrR levels are fairly high under our normal growth conditions (Fig. 1C), this allowed analysis of both the direct and indirect (long-term) effects of MgrR. In both sets of experiments, cells were grown in LB at 37°C; each experiment was carried out twice. The array signals for all genes are available at the following URL (to be included upon acceptance).

Only genes where the expression level for either condition 1 or condition 2 was reproducibly changed more than 2-fold between $mgrR^+$ and $mgrR^-$ conditions in duplicate experiments were considered significant, even if some inconsistency was observed between the actual values in the duplicate experiments or two different conditions. The 20 negatively regulated and 61 positively regulated genes that pass these filters are listed in Table 2 (with the expression levels for these genes in Table S1) and Table S2, respectively. In general, many more positively regulated genes showed >2 fold change only in the long-term growth experiment (condition 2); thus, many of these changes may reflect indirect effects. While some experiments were done to examine these changes, further work was focused on the apparent direct effects, and therefore focused on the negatively regulated genes.

Genes negatively regulated by MgrR

Of the 20 genes listed in Table 2, MgrR overexpression led to the most dramatic decreases in expression for *eptB* (b3546) and *ygdQ* (b2832) mRNAs, with an 8–9 fold decrease in mRNA for *eptB* and greater than 9-fold changes for *ygdQ* (Table 2). *eptB* encodes a phosphoethanolamine transferase, which modifies LPS at the outer KDO residue with phosphoethanolamine (Reynolds *et al.*, 2005). *ygdQ* encodes an uncharacterized inner membrane protein (Rapp *et al.*, 2004), with homology to the TerC family of integral

membrane proteins implicated in export of tellurium ions. The expression of these two genes was also observed to increase in Δ*mgrR* cells, compared to wild-type, although the fold difference was less dramatic (Table 2). Since the expression of MgrR is activated by PhoP, we also examined whether the expression of *eptB* and *ygdQ* were altered in previously published *phoP*$^{-}$ microarray profiles. The expressions of these two genes were modestly up-regulated in *phoP*$^{-}$ mutants in one microarray data set (approximately 2 fold difference for each) (Oshima *et al.*, 2002). These observations indicated that *eptB* and *ygdQ* were possible direct target genes for MgrR. This was confirmed (see below).

Of other genes that showed decreased expression after MgrR overexpression, effects were generally close to the 2-fold cut-off. Examination of the mRNAs of some of these genes by Northern blot (*mscC, pitA, yniC,* and *ndh*; data not shown) did not show a detectable change in abundance upon MgrR overexpression and were not further explored. Others showed changes only in the comparison of wild-type to Δ*mgrR* (for instance, *pdhR, ndh, ydgI* and *yfiD*) and not in the MgrR overexpression experiment. We assume the effects in these cases are indirect, although the mechanism of this indirect regulation is not currently known.

An intriguing observation was that some Hfq-binding small RNAs were modestly down-regulated when MgrR was present. For instance, the expression of GlmZ (*ryiA*) was decreased when MgrR was overexpressed. Spot42 (*spf*) levels were lower in wild-type strains than in the *mgrR* deletion, and in one of two experiments, decreased when MgrR was overexpressed. This has not been further explored in this paper. It is worth noting that these effects might not have been noted with earlier Affymetrix *E. coli* microarray experiments in which the sRNAs were not specifically annotated and therefore were more difficult to track.

Positively regulated genes

Table S2 lists genes with increased mRNA signals in conditions where MgrR was present, by comparison to the deleted strain, and/or genes in which mRNA signals increased upon MgrR overexpression. The strongest positive effects, including a number of operons (*dppABCDF*, *lldPRD*, and *paaABCDEFGHIJK*) and other co-regulated genes (*putA* and *putP*) were seen in comparing the wild-type to the *mgrR* deletion (long-term effects), and only a few of these showed any consistent increase under overexpression conditions; this is consistent with indirect effects of MgrR, or possibly with moderate changes in mRNA stability, more apparent over longer times.

Among these possible targets, *nanC* and *lldP* expression were tested by Northern blot, but the mRNA could not be detected under our growth conditions (data not shown). *nanC* regulation was also examined by assaying β-galactosidase activity in wild type and Δ*mgrR* strains carrying a *nanC-lacZ* translational fusion; this fusion was driven by the pBAD promoter (see Materials and Methods). However, no significant differences in activity of the fusion were observed when MgrR was present compared to its absence (data not shown). We also found no significant differences in the expression of *rpoS-lacZ* transcriptional and translational fusions between cells expressing or missing MgrR (data not shown).

Confirmation of specific targets

The microarray results suggested that *eptB* and *ygdQ* were the two major direct targets of MgrR negative regulation. Northern blot analysis was performed with the Δ*mgrR* cells containing either pBR-Plac-*mgrR* or the pBR-Plac vector control. The message for *eptB* was present before MgrR induction, and disappeared within 2 min in the presence of MgrR (Fig. 5B). The same pattern was observed for *ygdQ* (Fig. 5B); this rapid disappearance of message is consistent with direct regulation by MgrR. Since the expression of MgrR from the chromosomal copy is abundant during growth in LB, we also compared the expression

of *eptB* and *ygdQ* by Northern blot analysis in *mgrR*$^+$ and *mgrR::kan* cells (Fig. 5C). Both the *eptB* and *ygdQ* messages were undetectable in the *mgrR*$^+$ cells, but could easily be detected in *mgrR::kan* mutant cells, indicating that MgrR down-regulates the message of these two genes (Fig. 5C), and suggesting that they are transcribed at reasonable levels in wild-type cells but then destroyed after interaction with MgrR.

The 5' end for both genes was determined; *eptB* has a leader of 106 nts and *ygdQ* has a leader of 26 nts. Mfold predicted pairing between the highly conserved area in MgrR and both target genes (Fig. 5A). For *eptB* mRNA, the possible pairing with MgrR was close to the ribosomal binding site and included the ATG start codon; this region of *eptB* and the potential pairing is conserved in *Salmonella* and *Enterobacter* (Fig. S5). Possible base pairing was observed inside of the coding region of *ygdQ* (between +8 to +18 from the start codon), a part of the gene that is highly conserved among various species (Fig. S5). Pairing early in the coding region has been observed in other cases and shown to interfere with ribosome binding (Bouvier *et al.*, 2008)

As mentioned above, MgrR was less stable in the absence of Hfq (Fig. 1C), and consistent with other Hfq-bound sRNAs, we would expect MgrR to be unable to act in the absence of Hfq. Consistent with this, Figueroa-Bossi *et. al.* (2006) reported that the β-galactosidase activity of an *eptB-lacZ* translation fusion was increased in *hfq*$^-$ cells, although much of this increase could be ascribed to activation of the sigma E response (see below; Figueroa-Bossi *et al.*, 2006). The fate of the *eptB* and *ygdQ* messages was directly examined in the absence of Hfq. Northern blot showed that the messages of both *eptB* and *ygdQ* were increased in the *hfq*$^-$ cells, comparable to that seen in an *mgrR* mutant (Fig. 5D). *phoP* mutants, in which *mgrR* is not transcribed (Fig. 2), also showed elevated levels of both *eptB* and *ygdQ* (Fig. 5D).

The *mgrR* deletion mutant is more resistant to the antimicrobial peptide Polymyxin B

mgrR is regulated by PhoQ/PhoP, which are known to positively regulate genes involved in modification of LPS, resulting in reduced sensitivity of cells to antimicrobial peptides (reviewed in (Gunn, 2008). Interestingly, we have found here that one of the genes negatively regulated by PhoQ/PhoP, via MgrR, is *eptB*, also involved in LPS modification, putting a phosphoethanolamine onto a terminal KDO (Reynolds et al., 2005). We examined the role of *mgrR* and *eptB* in polymyxin B killing. Cells were grown to OD_{600} of 0.5, challenged with a final concentration of 4ug/ml of Polymyxin B for one hour, and plated for surviving cells. Although none of the strains was very resistant to this treatment, the *mgrR* mutant was 10 times more resistant than the wild type (Fig. 6A). The *eptB* deletion mutant showed a similar sensitivity to the wild type. A double *eptB mgrR* mutant strain had the sensitivity of the wild-type strain, consistent with *eptB* as the major target of MgrR, at least with respect to sensitivity to Polymyxin B. Complementation of the *eptB mgrR* double mutant with a plasmid expressing *eptB* restored the higher-level resistance seen in the *mgrR* mutant alone (Fig. 6B). In other experiments, complementation of the *mgrR* mutation, either from its own promoter (Fig. 6C) or from an inducible Plac promoter (data not shown), restored Polymyxin sensitivity.

The *eptB*-dependent change in polymyxin sensitivity presumably reflects LPS modification in the *mgrR* mutant. We wondered whether such a modification might change sensing of external Mg^{2+} and/or antimicrobial peptides, therefore changing signaling to the PhoP/PhoQ regulon. To assess this, Real time PCR signals for various PhoP-regulated genes were compared in a wild-type and a *mgrR*$^-$ strain, at various Mg^{2+} concentrations. At moderate Mg^{2+} concentrations (10 μM), the *mgtA* and *mgrB* RNA levels were slightly higher in the *mgrR* deletion strain than in wild-type (Fig. S6), suggesting that loss of *mgrR* and the resultant expression of *eptB* decreases Mg^{2+} levels seen by PhoQ.

Discussion

The PhoQ/PhoP two-component system plays a major role in pathogenesis in *Salmonella* and *E. coli* (Bishop *et al.*, 2000, Groisman, 2001, Miller *et al.*, 1989). The regulon is generally activated under low Mg^{2+} conditions or by antimicrobial peptides, and has previously been shown to include genes involved in magnesium transport (for instance, *mgtA* (Soncini *et al.*, 1996)) and genes leading to modification of LPS, increasing resistance to antimicrobial peptides (Ernst et al., 2001). Here we find that a novel Hfq-binding sRNA, MgrR, is part of the PhoQ/PhoP regulon, and that it acts to down-regulate a gene for an alternative LPS modification, under low to moderate Mg^{2+} growth conditions.

Regulation of MgrR by PhoQ/P expands the list of global regulators of sRNAs in *E. coli*. Among these are Crp and cAMP, regulating CyaR (De Lay & Gottesman, 2009) and Spot 42 (Polayes *et al.*, 1988), the EnvZ/OmpR two-component system, regulating OmrA/B (Guillier *et al.*, 2006), σ^E for MicA and RybB (Figueroa-Bossi *et al.*, 2006, Johansen *et al.*, 2006, Papenfort *et al.*, 2006, Thompson *et al.*, 2007), OxyR regulated OxyS (Altuvia *et al.*, 1997), GcvA, regulating GcvB (Urbanowski *et al.*, 2000), and the RcsC/D/B phosphorelay for RprA (Majdalani *et al.*, 2002). This finding reinforces the idea that most major regulons are likely to include an sRNA.

In addition, our findings extend the observations that Hfq-regulated sRNAs have significant roles in regulating the cell surface. Past studies have demonstrated effects of sRNAs in negatively regulating a large number of outer membrane proteins (reviewed in (Guillier et al., 2006). The work presented here provides the first demonstration of a role of sRNAs in regulating LPS modification.

Response of the *mgrR* promoter to Mg^{2+} levels

PhoP, a response regulator, is phosphorylated by PhoQ and binds to the direct repeats (G/T)GTTTA(A/T) separated by a 5 nucleotide spacer. The location of these repeats relative to the transcription start site can vary, as can the degree of similarity to consensus. We found that the *mgrR* promoter differs from other PhoP dependent promoters, such as *iraM*, *yneM*, and *mgrB*, in that it is expressed even at fairly high [Mg^{2+}] concentrations. For these other promoters, the [Mg^{2+}] providing the half-maximal level of each transcript ([Mg^{2+}]$_{50\%}$) was less than 10μM. On the other hand, the expression level of MgrR remained high in 2mM Mg^{2+} and the [Mg^{2+}]$_{50\%}$ was approximately 5mM. The simplest explanation for our finding is that low stimulus or alternative pathways are able to activate the *mgrR* promoter; the incomplete dependence upon PhoQ (Fig. 3C) is consistent with this idea. The ability to respond to PhoP at relatively high Mg^{2+} levels was not due to the extended −10 (Table 1). Rather, the presence of the extended −10 contributes to robust synthesis of MgrR RNA under inducing conditions. In addition, we found that the sequence for the promoter of *Salmonella enterica mgrR* behaves similarly to that seen in *E. coli*, in terms of general levels of expression and dependence on PhoP, strongly suggesting that MgrR will be similarly regulated in *Salmonella*.

Miyashiro *et al.* (2007) recently reported that *mgrB* and *phoP* showed identical transcriptional profiles at steady state, but that *mgtA* was different, being higher than others at lower stimulus. While we saw rapid repression of *mgtA* ORF mRNA as Mg^{2+} levels rose, their general conclusions that the differences in the transcriptional profile might be due to the lower dissociation rate of PhoP-P from some promoters, and also that sequences adjacent to the consensus PhoP box could change the activity are consistent with our observations. We have not determined why our results for *mgtA*, in terms of sensitivity to Mg^{2+} levels, differs from those of Miyashiro *et al*, but we note that they measure the activity of a promoter fusion, while we are measuring the mRNA, which includes a Mg^{2+} - responsive

riboswitch (Cromie *et al.*, 2006). Thus, while the promoter may be fairly permissive for high levels of Mg^{2+}, the riboswitch, measuring internal Mg^{2+}, provides a second level of tight regulation.

Have *mgrR* and *yneM* been co-evolving?

The small protein YneM is encoded in the same intergenic region with *mgrR* but is made from the opposite strand. Hemm and coworkers (Hemm *et al.*, 2008) reported that *yneM* encodes a small hydrophobic protein (31 amino acids) of unknown function and predicted it is a transmembrane protein.

Based on the 5' RACE of a sample from exponential phase cells, the transcript of *yneM* was 210 nt in size. This agrees with the longer of two transcripts described by Wassarman *et al.* (2001). The 5' UTR and coding regions of *yneM* were relatively conserved among various species (Fig. S3). In some of these species, the genes outside of the *yneM/mgrR* region vary (Fig. S1). Nevertheless, *yneM* and *mgrR* have remained adjacent in these species, suggesting that these genes may have coevolved.

This observation led us to ask whether the expression of *yneM* could be regulated by MgrR or in parallel to *mgrR*. We found that the regulation of *yneM* is independently activated by PhoP (Fig. 3C, Fig. S3). In fact, another possible PhoP box with a half repeat of the conserved sequences was observed in the region upstream of the *yneM* transcription start site (Fig. S3). However, the pattern of expression at different Mg^{2+} concentrations was rather different (Fig. 4), and may help to explain observations in *Salmonella* (Sittka et al, 2008), where the RNA levels for these two genes was reciprocal (*yneM* mRNA was highest under conditions in which the *mgrR* transcript was low, late in stationary phase). We found no evidence that *yneM* affected sensitivity to Polymyxin B. The *mgrR* deletion phenotype can be complemented by a plasmid expressing only *mgrR*, and a deletion of both *yneM* and *mgrR* had a similar phenotype, also complemented by expression of MgrR alone (data not shown). However, while the role of YneM remains to be defined, the retention of these two linked genes in many organisms, both regulated by PhoQ/PhoP, suggests that these two genes may have co-evolved as functionally related elements of the PhoQ/PhoP regulon.

Physiological role of MgrR

The only target of MgrR with a known function that we have confirmed is *eptB*. Reynolds *et al.* (2005) reported that EptB utilizes phosphatidylethanolamine (PE) as its donor substrate, and modifies the 3-deoxy-D-manno-octulosonic acid (Kdo) residue of LPS. The addition of a phosphoethanolamine (pEtN) moiety to the Kdo residue of lipopolysaccharide (LPS) reduces the net anion charge of molecules and decreases electrostatic repulsion between neighboring LPS molecules, thus eventually leading to polymxyin B resistance (Baker *et al.*, 1999, Gunn *et al.*, 2000, Helander *et al.*, 1996). The sequence of this gene is similar to PmrC (EptA) in *Salmonella* (Lee et al., 2004). PmrC in *Salmonella* is positively regulated indirectly by PhoQ/P and has been shown to modify LPS, and, consequently, to provide resistance to antimicrobial peptides, such as polymyxin B.

In this study we found that MgrR negatively regulates *eptB*, and a deletion of *mgrR* increases polymxyin B resistance. Thus, while other PhoQ/P regulated genes lead to increased resistance to antimicrobial peptides (Bader *et al.*, 2005, Shi *et al.*, 2004), *mgrR* has the opposite effect. This raises a number of questions to which we do not yet have the answers.

The first question is whether there are conditions under which *eptB* mRNA is not destroyed by MgrR. For instance, if transcription of *eptB* is increased, we might expect it to overcome MgrR negative regulation, leading to modification of LPS. It has previously been reported

that an *eptB* translational fusion is regulated as part of the sigma E regulon (Figueroa-Bossi et al., 2006), although in another study an increase in transcription was found when the sigma E regulon was induced, but no sigma E promoter was found (Rhodius *et al.*, 2006). Our alignment of the *eptB* promoter, based on the start site that we determined, also is inconsistent with direct regulation by sigma E; sequences show a conserved extended −10 typical of a sigma 70 promoter (Fig. 2), and assays of a *eptB* promoter fusion also failed to yield evidence of sigma E regulation (data not shown). These results suggest that the effect of sigma E may be indirect, occurring at some point past transcription initiation. Nonetheless, the results of Figueroa-Bossi et al strongly suggest that under conditions of sigma E activation, *eptB* is expressed, and thus the EptB-directed modification may be important under these conditions.

A second question is why this LPS modification is detrimental under growth conditions where PhoP/Q is active (when MgrR is suppressing *eptB* expression). Possibly this LPS modification interferes with other modifications. A possible role for this modification in signaling is provided by our observation that cells act as if they see somewhat reduced Mg^{2+} concentrations when *mgrR* is deleted (Fig. S6). Thus, in the transition from low signal (MgrR on, PhoQ/PhoP regulon on, *eptB* off) to high signal (MgrR and PhoQ/PhoP regulon all off), MgrR may help to set the transition point by regulating modification of the cell surface.

Consistent with a broader role for MgrR are the many changes seen in the microarrays in a comparison of the wild-type strain with a *mgrR* deletion strain (Table 2 and Tables S1 and S2). Among these, three of the negatively regulated genes (*pdhR*, *ndh* and *yfiD*) are known to be negatively regulated by PdhR (Buck & Guest, 1989,Ogasawara *et al.*, 2007), suggesting that a change in PdhR levels or activity may be found in *mgrR* mutant cells. Many of the positively regulated targets are genes that respond to various carbon sources and nutrients, possibly suggesting a change in preferred nutrient sources and/or a change in ability of these nutrients to be sensed during growth in LB in *mgrR* mutants. While we can show that *eptB* is necessary for the Polymyxin B resistance phenotype, it is not yet known whether *eptB*, *ygdQ*, or some other target(s) are important for the indirect effects of an *mgrR* deletion. This will be a useful future direction for research.

Experimental procedures

Bacterial strains and plasmids

Strains and plasmids used in this study are listed in Table 3. The recipient strain for standard cloning procedures was DH5 α. Antibiotic concentrations (in micrograms per milliliter) were as follows: ampicillin on plates, 50; ampicillin in liquid cultures, 100; kanamycin 25; chloramphenicol, 25. The chromosomal mutations Δ*mgrR*::*kn* and Δ*eptB*::*cm* were constructed using the λ Red recombination system (Yu *et al.*, 2000). A PCR fragment was obtained by amplifying either the kanamycin resistance cassette of the strains MC4100 *ybeW*::GBKn cassette (J.M. Ghigo, Institut Pasteur, Paris, France) or the chloramphenicol cassette of the strain TKC (D. Court, NCI, NIH). To amplify the cassettes, the forward (F) and reverse (R) primers listed in Table S3 were used with the Expand High Fidelity PCR System (Roche). The resulting PCR products were recombined into the chromosome of strain KM62, an MG1655 derivative carrying the mini- λ prophage encoding the λ Red functions (Court *et al.*, 2003) for the constructions with the kn cassettes (Spk knF and Spk knR). The transformed cells were selected on LB plates containing 25 μ g ml-1 kanamycin at 32°C. Recombinant products were verified by PCR and the mutations were transferred into MG1655 by P1 transduction.

Strains carrying *lacZ* transcriptional fusions were constructed using the PM1205 system (Mandin & Gottesman, 2009), which contains the P_{BAD}-*catsacB* segment upstream of *lacZ* at the chromosomal *lacZ* site. First, the PCR products were generated to amplify the PCR products having the sequences either from −60 to +10 or −35 to +10 of MgrR (see primers listed below). For a transcriptional fusion to yneM, the primers yneM 75upF and yneM LacZr (Table S3) were used to amplify −75 to +88 of *yneM*. For a translational fusion, the 5' primer (pBADyneMF) was homologous to the upstream region of P_{BAD} to allow recombination. The downstream primer was yneM_lacZTLr. Each PCR product was recombined into the chromosome of strain PM1205 by lambda Red recombinase-mediated gene replacement, selecting for sucrose resistance and screening for loss of chloramphenicol resistance, to switch P_{BAD}-*catsacB* with the different regions of the *mgrR* promoter, resulting in KM112 (primers 60up Spk F and Spk lacZ R; locations from −60 to +10) and KM113 (primers 35up Spk F and Spk lacZ R; locations from −35 to +10). To introduce the *mgrR* promoter region of *S. enterica* into the *E. coli* reporter fusion, the PCR product having the sequence from −60 to +10 of *mgrR* and was amplified using primers Styphi Spk 60upF and Styphi Spk lacZR from *S. enterica* genomic DNA (provided by Jim Slauch, U. of Illinois) and introduced into PM1205 as described above. To mutate the extended −10, the PCR product was amplified using the genomic DNA from KM112 (primer 60up Spk F) and a reverse primer (Spk Ex10 lacZR) which contained the mutated nucleotides (TG→AC). In order to construct the P_{BAD}-*nanC*-*lacZ* translational fusion, the PCR product harboring the sequence from +1 to +51 of *nanC* under P_{BAD} was amplified using primers pBAD nanCF and nanC_51 lacZR, resulting in 40 nt homologous to the P_{BAD} promoter at the 5'end. The PCR product was introduced into the PM1205 chromosome as described above.

For constructing Plasmid pGFK1013, a region of approximately 250bp region, including the *mgrR* promoter, was amplified with primers Spk MscI F and Spk EcoRI R, using the Expand High Fidelity PCR System (Roche). The PCR fragment was then cloned into pCR2.1 (Invitrogen) based on the manufacturer's protocol. pGFK1018 was constructed by PCR amplification of *mgrR* from strain MG1655 using the Spk AatII F and Spk EcoRI R primers and subsequently cloning the PCR product into the pBR-Plac vector digested with AatII and EcoRI. To construct pGFK1024, the coding region of *eptB* was amplified by PCR (high fidelity PCR system; Roche) using a forward primer, eptB_EcoRSD F, containing an EcoRI site and Ribosome binding sequence (AGGAGG) and a reverse primer eptB-HindR2. The PCR product was cloned into the pBAD30 vector digested with EcoRI and HindIII. The transcript of *eptB* from pGFK1024 was checked by Northern blot.

RNA Half-life determination

Cells were grown at 37°C to an OD_{600} of ≈ 0.7 in LB media. Rifampicin stock solution in dimethyl formamide was added to a final concentration of 300 μg ml^{-1} (Majdalani *et al.*, 2001). Total RNA was extracted from 1 ml aliquots of culture taken 0, 2, 4, 6, 8, 10 and 20 min after the addition of rifampicin. To examine the MgrR turnover rate by washing out an inducer, MgrR was first induced from pBR-Plac derivative with IPTG at 37°C. After 15 min induction, the inducer was removed by filtration with prewarmed LB medium and cells were transferred into fresh medium without IPTG. The samples were collected at 0, 2, 4, 6, 8, 10 and 20 min after the wash for RNA extraction. Northern blot analyses were performed using an MgrR probe and the quantification of the signal was performed with Multi Gauge software (Fuji).

Hfq immunoprecipitation

Immunoprecipitations were carried out as previously described (Wassarman, 2002) using rabbit antisera against Hfq. Typically, 20 μ l of extract, 0.1–5 μ l of serum, and 2–5 mg of protein A Sepharose (CL-4B, Pharmacia) were used and extract was incubated with protein

A Sepharose antibody pellets overnight at 4°C. After immunoprecipitation, RNA was extracted by phenol:Chloroform:IAA followed by ethanol precipitation.

Beta-galactosidase assays

The β -galactosidase activity of strains carrying *lacZ* fusions was assayed on a SpectraMax 250 (Molecular Devices) microtitre plate reader as described previously (Majdalani *et al.*, 1998). Specific activities are represented as the Vmax divided by the OD600, and these units are about 25 times lower than standard Miller units.

For starvation experiments, β -galactosidase assays were as described previously (Bougdour *et al.*, 2008). Briefly, cells were grown overnight in M9 minimal medium containing 2 mM $FeSO_4$ and 100 μ M $CaCl_2$ (complete M9 medium), then subcultured into fresh complete medium at an OD600 of 0.01 (~1:400 dilution) and grown to mid-logarithmic phase (OD600 of ~0.3). The cells were then washed twice by filtration with prewarmed (37°C) starvation medium, re-suspended in the same volume of starvation medium, and incubation at 37°C was continued as indicated. As a control, a sample of the culture was kept in complete M9 medium and grown as indicated (with Mg^{2+}/Ca^{2+}). The results report the average of three experiments.

5' RACE

5' RACE was performed by using the method described by (Argaman *et al.*, 2001). One microgram of RNA was ligated to the 5' universal RNA adapter (5'-GAU AUG CGC GAA UUC CUG UAG AAC GAA CAC UAG AAG AAA-3') and was reverse transcribed with Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The cDNA was then amplified by PCR using the RACE universal primer (RACE Universal F) and the *mgrR* specific primer Ig957 302R. The PCR products with or without TAP (tobacco acid pyrophosphatase) treatment were checked on a 1.8% Agarose gel. The PCR product was sequenced with the *mgrR* specific primer, SPK 302R. The PCR products were then TOPO cloned into the pCR2.1 (Invitrogen), and the resulting plasmids were transformed into Top10 cells. Plasmids containing the amplified cDNA from the tobacco acid pyrophosphatase-treated RNA were sequenced using the M13For (−20) primer supplied with the TOPO cloning kit (Invitrogen).

Northern Blot

To examine the effect of MgrR from a multicopy vector, cells were grown in LB medium supplemented with ampicillin at 37°C to an OD600 of about 0.5. MgrR was induced from pBR-plac derivatives using 100 μM IPTG and cells were collected at 0, 2, 5, 10, 20 and 30min. To examine chromosomally expressed MgrR, cells were grown in LB at 37°C and collected at different ODs as indicated.

For the effect of different [Mg^{2+}], cells were grown in M9 minimal medium described previously (Bougdour *et al.*, 2008) containing 2mM Mg for overnight growth. Cells then were diluted about 400-fold into fresh M9 minimal medium containing 2 mM $MgSO_4$ and 100 μ M $CaCl_2$ and grown to mid-logarithmic phase (OD600 of ~0.3). Cells were washed twice with medium with different [Mg^{2+}] ([Ca^{2+}] remained constant) followed by transfer into fresh medium with the designated [Mg^{2+}].

Total RNA was extracted at different time points by the hot-phenol method as previously described (Masse *et al.*, 2003) using 650 μl of culture mixed with 94 μl of lysis solution and 750 μl phenol/water. After precipitation with ethanol, RNA was resuspended in DEPC-water and its concentration was determined by measuring the OD at 260 nm. Analysis of MgrR by Northern blot was performed with 5 μg total RNA separated on an 10% urea-

acrylamide sequencing gel in 1 × TBE at 75 V for about 2 hour and 30 min and transferred onto a positively charged nylon membrane at 200 mA for 1 hour and 30 min. When RNA was extracted from cultures in minimal medium, only 2μg total RNA was used to visualize MgrR. For *eptB* and *ygdQ* mRNA analysis, 15 μg total RNA was loaded on a 1.2 % MOPS agarose gel and transferred onto a positively charged nylon membrane as in Massé & Gottesman, 2002). Detection was as above with the corresponding biotinylated probes.

Microarrays

For condition 1, strain KM132, transformed with pBR-*plac-mgrR* or the empty vector pNM12, was grown overnight at 37°C in LB containing Ampicillin. IPTG was then added at a final concentration of 100uM for 15 min and RNA was extracted as described above using 800μl of culture. The expression level of MgrR was verified by Northern blot. The induction of MgrR from the inducible promoter, pBR-plac, was 5 fold higher than the expression from the chromosomal copy (Fig. S4). For condition 2, fresh colonies of the wild-type strain (NM22540) and the Δ*mgrR* mutant (KM129) were inoculated into LB and grown to OD600 0.5. The total RNA was extracted, processed, and assayed on Affymetrix arrays. After removing genomic DNA, RNA was phenol-extracted, precipitated with ethanol and resuspended in DEPC-water. After a new quantification, 15 μg RNA was used for cDNA synthesis, fragmentation, labeling and subsequent hybridization to the *E. coli* 2.0 Arrays (Affymetrix) following the manufacturer's instructions. Each experiment was done in duplicate.

Data analysis was done as previously described by Massé *et al.* (Massé *et al*., 2005). In this study, genes whose expression level were indicated as “absent” under all conditions by Affymetrix Microarray Suite 5.0 software were discarded. The genes whose expression patterns changed by two-fold or more either in both experiments comparing Plac-mgrR plasmid to vector or in both experiments comparing wild type to deletion mutant were included in Table 2 and Tables S1 and S2 for further consideration.

Real time RT-PCR analysis

To determine more quantitatively the expression of each PhoP-dependent gene at various Mg^{2+} concentrations, real-time RT-PCR was performed on RNA obtained from both the wild type MG1655 and *mgrR* deletion strain (KM201) grown in M9 minimal medium at various Mg^{2+} concentrations as described above. After Turbo DNase treatment to remove genomic DNA contamination, RT-PCR analysis was performed using SsrA as an internal standard. RT-PCR products were visualized on 2% agarose gels.

From the purified RNA, cDNA was generated using 1 μg of RNA in a total volume of 20 μl plus random hexamers [d(N)6; New England Biolabs (NEB)] as primers and the Moloney murine leukemia virus reverse transcriptase (NEB) (Moon *et al*., 2005).

Real-time PCR was done using an iQcycler (Bio-Rad). Expression of the SsrA gene was used as an internal standard, and SYBR Green Supermix was used as a signal reporter. Reactions were done in a 96-well microtiter PCR plate using 5 μl of cDNA and final concentration 0.4 μM sense and antisense primers for amplifying SsrA, *mgtA, mgrB, iraM, phoP, yneM* and MgrR, 3 μM $MgCl_2$, and 1× iQ SYBR Green Supermix (Bio-Rad). Cycling conditions were as follows: denaturation (95°C for 3 min), amplification and quantification (95°C for 30 s, 55°C for 25 s, and 72°C for 30 s, with a single fluorescence measurement at both 55°C and 72°C for 30-s segments) repeated 40 times, a melting curve program (55 to 95°C with a heating rate of 0.1°C/s and continuous fluorescence measurement), and a cooling step to 50°C. Each sample was tested in triplicate.

Data were analyzed using the iQcycler analysis software (Bio-Rad). The expression level of each sample were obtained by the standard curve for each gene and was normalized by the level of the internal control, SsrA. The result is shown as the percentage of the expression level of the sample without any Mg^{2+} in the medium.

Polymxyin B Survival assay

Each strain was inoculated in fresh LB medium and grown to an OD600 of ~0.5 at 37C. The cultures were challenged with polymyxin B (Sigma-Aldrich) at a final concentration of 4ug/ml, incubated at 37°C with shaking at 225 rpm for 1 h, and plated on LB plates after serial dilutions. After overnight incubation, the surviving colonies were counted. The percentage survival was calculated as follows: survival (%) = cfu of surviving cells /cfu of initially challenged cells × 100. Each experiment was repeated at least three times.

Acknowledgments

We thank members of the Gottesman lab for advice and materials. We thank A. Battesti, P. Milanesio, P. Mandin, N. De Lay, N. Majdalani and G. Storz for comments on the manuscript. This research was supported by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.

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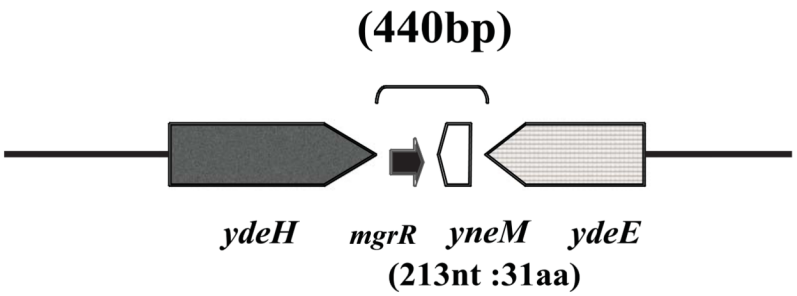
A.



B.

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+1

AC**G**ATTCGTTATCAGTGCAGGAAAATGCCTGTTAGCGTAAAAGCAAAACACAAATCTATCCATGCAAGC

ATTCACCGCCGGTTTACTGGCGGTTTTTTTTCGCCGTCATAAAAATCAGGCCCCTTGTACACAACTGTA

ACAAGGGGCCGGTTAGGTGAGGGATTATCTCCGTTCA*TTA* GTCATCCCATTTGTGGCTGAAATACGCG

yneM stop codon

GCCAGAAAACCAGAAAATAAAATTATTCCCAGTACGGCCATAAAAACATTCATATTACCCAG *CAT* TATT T

yneM start codon

+1

GCCTCCGTTTATGGTGTGGTGTGTTTCCTCGCTTACC TTAATTTTCATCTCA **A**TGAGAAAGATA TTAGCA

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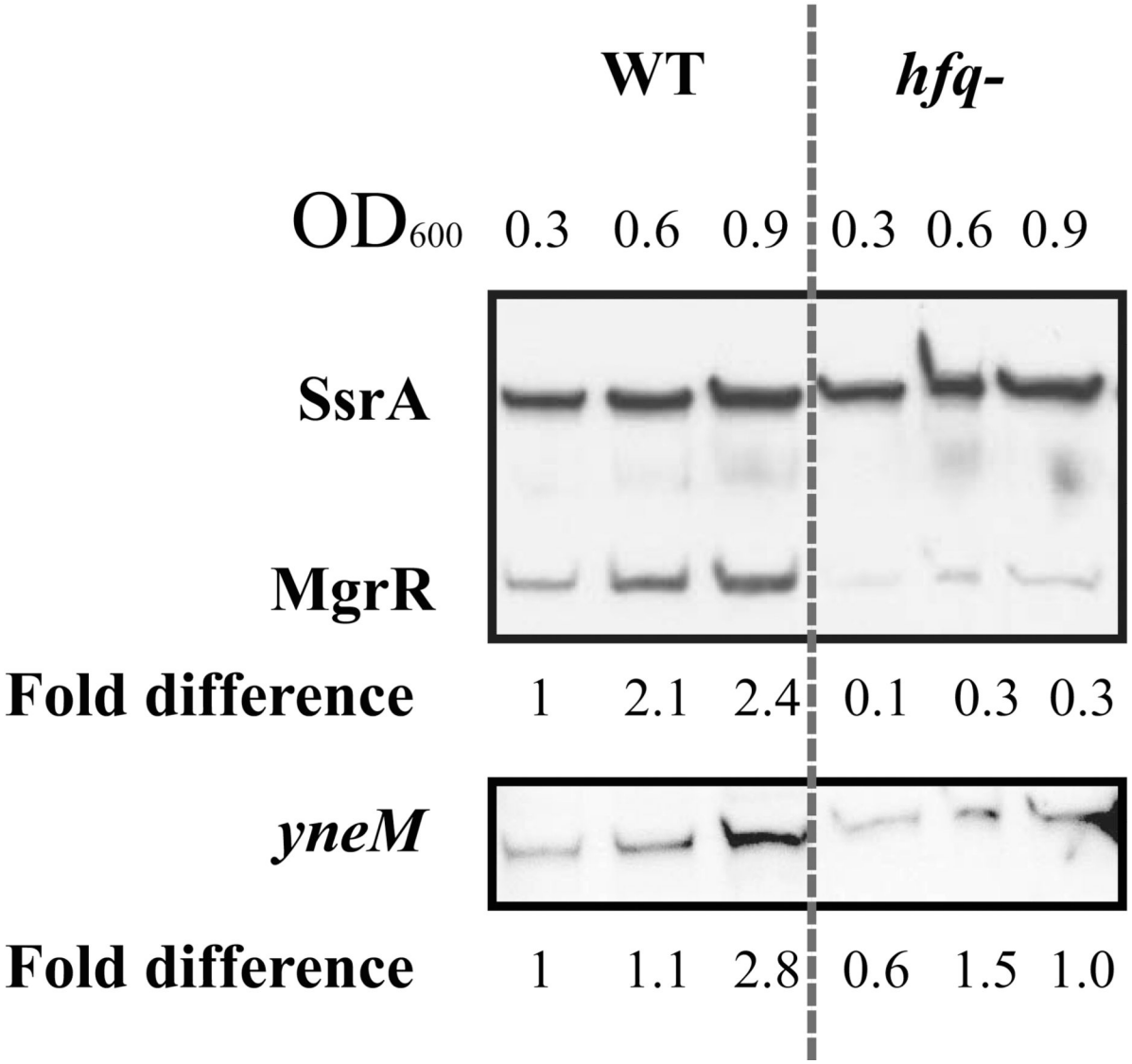

Figure 1. The organization of *mgrR* and neighboring genes and the sequence of the intergenic region

A. The intergenic region between *ydeE* and *ydeH* contains both *yneM* and the gene for the non-coding RNA MgrR (dark gray arrow). *yneM* encodes a hypothetical protein of 31 amino acids. Note that these genes are all shown in inverted order to that found in the *E. coli* chromosome, to make it easier to see the *mgrR* gene organization.

B. The underlined GTTTA repeat in bold italics is a possible PhoP Box for *mgrR* and the underlined sequence in bold represents a possible PhoP box for *yneM*. The boxed sequence is a predicted extended −10 region for *mgrR*. Underlined G represents the transcriptional start site for *mgrR* and underlined A the start site for *yneM*. Black arrows and dashed arrows

represent the inverted repeats of the rho-independent terminators for *mgrR* and *yneM* respectively. The half bracket shows the gap between the predicted ends of the *mgrR* and *yneM* transcripts.

C. Expression of MgrR and *yneM*. Samples were collected from wild type (MG1655) and *hfq*$^{-}$ strains at different OD_{600} as indicated. 5ug of total RNA was loaded in each lane and 5' biotinylated Spk probe and Ig957-88R were used to detect MgrR and *yneM* transcripts, respectively; SsrA (tmRNA) was used as an internal control.

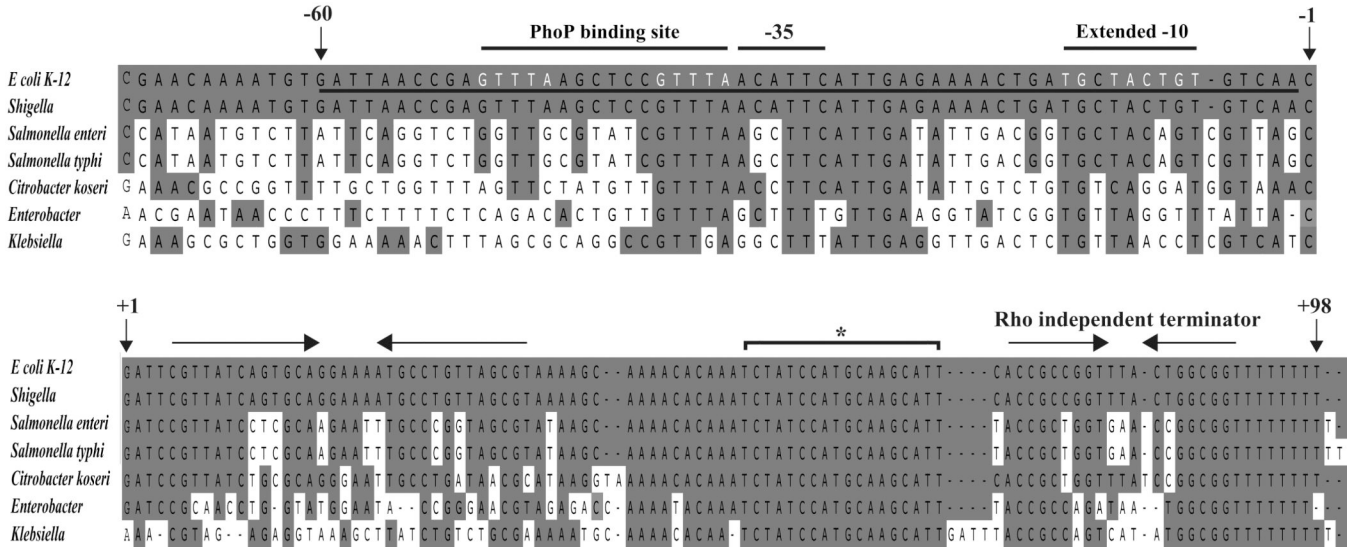

Figure 2. Conservation of *mgrR* and its regulatory regions

Alignment of *mgrR* and upstream sequence from various *Enterobacteriacae*. Sequences identical to the *E. coli* K12 sequence are shaded in gray. The predicted PhoP binding site in *E. coli* K12 is in white type and the extended −10 is also in white; the conserved sequence within the spacer (CATTGA) may also contribute to regulation. The asterisk (*) indicates the highly conserved sequences among various gammaproteobacteria and the arrows show the inverted repeats within MgrR.

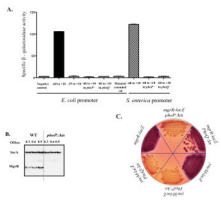

Figure 3. Transcriptional Regulation of *mgrR* by PhoP

A. β-galactosidase assays were performed with different fusions either in the wild type background (KM112, derived from PM1205), the *phoP*$^{-}$ background (KM114), or *phoQ*$^{-}$ background (KM195). The mutated extended −10 (ACcTACTGT) was introduced into the wild type background, generating the strain KM188. The *S. typhi mgrR* promoter was also introduced into either the wild type background, phoP^{-} background, or *phoQ*$^{-}$ background, resulting in strains KM187, KM189, and KM196, respectively. Cells were grown in LB at 37°C and 100μl samples were taken at mid-log phase (OD_{600} = 0.6) to measure enzyme activity. The negative control represents the background strain without any promoter region (PM1205). The results reported are representative of at least three experimental trials. Error bars indicate standard deviations.

B. Northern blot analysis of wild type (MG1655) and *phoP*$^{-}$ (EG12976) cells grown in LB at 37°C. Cells were harvested at each OD_{600} point as indicated. SsrA (tmRNA) was used as an internal control.

C. *mgrR-lacZ* and *yneM-lacZ* fusion strains in *phoP*$^{-}$ and *phoQ*$^{-}$ mutants. For full length *mgrR-lacZ* fusion in different backgrounds, KM112, KM114, and KM195 were used. For full length *yneM-lacZ* fusion, sequence from −75 to +88 relative to the *yneM* start site was introduced into PM1205 (See Methods and Materials), generating transcriptional fusion strain KM194. Then, either *phoP* or *phoQ* null mutations were introduced by P1 transduction, generating KM197 (*phoP*$^{-}$) and KM198 (*phoQ*$^{-}$). Each strain was streaked on MacConkey plates and incubated overnight at 37°C.

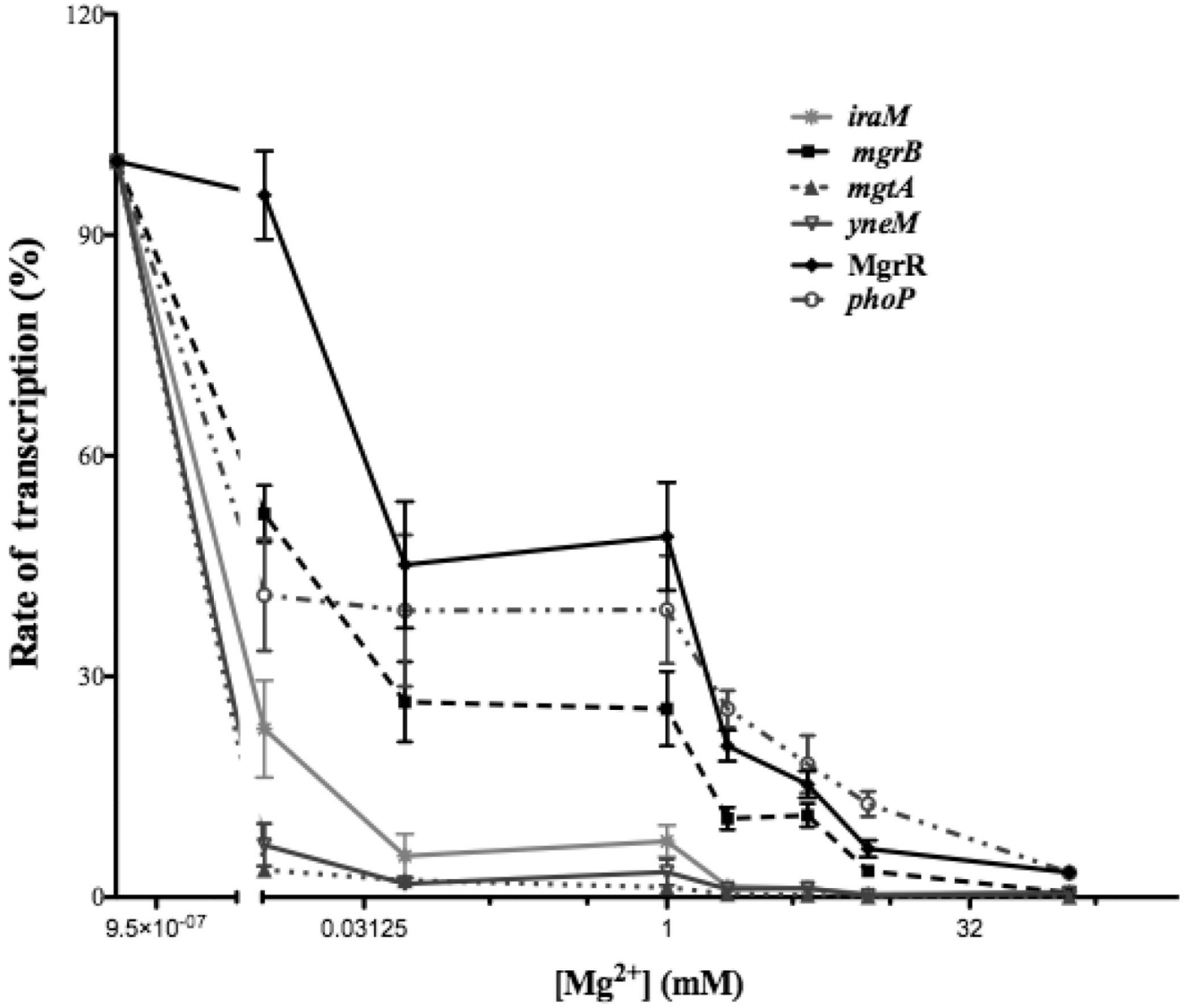

Figure 4. Activity of the PhoP regulated promoters as a function of Mg^{2+} concentration

Wild type (MG1655) was grown at M9 minimal medium containing 2mM Mg^{2+}, then switched to the same medium at various Mg^{2+} concentrations. The samples were collected at 50 min after changing to the indicated concentrations. RNA was extracted based on hot phenol extraction method and was treated with Turbo DNase (Ambion) to prepare cDNA for real time RT-PCR. The normalized mRNA level of the sample with 0uM Mg^{2+} was designated as 100% and the samples with other Mg^{2+} concentrations were compared to this value as indicated. Error bar shows standard deviation among 3 different trials.

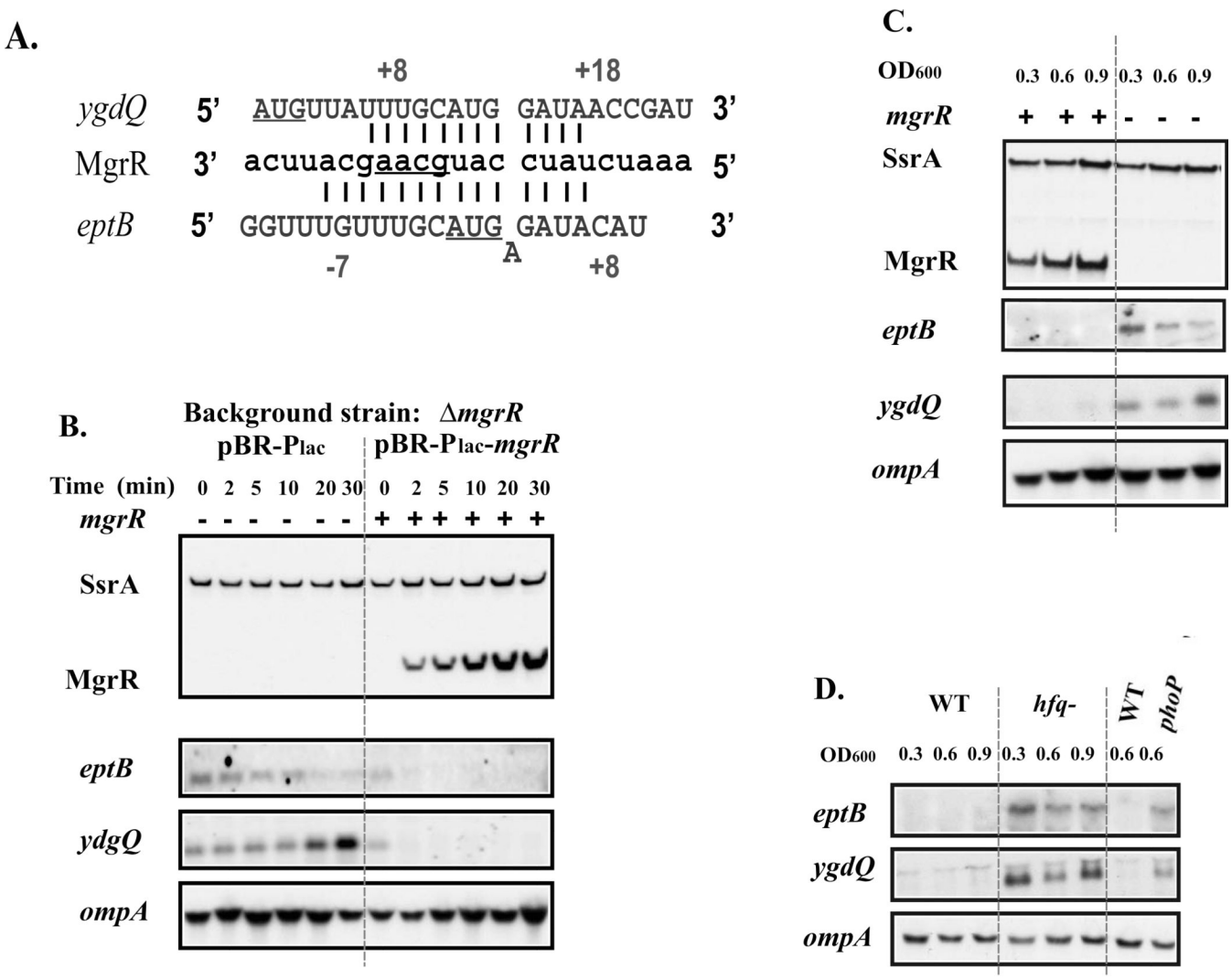

Figure 5. *eptB* and *ygdQ* are negative targets of MgrR

A Predicted base pairing between MgrR and *eptB* or *ygdQ* mRNA. 5' to 3' direction is indicated for each RNA. Numbering refers to translation initiation codon for *eptB* and *ygdQ*. The region of MgrR for base pairing is the highly conserved region shown bracketed in Figure 2. The underlined AUG represents the start codon of each gene.

B. The *mgrR*::*kn* strain (KM132) was transformed with Plac-*mgrR* or the corresponding empty vector, and cells were grown in LB-Ampicillin to exponential phase. IPTG (100μM final concentration) was added to both cultures and samples were taken at the indicated times after IPTG addition.

C. To examine the effect of expression from the chromosomal copy of *mgrR*, wild type (MG1655) and isogenic *mgrR*::kn (KM129) strains were grown in LB and the samples were taken at the indicated ODs.

D. Effect of Δ*hfq* (NM22562) and Δ*phoP* (EG12976) mutants on the expression of negative targets. Cells were grown in LB at 37°C and RNA samples were collected at various ODs as indicated. *ygdQ* and *eptB* mRNAs as well as MgrR sRNA were analyzed by Northern blot. *ompA* mRNA and SsrA (tmRNA) were used as internal controls.

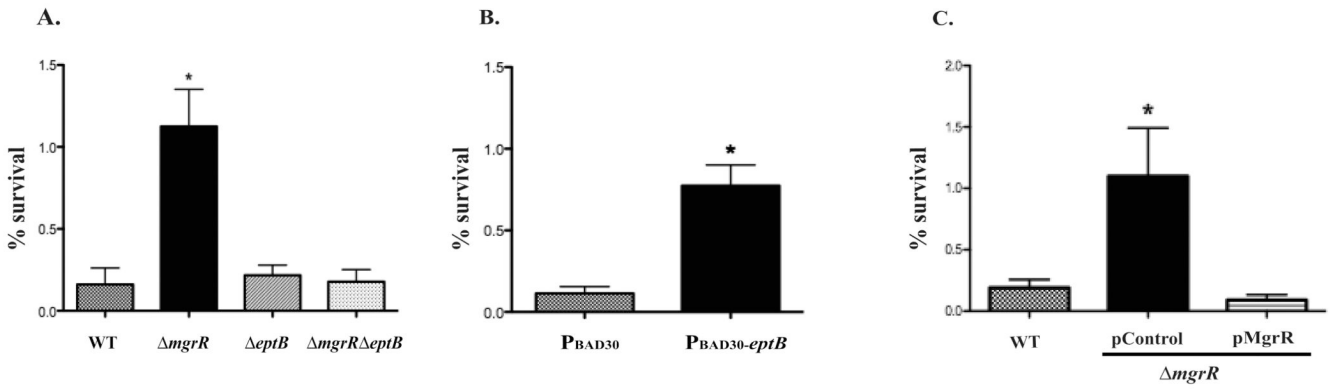

Figure 6. Effect of MgrR on polymyxin B resistance

A. The indicated strains (WT: NM22540; Δ*mgrR*: KM129; Δ*eptB*: KM176; Δ*mgrR* Δ*eptB*: KM177) were exposed to 4 μg/ml polymyxin B sulfate for 1 h at 37°C and then were plated on LB plates to determine CFU. Polymyxin sensitivity is expressed in terms of percent survival.

B. For the complementation assay, a plasmid containing *eptB* expressed from the arabinose promoter (pGFK1024) was first transformed into the Δ*mgrR*Δ*eptB* strain (KM177). Cells harboring an empty vector (pBAD30) were used as a control. The cells were grown in LB and induced with 0.2% arabinose for 3 hours and then were challenged with 4 μg/ml polymyxin B sulfate for 1 h at 37°C, diluted and plated in the absence of arabinose and polymyxin to assess CFU. The error bars show variations in CFU between at least three independent experiments. * indicates significantly different from the other strains at $p<0.05$.

C. A plasmid carrying *mgrR* under its own promoter (pGFK1013) was transformed into the Δ*mgrR* strain (KM129). Wild type (NM22540) and cells harboring an empty vector (pCRII) were used as controls. The cells were grown in LB and challenged with 4 μg/ml polymyxin B sulfate for 1 h at 37°C as described above. The error bars show variations in CFU between at least three independent experiments. * indicates significantly different from the other strains at $p<0.05$.

Table 1

Effect of the extended −10 on expression of the *mgrR* promoter.

	0 min[b]	30 min	60 min	90 min	120 min
KM112 L[a]	65.5 (+/− 0.7)[c]	62.6 (+/− 3.5)	86.3 (+/− 4.9)	128.4 (+/− 9.0)	149.6 (+/− 4.8)
KM112 H[a]	65.5 (+/− 0.7)	43.3 (+/− 2.6)	44.2 (+/− 2.4)	27.6 (+/− 0.1)	33.7 (+/− 3.0)
KM188 L	2.9 (+/− 1.1)	2.0 (+/− 0.4)	2.8 (+/− 0.3)	4.3 (+/− 0.3)	5.0 (+/− 0.1)
KM188 H	2.9 (+/− 1.1)	1.9 (+/− 1.7)	1.6 (+/− 0.7)	0.7 (+/− 0.3)	0.8 (+/− 0.2)

[a] KM 112 contains the wild-type *mgrR-lacZ* fusion; KM188 contains the mutant fusion in which the TG in the extended −10 has been changed to AC. All cells were initially grown in glucose minimal media with 2 mM [Mg^{2+}], filtered and resuspended in medium with 0mM [Mg^{2+}] (L) or 10mM [Mg^{2+}] (H).

[b] Time was measured from the moment cells were switched into media with either Low or High concentration of [Mg^{2+}] after washing by filtration.

[c] Each number was obtained from the average of three different individual cultures and the number in the parenthesis indicates the standard deviation.

Table 2

Potential negative targets of MgrR

Gene	locus	pBR-Plac vs. pBR-Plac-*mgrR*		Δ *mgrR* vs. WT		Descriptions
		Fold change	Fold change	Fold change	Fold change	
		Exp 1	Exp2	Exp1	Exp2	
pdhR	b0113	−2.2	−1.1	−8.5	−5.6	transcriptional regulator for pyruvate dehydrogenase complex
rhlE	b0797	−2.6	−1.8	−2.7	−2.2	putative ATP-dependent RNA helicase
ycaD	b0898	−2.6	−1.3	−2.2	−2.0	Putative MFS transporter
aroA	b0908	−3.0	−2.0	−2.0	1.3	5-enolpyruvylshikimate-3-phosphate synthetase
ndh	b1109	−2.6	−1.7	−5.2	−3.6	respiratory NADH dehydrogenase
trpH	b1266	−2.1	−2.1	−2.1	−1.0	putative enzymes
ydgI	b1605	−2.2	−1.2	−3.1	−2.8	putative arginineornithine antiporter
ydgL	b1627	−2.5	−2.2	−1.7	−1.2	hypothetical protein
yniC	b1727	−2.7	−2.3	−1.5	−0.8	2-deoxyglucose-6-P phosphatase
yfhL	b2562	−2.7	−2.2	−2.2	−1.6	Predicted Fe-S cluster containing protein
yfiD	b2579	1.1	−1.2	−5.2	−4.2	Pyruvate formate-lyase subunit
ygdQ	b2832	−15.0	−9.2	−3.0	−4.3	putative transport protein
mscS (*yggB*)	b2924	−2.3	−2.1	−1.8	−1.4	Subunit of mechanosensitive channel
pitA	b3493	−2.7	−2.7	−1.5	−1.5	low-affinity phosphate transport
eptB	b3546	−9.7	−8.7	−1.8	−2.8	phosphoethanolamine transferase
yibK	b3606	−2.8	−2.7	−1.7	−1.1	predicted rRNA methylatse
yieG	b3714	−2.6	−2.3	−1.4	−1.3	putative membrane transport protein
spf	b3864	−2.3	1.3	−3.1	−2.0	Spot 42 RNA
yjaA	b4011	−3.9	−2.6	−3.0	−1.8	hypothetical conserved protein
ryiA	b4456	−2.1	−2.3	−1.2	−1.4	GlmZ small RNA

Table 3

Strains and plasmids used in this study

Strains or plasmids	Description	Reference or Source
Strains		
MG1655	Wild-type *E. coli*	
NM22540	MG1655 Δ*araBAD*, *leu*$^+$	N. Majdalani
NM22562	MG1655 Δ*araBAD*, *leu*$^+$, Δ*hfq*::cm	N. Majdalani
PM1205	*mal*::*lacI* ^{q}, *araC*$^+$, pBAD-*cat-sacB-lacZ*, mini-λ -Red-tet	(Mandin & Gottesman, 2009)
KM62	NM22540 mini- λ -Red-tet	This study
KM100	*mal*::*lacI* ^{q}, Δ*araBAD*, *leu* $^+$, *araC*$^+$, *araE*	This study
KM112	PM1205 *mgrR-lacZ* transcriptional fusion (positions −60 to +10 of *mgrR* promoter)	This study
KM113	PM1205 *mgrR-lacZ* transcriptional fusion (positions −35 to +10 of *mgrR* promoter)	This study
KM114	KM112 Δ *phoP*::*kn*	This study
KM129	NM22540 Δ *mgrR*::*kn*	This study
KM132	KM100 Δ *mgrR*::*kn*	This study
KM176	NM22540 Δ *eptB*::*cm*	This study
KM177	NM22540 Δ *mgrR*::*kn*, Δ *eptB*::*cm*	This study
KM187	PM1205 *mgrR-lacZ* transcriptional fusion from *S. enterica* (positions −60 to +10 of *mgrR* promoter)	This study
KM188	PM1205 *mgrR-lacZ* transcriptional fusion with mutated extended −10 (positions −60 to +10 of *mgrR* promoter)	This study
KM189	KM187 Δ *phoP*::*kn*	This study
KM194	PM1205 *yneM-lacZ* transcriptional fusion (positions −75 to +88 of *mgrR* promoter)	This study
KM195	KM112 Δ *phoQ*::*kn*	This study
KM196	KM187 Δ *phoQ*::*kn*	This study
KM197	KM194 Δ *phoP*::*kn*	This study
KM198	KM194 Δ *phoQ*::*kn*	This study
EG12976	MG1655 Δ *phoP*::*kn*	(Zwir *et al.*, 2005)
Plasmids		
pCR2.1	Amp^r; Cloning vector having pBR322 origin	
pBR-plac	Amp^r; lac promoter-based expression vector having a pBR322 origin	
pBAD30	Amp^r; araBAD promoter-based expression vector having a pACYC origin	(Guzman *et al.*, 1995)
pGFK1013	Approximately 250bp *mgrR* region containing its own promoter	This study
pGFK1018	AatII-EcoRI *mgrR*-containing fragment cloned into the same sites in pBR-plac	This study
pGFK1024	*eptB* coding region cloned into pBAD30	This study