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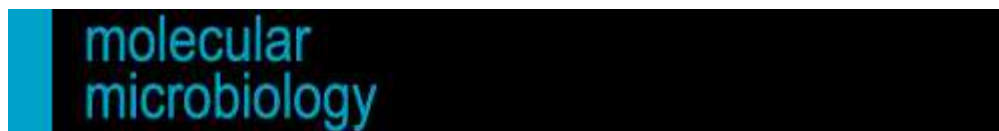
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MicA sRNA links the PhoP regulon to cell envelope stress

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Abstract

Numerous small RNAs regulators of gene expression exist in bacteria. A large class of them binds to the RNA chaperone Hfq and act by base-pairing interactions with their target-mRNA, thereby affecting their translation and/or stability. They often have multiple direct targets, some of which may be regulators themselves, and production of a single sRNA can therefore affect the expression of dozens of genes.

We show in this study that the synthesis of the *E. coli* pleiotropic PhoPQ two-component system is repressed by MicA, a σ E-dependent sRNA regulator of porin biogenesis. MicA directly pairs with *phoPQ* mRNA in the translation initiation region of *phoP* and presumably inhibits translation by competing with ribosome binding. Consequently, MicA down-regulates several members of the PhoPQ regulon. By linking PhoPQ to σ E, our findings suggest that major cellular processes such as Mg^{2+} transport, virulence, LPS modification or resistance to antimicrobial peptides are modulated in response to envelope stress.

In addition, we found that Hfq strongly affects the expression of *phoP* independently of MicA, raising the possibility that even more sRNAs, that remain to be identified, could regulate PhoPQ synthesis.

1 Introduction

2 A major challenge faced by bacteria is the necessity to adapt rapidly to ever-
3 changing environments, which partly relies on their ability to regulate gene expression as
4 a function of the environmental conditions. Two-component systems (TCS) are key
5 components of this process. The model organism *Escherichia coli* has more than 30 TCS,
6 which directly regulate transcription of hundreds of genes. Typically, TCS consist of a
7 sensor kinase that autophosphorylates in response to a given external stimulus and then
8 transfers its phosphate group to its cognate response regulator. The activated response
9 regulator then binds to promoter regions and regulates transcription of its targets. In most
10 cases, the genes encoding the regulator and the kinase are organized within an operon,
11 which allows their synthesis to be coordinated. It is also common that expression of TCS
12 operons is subject to feedback control. In the case of the *E. coli* EnvZ-OmpR TCS, this
13 feedback is not direct, but is mediated by two homologous small RNAs (sRNAs), OmrA
14 and OmrB, whose transcription is activated by EnvZ-OmpR and which in turn repress the
15 expression of multiple genes, including the *ompR-envZ* operon itself (Guillier and
16 Gottesman, 2008).

17 sRNAs are another class of widespread regulators in bacteria (Waters and Storz,
18 2009 for a recent review). Different experimental searches led to the identification of
19 almost a hundred of them in *E. coli* (Sharma and Vogel, 2009). In general they are
20 synthesized as discrete transcripts whose size varies between 50 and 400 nts in length.
21 Most of them are post-transcriptional regulators that can act according to two major
22 mechanisms: sRNAs that titrate a protein and therefore modulate its activity or sRNAs
23 that directly base-pair with target-mRNAs. In this later class, one can distinguish sRNAs

1 that are encoded on the opposite DNA strand to their target, and therefore share perfect
2 complementarity with their target-mRNA, and sRNAs referred to as “*trans*-encoded” that
3 often regulate multiple targets via limited complementarity. All *trans*-encoded sRNAs
4 studied so far in *E. coli* bind the RNA chaperone Hfq, which has been shown to stabilize
5 several sRNAs as well as facilitating sRNA-mRNA duplex formation by a mechanism
6 that is still unclear (Brennan and Link, 2007; Valentin-Hansen *et al.*, 2004). Most, but not
7 all, *trans*-encoded sRNAs are transcribed under specific conditions as part of major
8 regulons. For instance, MgrR, RyhB and CyaR sRNAs belong respectively to the
9 regulons of PhoPQ TCS (Moon and Gottesman, 2009), Fur (Massé and Gottesman, 2002)
10 and CRP (De Lay and Gottesman, 2009; Johansen *et al.*, 2008; Papenfort *et al.*, 2008).
11 Pairing of sRNAs to their targets leads to regulation of gene expression by affecting
12 translation and/or stability of the mRNA, either positively or negatively. Genes subject to
13 post-transcriptional control by sRNAs are involved in numerous cellular processes, such
14 as iron homeostasis, envelope homeostasis and quorum sensing, to name just a few. The
15 expression of several regulators has also been shown to be controlled by sRNAs: for
16 example, at least three of them directly activate translation of *rpoS*, the gene for the
17 stationary phase sigma factor (Lease *et al.*, 1998; Majdalani *et al.*, 1998; Majdalani *et al.*,
18 2001; Mandin and Gottesman, manuscript in preparation; Majdalani *et al.*, 2002) and, as
19 mentioned above, OmrA/B repress expression of the EnvZ-OmpR TCS.

20 Interestingly, regulation of TCS by sRNAs is not restricted to EnvZ-OmpR. Other
21 examples include regulation of *E. coli dpiBA*, encoding a TCS induced by β -lactams and
22 involved in the SOS response, by RybC (Mandin and Gottesman, 2009), as well as

1 regulation of *luxOU*, encoding the response regulator LuxO and the phosphotransfer
2 protein LuxU, by Qrr1-4 sRNAs in *Vibrio cholerae* (Svenningsen *et al.*, 2009).

3 In this study, we show that yet another TCS, PhoPQ (where PhoQ is the sensor
4 kinase and PhoP the cognate response regulator), is subject to direct post-transcriptional
5 control by MicA sRNA. PhoPQ has been extensively studied in bacteria and especially in
6 *Salmonella*. It is induced in response to low Mg^{2+} and Ca^{2+} , as well as in the presence of
7 antimicrobial peptides. Genes that are regulated by PhoPQ are involved in Mg^{2+} transport,
8 resistance to antimicrobial peptides, acid resistance, LPS modification and virulence
9 (Groisman, 2001). MicA is an Hfq-binding sRNA that was previously shown to directly
10 repress synthesis of at least two bacterial outer membrane proteins (OMPs), OmpA and
11 LamB (Bossi and Figueroa-Bossi, 2007; Rasmussen *et al.*, 2005; Udekwu *et al.*, 2005).
12 MicA transcription is dependent on the alternative sigma factor σE which, in turn,
13 responds to envelope stress (Johansen *et al.*, 2006; Papenfort *et al.*, 2006; Udekwu and
14 Wagner, 2007). Our results show that expression of *phoPQ* is repressed in a MicA-
15 dependent manner upon activation of σE , and that several PhoP-regulated genes are also
16 controlled by MicA in a PhoP-dependent fashion. Therefore, expression of the PhoP-
17 PhoQ regulon, or at least part of it, is likely to be modulated in response to cell envelope
18 stress. Finally, preliminary findings indicate that, in addition to MicA, other sRNAs
19 might also be regulators of PhoPQ synthesis.

20

Results

Hfq and MicA affect the expression of *ompT* in the absence of OmrA and OmrB sRNAs

Expression of the outer membrane protease OmpT was previously shown to be regulated by two redundant Hfq-binding sRNAs, OmrA and OmrB, via a direct interaction between the conserved 5' end of OmrA/B and the early coding region of the *ompT* mRNA (Fig. 1, Guillier and Gottesman, 2006; Guillier and Gottesman, 2008). However, in a strain lacking OmrA/B, the expression of an *ompT-lacZ* translational fusion is still 1.3-fold higher in an *hfq* mutant compared to a wild-type strain (Fig. 2A, Table S1). One possible explanation for this observation is that, in addition to and independently of OmrA/B, other sRNAs could control OmpT synthesis.

σ E is an alternative sigma factor whose activity is induced under conditions of envelope stress leading to the accumulation of unfolded OMP in the periplasm (Ruiz and Silhavy, 2005). σ E activates the transcription of numerous genes, including genes for periplasmic chaperones and proteases (Mutalik *et al.*, 2009; Rhodius *et al.*, 2006). It also activates transcription of two Hfq-binding sRNAs, RybB and MicA, that down-regulate the synthesis of major OMPs, such as OmpC and OmpA in *E. coli*, thereby reducing the accumulation of unassembled periplasmic OMPs (Johansen *et al.*, 2006; Papenfort *et al.*, 2006; Rasmussen *et al.*, 2005; Thompson *et al.*, 2007; Udekwu *et al.*, 2005; Udekwu and Wagner, 2007). We reasoned that, since OmpT is a rather abundant OMP (Molloy *et al.*, 2000), its expression could be repressed by σ E-dependent sRNAs.

1 The activation of σ E relies on a proteolytic cascade that leads to the degradation
2 of RseA, an anti-sigma factor that sequesters σ E at the inner membrane under non-
3 inducing conditions (Fig.1, Ades, 2008). To test whether the expression of *ompT* was
4 affected by σ E, we compared the activity of the *ompT-lacZ* fusion in *rseA*⁺ and *rseA*⁻
5 strains. There is a decrease of about 1.2 fold in the β -galactosidase activity in the *rseA*⁻
6 strain (Fig. 2B and Table S1). Even though this effect is very modest, it was enough to
7 convince us to look for σ E-dependent sRNAs involved in *ompT* regulation.

8 To date, two such sRNAs have been identified in *E. coli* and related
9 enterobacteria, MicA and RybB (see above). The effect of these two sRNAs on the
10 *ompT-lacZ* fusion was therefore analyzed by measuring the β -galactosidase activity of
11 strains transformed with a plasmid overexpressing either MicA or RybB. In these
12 experiments, overexpression of MicA resulted in a 3-fold decrease in the β -galactosidase
13 activity compared to the vector control, whereas overexpression of RybB had no
14 noticeable effect on the expression of the fusion (Fig. 2C). Consistent with what was
15 found in previous studies, overexpression of OmrA, a direct regulator of *ompT*, down-
16 regulated the expression of the *ompT-lacZ* by only 1.4-fold. The experiments shown in
17 Fig. 2C were carried out in a strain deleted for the chromosomal copies of OmrA and
18 OmrB, and the effect of MicA is therefore independent of these two sRNAs. In addition,
19 MicA is responsible for the decrease in *ompT-lacZ* activity observed in an *rseA*⁻ strain
20 (see above), as this effect is abolished in a *micA*⁻ *rseA*⁻ double mutant (Fig. 2B, Table S1).

21 Repression of *ompT* by MicA is dependent on the PhoP transcriptional regulator

MicA (previously SraD) is an Hfq-binding sRNA that was identified in several searches for bacterial sRNAs (Argaman *et al.*, 2001; Zhang *et al.*, 2003). It was shown to inhibit synthesis of two OMPs, OmpA and LamB, by pairing in the translation initiation region of their mRNAs (Bossi and Figueroa-Bossi, 2007; Rasmussen *et al.*, 2005; Udekwi *et al.*, 2005). However, no obvious complementarity was found between MicA and the *ompT* mRNA region present in our fusion, suggesting that control of *ompT* by MicA might be indirect. The translational *ompT-lacZ* fusion used in this study carries 220 nts of *ompT* upstream of the start codon and its transcription is driven by the *ompT* promoter. Therefore, this reporter fusion should be sensitive to regulation exerted either at the level of transcription and/or translation initiation.

ompT transcription was shown to be activated by PhoP, the response regulator of the PhoP-PhoQ TCS, probably in a direct manner (Eguchi *et al.*, 2004; Zwir *et al.*, 2005; Fig. 1). Therefore, one possible explanation of our results is that MicA controls *ompT* through PhoPQ. To test this hypothesis, we measured the effect of MicA sRNA on the expression of the *ompT-lacZ* fusion in a strain where *phoP* was inactivated by a transposon insertion. As expected, the activity of the fusion was strongly decreased compared to the *phoP*⁺ background, but was still sufficient to observe a potential repression. The effect of MicA dropped from 3.3-fold in the wt strain to 1.2-fold in the *phoP* mutant, whereas repression by OmrA was similar in the wt and mutant strains (roughly 1.4-fold) (Fig. 3 and Table S1). These results clearly show that repression of *ompT* by MicA requires PhoP.

MicA regulates the expression of *phoPQ* through direct base-pairing interaction

1 The next logical question was to ask whether MicA directly controlled PhoPQ
2 synthesis. As is the case for most two component-systems, the genes for the response
3 regulator and the sensor kinase are organized in a bicistronic operon, here *phoP-phoQ*,
4 and interestingly, a base-pairing interaction can be predicted between MicA and the
5 *phoPQ* mRNA in the *phoP* translation initiation region (Fig. 4A). Most of this pairing is
6 conserved in several enterobacteria, including *Salmonella*, *Shigella* and *Enterobacter*.
7 However, in some members of Enterobacteriaceae where both *micA* and *phoP* genes are
8 present, complementarity between MicA and the *phoP* translation initiation region is
9 poorer. This is the case in *Yersinia pestis*, *Klebsiella pneumoniae* and *Photobacterium*
10 *luminescens* (data not shown).

11 In order to determine whether the predicted interaction in *E. coli* takes place *in*
12 *vivo*, we constructed a translational *phoP-lacZ* reporter fusion. This fusion encompasses
13 nts -36 (i.e. transcription start site of the *phoP* promoter) to +30 of the *phoP* mRNA
14 placed at the *lacZ* locus in frame with the 9th codon of *lacZ*. Transcription of this fusion is
15 driven by a P_{BAD} promoter; only control at the post-transcriptional level is therefore
16 expected to affect its expression.

17 Overproduction of MicA down-regulated this fusion by 3.6-fold, whereas OmrA
18 had no inhibitory effect, but rather increased expression of the fusion by 1.4-fold (Fig.
19 4B). A slight increase in expression was also observed upon overproduction of MicA_{mut},
20 a mutant derivative of MicA where 4 nts involved in the predicted interaction with *phoP*
21 mRNA were mutated (Fig. 4A). This mutant was shown to be present at a level similar to
22 that of wt MicA in a previous experiment and, in agreement with MicA modulating *ompT*
23 expression through PhoP, we found that MicA_{mut} overproduction does not affect the

activity of an *ompT-lacZ* fusion (Fig. 2C). A *phoP-lacZ* fusion carrying the compensatory changes was no longer controlled by wt MicA, whereas control was partially restored by MicA^{mut} (repression of 1.1- and 2-fold respectively) (Fig. 4B and Table S1). As for the wt *phoP-lacZ* fusion, overproduction of OmrA up-regulated expression of the mutant fusion by 1.4-fold. The reason for the positive action of OmrA on these fusions, as well as of MicA^{mut} on the wt fusion, is not clear and we suspect that these effects are most likely indirect, possibly through Hfq titration. Together, these results unambiguously show that MicA base-pairs with *phoPQ* mRNA around the *phoP* start codon. This leads to down-regulation of *phoP* expression. *phoQ*, in an operon with *phoP*, is also down-regulated, as measured with a *phoPQ-lacZ* fusion (data not shown).

MicA sRNA links PhoP synthesis to σ E activity

In the previous experiment, MicA was expressed from a multicopy plasmid and its transcription was driven by an IPTG-inducible promoter. Because MicA is strongly overexpressed in these experiments (see lane pMicA of Fig. 4C), we decided to look at the effect of MicA induction on *phoP* expression, when MicA was transcribed from its own promoter under more physiological conditions. In a first experiment, σ E was activated using an *rseA*⁻ strain. Consistent with a control of *phoPQ* by MicA, this resulted in a 1.5-fold decrease in the expression of the P_{BAD}-*phoP-lacZ* fusion. This effect was due to MicA, since activity was no longer decreased in an *rseA*⁻ *micA*⁻ double mutant (Fig. 4D and Table S1). We then used different envelope stresses to induce σ E. Surprisingly, we found that several of them had no effect on *phoP* expression and so far, we do not have a clear explanation for this. However, other stresses, such as addition of procaine for

instance, slightly decreased *phoP* expression in a reproducible manner (data not shown). Procaine most likely induces σ E through EnvZ-OmpR activation, which is expected to result in an elevated synthesis of OMPs. In agreement with this, Thompson and Gottesman isolated a plasmid carrying *envZ-ompR* as a multicopy activator of the σ E-dependent *rybB-lacZ* fusion in a genetic screen (Thompson *et al.*, 2007). This plasmid is referred here as pEnvZ-OmpR.

In the presence of pEnvZ-OmpR, MicA was produced to a much lower level than in the *rseA* mutant (Fig. 4C, compare lanes pEnvZ-OmpR and *rseA*⁻). Nevertheless, this was sufficient to observe a slight decrease in the expression of *phoP*: 1.4-fold at the best (Fig. 4E and Table S1). Since this effect is modest, we repeated this experiment 7 times, and results were similar. Shown in Fig. 4E is a representative experiment where β -galactosidase activity was measured on two duplicate samples. As expected, this decrease is reproducibly dependent on MicA as it is not observed in the *micA* mutant strain. In contrast, pEnvZ-OmpR activates the expression of the σ E-dependent *rybB-lacZ* fusion in a MicA-independent manner (induction of 5.4- and 5.8-fold respectively in *micA*⁺ and *micA*⁻ cells) (Fig. 4E and Table S1). Note that these experiments were performed in the absence of *ompA*, a major target of MicA. However, in several experiments, a similar effect of pEnvZ-OmpR on *phoP* was observed in *ompA*⁺ cells.

Regulation of different members of the PhoP regulon by MicA

Expression of the porin-encoding genes *ompC* and *ompF* is regulated by the EnvZ-OmpR TCS. Surprisingly however, their expression was shown to be rather constant over a large range of OmpR and EnvZ levels (Batchelor and Goulian, 2003).

One can therefore wonder what the effect of regulating a TCS will be on the expression of its target-genes. To gain insight into this question, we looked at how MicA overproduction affects the expression of different genes whose transcription is controlled by PhoQ-PhoP, in addition to *ompT*. We focused on two targets of PhoPQ: MgrR, a sRNA regulator of LPS modification (Moon and Gottesman, 2009), and *yneM*, that encodes a small membrane protein (Hemm *et al.*, 2008). Interestingly, MgrR and *yneM* are differentially regulated by PhoPQ in response to Mg^{2+} levels, with MgrR being expressed even at rather high Mg^{2+} (half-maximal expression occurs at $[Mg^{2+}]_{50\%} \sim 5$ mM) whereas *yneM* is expressed only at fairly low Mg^{2+} ($[Mg^{2+}]_{50\%} \sim 0.005$ mM), which is closer to what is observed for other PhoP targets (Moon and Gottesman, 2009, and Moon and Gottesman, personal communication).

Despite their differential responses to Mg^{2+} , repression of *lacZ* fusions to these two genes by MicA was similar (repression factors of 3.1 and 2.7 for *mgrR-lacZ* and *yneM-lacZ* respectively), whereas, as expected, activity of these fusions was either not affected or slightly increased by ectopic expression of either MicA^{mut} or OmrA (Fig. 5A, 5B, and Table S1). It is interesting that MicA also repressed the activity of *ompT-lacZ* and *phoP-lacZ* fusions to a similar extent (repression of 3.1 and 3.6-fold respectively, see above). Therefore, as for *ompT*, MicA represses *mgrR* and *yneM* expression, presumably through *phoPQ*. In agreement with this, we found that MicA no longer controls the expression of an *mgrR-lacZ* fusion expressed from the PhoP-independent promoter P_{BAD} (Fig. 5C and Table S1). It will be interesting to determine whether MicA also controls other targets of this TCS that can be differentially regulated as a function of PhoPQ stimulation (Miyashiro and Goulian, 2007).

1

2 **Additional sRNAs regulating *ompT* and/or *phoP* expression ?**

3 Starting from the observation that *ompT* expression increases in an *hfq* mutant
4 strain in the absence of OmrA/B, we showed in this study that MicA, an Hfq-dependent
5 sRNA, directly represses the expression of *phoPQ*, a TCS regulator of *ompT*
6 transcription. However, this does not necessarily mean that the effect of Hfq on *ompT* is
7 due to MicA. To determine this, we compared the activities of *ompT-lacZ* in *hfq*⁺ and *hfq*⁻
8 strains in the presence and absence of MicA and/or the OmrA/B sRNAs. In all cases,
9 expression of the fusion was increased in the *hfq* mutant (between 1.2 and 1.4-fold
10 compared to the *hfq*⁺ strain, Fig. 6A and Table S1), clearly showing that, as OmrA/B,
11 MicA does not account for the full effect of Hfq on *ompT-lacZ*. Even though Hfq can
12 affect gene expression independently of sRNAs (Folichon *et al.*, 2003; Hajnsdorf and
13 Regnier, 2000), a possible explanation for this observation is that additional sRNAs act
14 on *ompT-lacZ*. If this is true, they could either affect transcription of the fusion, by
15 regulating *phoPQ* for instance, or act at the post-transcriptional level.

16 If the effect of Hfq on *ompT-lacZ* is through sRNA regulation of *phoPQ*, then the
17 expression of the P_{BAD}-*phoP-lacZ* fusion should also be increased in an *hfq* mutant. To
18 verify this, we measured the activity of this fusion in *hfq*⁺ and *hfq*⁻ strains: *hfq*
19 inactivation resulted in a 3.7-fold increase in β -galactosidase activity (Fig. 6B, Table S1).
20 Somewhat surprisingly, this effect was completely independent of MicA, since a similar
21 increase (3.85 fold) was observed in a *micA*⁻ strain. Again, it is conceivable that yet
22 another Hfq-dependent sRNA regulator of *phoPQ* is responsible for this effect (Fig. 1),
23 but it is not the only potential explanation.

1 This large MicA-independent effect of *hfq* on *phoPQ* translation would be
2 sufficient to explain the effects of an *hfq* mutant on *ompT-lacZ*. However, to directly test
3 this, the activity of *ompT-lacZ* in both *hfq*⁺ and *hfq*⁻ cells was determined in the absence
4 of a functional PhoP regulator. Despite a lower level of expression, the results were
5 similar to those obtained in a *phoP*⁺ strain : the activity was increased by 1.3-fold in the
6 *hfq* mutant. This is independent of the sRNAs known to directly regulate *ompT* at the
7 post-transcriptional level, OmrA/B, since the increase was almost 1.5-fold in an *omrAB*⁻
8 background (Fig. 6C and Table S1). Therefore, *ompT* expression is regulated by Hfq in a
9 way that is, at least partially, independent of PhoP. This could be due to one or more
10 unknown sRNAs acting directly on *ompT* (Fig. 1).

12 Discussion

13 We show here that PhoPQ synthesis is negatively regulated at the post-
14 transcriptional level by MicA, a σ E-dependent sRNA. Consistent with this observation,
15 MicA overproduction represses expression of several PhoP-regulated genes, such as
16 *ompT*, *mgrR* and *yneM*. Regulation of *ompT* by MicA is abolished in a strain where PhoP
17 is inactivated (Fig. 3). Similarly, the involvement of PhoPQ in the regulation of *mgrR* by
18 MicA is strongly suggested by our result showing that MicA does not affect expression of
19 an *mgrR-lacZ* fusion under the control of the araBAD promoter (Fig.5). This is
20 presumably true for *yneM* as well.

21 Compensatory changes clearly showed that MicA pairs with *phoPQ* mRNA
22 around the *phoP* start codon (Fig. 4B). Therefore, MicA most likely represses expression
23 of *phoPQ* operon by competition with ribosome binding, as was shown to be the case for

1 several negatively acting sRNAs (Chen *et al.*, 2004; Udekwu *et al.*, 2005 for instance).
2 This is likely to be accompanied by degradation of the mRNA, since a decrease in *phoP*
3 mRNA level was observed by transcriptome analysis after MicA induction (Gogol and
4 Gross, manuscript in preparation). Again, this is true for many sRNAs acting by pairing
5 (Aiba, 2007).

6 For Hfq-dependent sRNAs that directly regulate multiple targets, it is also
7 common to find that the same region of the sRNA is involved in the pairing with different
8 mRNAs (Guillier and Gottesman, 2008; Sharma *et al.*, 2007). This seems to be the case
9 for MicA since nts that are paired with *phoPQ* mRNA are also predicted to pair with
10 *ompA* and *lamB* messages (Bossi and Figueroa-Bossi, 2007; Rasmussen *et al.*, 2005;
11 Udekwu *et al.*, 2005). However, it is worth noting that, whereas MicA no longer
12 controls *phoPQ* expression (Fig. 4B), it still causes a decrease in *ompA* mRNA levels
13 when overproduced (data not shown). In this case, the stretch of 12 consecutive bp
14 between nts 13 to 24 of MicA and nts -11 to -22 of *ompA* mRNA, some of which were
15 shown to be involved in the repression *in vivo* by compensatory changes (Udekwu *et al.*,
16 2005), is presumably sufficient for pairing. The effect of MicA on *lamB* expression
17 has not been tested so far.

18 In addition to *ompA* and *lamB* mRNAs, MicA was also shown to affect the level
19 of the message for another OMP, *ompX*, which is the direct target of yet another sRNA,
20 CyaR (De Lay and Gottesman, 2009; Johansen *et al.*, 2008; Papenfort *et al.*, 2008). We
21 therefore wondered whether CyaR could also be a regulator of *phoP* expression. This
22 does not seem to be the case however, since despite a complementarity of 7 bp between

1 CyaR and the TIR region of *phoP*, we found that overproduction of CyaR did not affect
2 the activity of our *phoP-lacZ* fusion (our unpublished results).

3
4 These results uncover a novel link between sigma factors and TCS, two major
5 classes of transcriptional regulators. To our knowledge, this is the first example of such a
6 connection, which highlights the complexity of regulation of TCS synthesis. Multiple
7 input signals are therefore integrated to modulate TCS expression and/or activity: signals
8 that are directly sensed by the sensor kinase, leading to phosphorylation of the regulator,
9 but also signals that modulate the synthesis of the TCS through production of a regulator,
10 such as a sRNA for instance. It is not clear how this latter control affects the level of the
11 active, ie phosphorylated, form of the regulator. Nonetheless, modulating the levels of
12 TCS regulators, such as PhoP and OmpR, without affecting the inducing signals, clearly
13 leads to regulation of target-genes (this study and Guillier and Gottesman, 2006 for
14 instance). The connection between regulation of expression and phosphorylation remains
15 to be investigated in detail.

16 More specifically, our results indicate that some conditions of cell envelope stress
17 known to induce the σ E response will lead to repression of the PhoPQ regulon, which
18 includes genes involved in magnesium transport, resistance to antimicrobial peptides,
19 acid resistance and LPS modification. From a physiological standpoint, it is not
20 surprising that resistance to antimicrobial peptides, acid resistance and LPS modification
21 could be affected by cell envelope stress. Other members of the PhoPQ regulon are
22 involved in magnesium transport and their expression could therefore be regulated by

MicA as well. The physiological interest of such a link, if it exists, between envelope stress and magnesium transport remains unclear.

Interestingly, Moon and Gottesman recently identified *eptB* as a major target of the PhoP-regulated sRNA MgrR. *eptB* is involved in LPS modification and is regulated by σ E, most likely in an indirect manner (Moon and Gottesman, 2009). While results from Moon and Gottesman demonstrate that there clearly is an effect of σ E on the transcription initiation of *eptB* (K. Moon and S. Gottesman, unpublished results), it is possible that down-regulation of MgrR by MicA (via PhoP) also contributes to the observed σ E up-regulation of *eptB*. Irrespective of the mechanism of this regulation, this provides another example of intertwined regulation of the σ E and PhoPQ regulons.

It is also worth noting that the extent of these controls is limited, as is most often the case for regulation exerted by sRNAs. For instance, whereas the activity of the *ompT-lacZ* fusion used in this study was decreased by almost 30-fold when PhoP was inactivated (Fig. 3), down-regulation of *phoPQ* by MicA resulted only in a 3.1-fold or 1.2-fold decrease in *ompT-lacZ* when MicA was overproduced from a plasmid or induced from the chromosome using an *rseA*⁻ mutant allele respectively (Fig. 2). Therefore, MicA likely participates in fine-tuning the regulation of *phoPQ* under conditions where the σ E regulon is induced, such as envelope stress and to a lesser extent stationary phase. The limited extent of this regulation is highlighted by the fact that induction of MicA from the chromosome has only a modest effect on *phoP* expression (Fig. 4E). It is possible however that other envelope stresses will have a stronger effect on MicA accumulation and *phoP* control.

Our observation of a strong effect of an *hfq* mutant on the *phoP-lacZ* fusion, even in the absence of MicA, raises the exciting possibility that sRNAs other than MicA will regulate *phoPQ*. Two-component systems are widely used in bacteria as a way to sense and adapt to the environment. More than 30 of them have been described in the *E. coli* genome and it is tempting to speculate that, in addition to PhoPQ, DpiBA and OmpR-EnvZ, many more will be subject to sRNA-mediated regulation. It will also be interesting to elucidate how these controls affect the expression of the downstream genes and the overall physiology of the cell.

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Experimental procedures

Bacterial strains

With the exception of DH5 α that was used as the recipient strain for cloning procedures, all strains used in this study are derivatives of MG1655. They were grown

1 aerobically in LB medium. When needed, antibiotics were added at the following
2 concentrations : kanamycin 25 µg/ml, chloramphenicol 10 µg/ml (for transduction of the
3 *hfq::cm* allele) or 25 µg/ml, tetracycline 10 µg/ml, ampicillin 150 µg/ml.

4 Construction of *ΔmicA::cm*, *ΔmicA::tet*, *ΔomrAB::tet* and *ΔompA::kan* was as
5 follows : antibiotic resistance cassettes were amplified by PCR using forward (for) and
6 reverse (rev) oligonucleotides (sequence in Table S2) and the Phusion polymerase
7 (Finnzymes) following manufacturer's instructions. These PCR products were then
8 recombined in strain NM300 for *ΔmicA::cm* and *ΔompA::kan* or NM1200 for *ΔmicA::tet*
9 and *ΔomrAB::tet*, that carry a mini-lambda (Court *et al.*, 2003), as described previously
10 (Yu *et al.*, 2000).

11 These alleles, as well as *hfq::cm* (Tsui *et al.*, 1997), *ΔomrAB::kan* (Guillier and
12 Gottesman, 2006), *phoP::Tn Cm*, *phoP::kan* (Bougdour *et al.*, 2008) and *ΔrseA::kan*
13 (Thompson *et al.*, 2007) were then moved by P1vir transduction as requested. Note that
14 in *ΔmicA::cm* and *ΔmicA::tet* constructs, the promoter region for the adjacent and
15 divergently transcribed *luxS* gene is removed; therefore, *luxS* should not be expressed in
16 these *micA* mutants.

17 Construction of the *ompT-lacZ* translational fusion was described previously
18 (Guillier and Gottesman, 2008). For the P_{BAD} -*phoP-lacZ* fusion, a PCR product carrying
19 nts -36 to +30 of *phoP* relative to the start codon was amplified from genomic DNA from
20 strain MG1655 using the Phusion polymerase and primers *phoP-lac* for and *phoP-lac* rev,
21 that carry homology regions to P_{BAD} promoter and *lacZ* ORF respectively (Table S2). It
22 was then recombined in the strain PM1205 (Mandin and Gottesman, 2009) to replace the
23 *cat-sacB* cassette of the P_{BAD} -*cat-sacB-lacZ* construct as described (Yu *et al.*, 2000).

Recombinants were selected on LB plates without NaCl supplemented with 6% sucrose. Chloramphenicol sensitive colonies were purified three times on the same medium and the P_{BAD} -*phoP-lacZ* construct was confirmed by sequencing. For the construction of the P_{BAD} -*phoPmut-lacZ* fusion, the PCR product was obtained as follows. First, a PCR fragment was amplified from genomic DNA of strain MG1655 using primers *phoP-lac* for and *phoPmut* (Table S2) and the Phusion polymerase. This reaction was then treated with DpnI enzyme, purified and the resulting PCR product was used as a template in a second PCR reaction, with primers *phoP-lacZ* for and *phoP-lacZ* rev and Phusion DNA polymerase. Recombination in strain PM1205 and selection of recombinants was then carried out as above.

To construct the P_{BAD} -*mgrR-lacZ* fusion (strain MG1484), a PCR product synthesized with the partially complementary primers 5' P_{BAD} -*mgrR* and 3'*mgrR-lacZ* was recombined in strain PM1205 as previously. Recombinants were selected on M9 plates containing 6% sucrose, 0.2% arabinose and 20 μ g/ml Xgal. Verification of recombinants was as above.

Plasmids

pBRplac and *pOmrA* (also referred to as *pBRplacOmrA*) have been previously described (Guillier and Gottesman, 2006).

pMicA and *pRybB* : *MicA* and *RybB* were PCR-amplified from genomic DNA of strain MG1655 using primers AatII-*MicA* and Hind-*MicA* or AatII-*RybB* and Eco-*RybB* respectively, and the Expand High-Fidelity System (Roche). PCR products were then

digested with EcoRI and AatII (RybB) or AatII and HindIII (MicA) and ligated in the pBRplac vector cut with the same enzymes. Plasmids were checked by sequencing.

pMicAmut : mutation was introduced in pMicA using the Quickchange Site-directed mutagenesis kit (Stratagene) with MicAmut for and MicAmut rev primers. After sequencing, the AatII-HindIII fragment was cloned in pBRplac to avoid any unwanted mutations.

The plasmid pEnvZ-OmpR (a.k.a pK4-55) was isolated from a genomic library constructed in pHDB3 (Ulbrandt *et al.*, 1997) as an activator of a *rybB-lacZ* fusion (Thompson *et al.*, 2007).

β-galactosidase assays

Cells were grown overnight in LB medium – supplemented with ampicillin, arabinose or IPTG when necessary, see below - and diluted 500-fold in fresh medium. When the OD₆₀₀ reached 0.4, the β-galactosidase activity was assayed as described in (Miller, 1992) using 0.5 ml of culture, or 0.2 ml for experiment shown in Fig. 5A.

Media used in the different experiments are LB (Fig. 2A, 2B, 6A and 6C), LB + ampicillin (Fig. 4E, *rybB-lacZ* fusion), LB + 0.02% arabinose (Fig. 4D and 6B), LB + ampicillin + IPTG 100 μM (Fig. 2C, 3, 5A and 5B), LB + ampicillin + IPTG 100 μM + 0.02% arabinose (Fig. 4B), LB + ampicillin + 0.02% arabinose (Fig. 4E, P_{BAD}-*phoP-lacZ* fusion) or LB + ampicillin + IPTG 100 μM + 0.004% arabinose (Fig. 5C).

Results are the average value of at least two (Fig. 2, 5A, 5B, 6B and 6C) or three (Fig. 3, 4B, 4D and 6A) independent experiments, except for Fig. 4E and 5C where a single representative experiment carried out on two duplicate samples is represented.

RNA extraction and Northern-Blot analysis

RNA was extracted from cells diluted 500-fold from an overnight culture and grown to exponential phase ($OD_{600} \sim 0.4$) in LB medium, supplemented if necessary. For Fig. 2C, RNA was extracted at the same time as samples taken to assess the β -galactosidase activity. Extraction was done as previously described using 650 μ l of culture (Guillier and Gottesman, 2006). A constant amount of total RNA (3 μ g for Fig. 2C and 2.5 μ g for Fig. 4C) was then separated on an 8% acrylamide TBE-urea gel, and transferred in TAE 1X to an Hybond-N+ membrane (Amersham). RNA was finally detected using specific biotinylated probes (sequence in Table S2) and the Brighstar Biodetect kit (Ambion). After boiling in SDS 0.5% for 15 minutes, the same membrane could be hybridized with a different probe.

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Figure Legends

Fig. 1. Model of regulation of *ompT* and *phoPQ* expression. sRNAs are shown in red and two component-systems are in blue. Positive and negative regulation events are indicated by arrows and horizontal bars respectively. Dashed lines correspond to putative regulation events. Note that overproduction of MicA should down-regulate the σ^E response by down-regulating the level of several OMPs.

Fig. 2. Both Hfq and MicA affect *ompT* expression in the absence of OmrA and OmrB.

(A) The β -galactosidase activity of an *ompT-lacZ* translational fusion is increased in an *hfq* mutant (strain MG1194) compared to the *hfq*⁺ isogenic strain (strain MG1188) in the absence of OmrA and OmrB sRNAs. (B) Activity of the same fusion is slightly decreased in an *rseA*⁻ strain, but not in an *rseA*⁻ *micA*⁻ double mutant. Strains used here are MG1173 (wt), MG1447 (*rseA*⁻) and MG1461 (*rseA*⁻ *micA*⁻). (C) The activity of the fusion in a strain lacking *omrA* and *omrB* chromosomal copies was measured after transformation of the MG1188 strain with the pBRplac empty vector (vect.) or its derivatives overexpressing different sRNAs. Numbers on the top of the bars correspond to the repression factors. Levels of the different sRNAs in this experiment were analyzed by Northern-Blot with SsrA used as a loading control.

Fig. 3. Control of *ompT* expression by MicA requires PhoP.

The effect of ectopic overexpression of MicA and OmrA sRNAs on the *ompT-lacZ* fusion was determined in both *phoP*⁺ (A) and *phoP*⁻ (B) backgrounds (strains MG1188 and MG1423 respectively).

Fig. 4. MicA directly represses *phoPQ* expression through base-pairing interaction.

(A) Base-pairing prediction between MicA and *phoPQ* mRNA in *Escherichia coli*. *phoP* translation initiation codon and putative Shine-Dalgarno sequence are in bold. Numbering of MicA refers to transcription initiation. The nature of the compensatory changes introduced in the RNAs is indicated by “mut”. (B) Compensatory changes in MicA and

phoP-lacZ mRNA restore the control of a P_{BAD} -*phoP-lacZ* construct. Strains used in this experiment are MG1430 (P_{BAD} -*phoP-lacZ* fusion) and MG1431 (P_{BAD} -*phoPmut-lacZ* fusion) and are deleted for the chromosomal *micA* gene. (C) Comparison of MicA levels by Northern-Blot when overexpressed from a plasmid or induced from the chromosome using EnvZ-OmpR overproduction or an *rseA*⁻ allele. Blot was also probed for SsrA as a loading control. RNA was extracted from strain MG1425 transformed with pBRplac or pMicA, MG1490 transformed with pHDB3 or pEnvZ-OmpR, or from strain MG1425, MG1459 or MG1460 as described in Experimental Procedures. (D, E) Activity of the P_{BAD} -*phoP-lacZ* fusion is decreased upon induction of *micA* chromosomal copy in a MicA-dependent manner. Strains are MG1425 (wt), MG1459 (*rseA*⁻) and MG1460 (*rseA*⁻ *micA*⁻) on panel D. On panel E, they are MG1490 and MG1491 (P_{BAD} -*phoP-lacZ*, *micA*⁺ and *micA*⁻ respectively), or MG1492 and MG1493 (*rybB-lacZ*, *micA*⁺ and *micA*⁻ respectively), transformed with pHDB3 (-) or pEnvZ-OmpR (+).

Fig. 5. Regulation of different members of the PhoP regulon by MicA.

The β -galactosidase activity of two PhoP-regulated fusions (Moon and Gottesman, 2009) was measured in presence of plasmids overexpressing MicA, MicA^{mut} or OmrA sRNAs. Strains used in this experiment are KM112 (panel A) and KM194 (panel B). (C) A similar experiment was performed with an *mgrR-lacZ* fusion under the control of a PhoP-independent promoter, P_{BAD} (strain MG1484).

Fig. 6. Additional sRNAs regulating *ompT-lacZ* and *phoP-lacZ* expression ?

(A) β -galactosidase activity of *ompT-lacZ* was compared in *hfq*⁺ and *hfq*⁻ isogenic strains, in the presence or absence of MicA and/or OmrA/B. Strains are MG1173 (wt), MG1196 (*hfq*⁻), MG1451 (*micA*⁻), MG1455 (*micA*⁻ *hfq*⁻), MG1188 (*omrAB*⁻), MG1194 (*omrAB*⁻ *hfq*⁻), MG1449 (*micA*⁻ *omrAB*⁻) and MG1450 (*micA*⁻ *omrAB*⁻ *hfq*⁻). The same experiment was done using the P_{BAD} -*phoP-lacZ* fusion in *micA*⁺ and *micA*⁻ strains (B), or the *ompT-lacZ* fusion in a *phoP::kan* context in the absence of MicA or OmrA/B (C). Strains used in panel B are MG1425, MG1453, MG1452 and MG1458 (wt, *hfq*⁻, *micA*⁻ and *hfq*⁻ *micA*⁻ respectively); and in panel C MG1446, MG1454, MG1456, MG1457, MG1465 and MG1466 (wt, *hfq*⁻, *micA*⁻, *hfq*⁻ *micA*⁻, *omrAB*⁻ and *hfq*⁻ *omrAB*⁻ respectively).

Table 1. Strains and plasmids used in this study

Table S1. β -galactosidase activities measured in this study. Shown in this table are the average and the standard deviation of at least two (Fig. 2, 5A, 5B, 6B and 6C) or three (Fig. 3, 4 and 6A) independent experiments. For Fig. 4E and 5C, a single representative experiment done with two duplicate samples is shown. Measurements were done on cells grown to exponential phase (A_{600} of about 0.4) in rich medium (LB (Fig. 2A, 2B, 6A and 6C), LB + ampicillin (Fig. 4E, *rybB-lacZ* fusion), LB + 0.02% arabinose (Fig. 4D and 6B), LB + ampicillin + IPTG 100 μ M (Fig. 2C, 3, 5A and 5B), LB + ampicillin + IPTG 100 μ M + 0.02% arabinose (Fig. 4B), LB + ampicillin + 0.02% arabinose (Fig. 4E, P_{BAD} -*phoP-lacZ* fusion) or LB + ampicillin + IPTG 100 μ M + 0.004% arabinose (Fig. 5C)).

Table S2. Oligonucleotides used in this study

1

Strain	Description	Construction or source
MG1655	Wild-type strain	F. Blattner
DJ480	MG1655 $\Delta lacX174$	D. Jin
DJ624	DJ480 <i>mal::lacI^q</i>	D. Jin
NM300	DJ480 <i>mini-λ tet^R</i>	N. Majdalani
NM1200	MG1655 <i>mini-λ cm^R</i>	N. Majdalani
PM1205	MG1655 <i>mal::lacI^q $\Delta araBAD$ araC⁺ lacI' :: P_{BAD}-cat-sacB-lacZ, mini-λ tet^R</i>	Mandin and Gottesman, 2009
MG1173	DJ624 λ RSompT-lacZ	This study
MG1188	DJ624 λ RSompT-lacZ $\Delta omrAB::kan$	Guillier and Gottesman, 2008
MG1194	MG1188 <i>hfq::cm</i>	This study
MG1196	MG1173 <i>hfq::cm</i>	This study
MG1423	MG1188 <i>phoP::Tn Cm</i>	This study
MG1425	MG1655 <i>mal::lacI^q $\Delta araBAD$ araC⁺ P_{BAD}-phoP-lacZ</i>	This study
MG1430	MG1655 <i>mal::lacI^q $\Delta araBAD$ araC⁺ P_{BAD}-phoP-lacZ</i>	This study
MG1431	MG1655 <i>mal::lacI^q $\Delta araBAD$ araC⁺ P_{BAD}-phoPmut-lacZ</i>	This study
	<i>$\Delta micA::cm$</i>	
MG1446	MG1173 <i>phoP::kan</i>	This study
MG1447	MG1173 <i>ArseA::kan</i>	This study
MG1449	MG1188 <i>$\Delta micA::tet$</i>	This study
MG1450	MG1194 <i>$\Delta micA::tet$</i>	This study
MG1451	MG1173 <i>$\Delta micA::tet$</i>	This study
MG1452	MG1425 <i>$\Delta micA::tet$</i>	This study
MG1453	MG1425 <i>hfq::cm</i>	This study
MG1454	MG1446 <i>hfq::cm</i>	This study
MG1455	MG1196 <i>$\Delta micA::tet$</i>	This study
MG1456	MG1446 <i>$\Delta micA::tet$</i>	This study
MG1457	MG1454 <i>$\Delta micA::tet$</i>	This study
MG1458	MG1453 <i>$\Delta micA::tet$</i>	This study
MG1459	MG1425 <i>ArseA::kan</i>	This study
MG1460	MG1459 <i>$\Delta micA::tet$</i>	This study
MG1461	MG1447 <i>$\Delta micA::tet$</i>	This study
MG1465	MG1446 <i>$\Delta omrAB::tet$</i>	This study
MG1466	MG1454 <i>$\Delta omrAB::tet$</i>	This study
MG1484	MG1655 <i>mal::lacI^q $\Delta araBAD$ araC⁺ P_{BAD}-mgrR-lacZ</i>	This study
MG1490	MG1425 <i>$\Delta ompA::kan$</i>	This study
MG1491	MG1452 <i>$\Delta ompA::kan$</i>	This study
MG1492	KMT12000 <i>$\Delta ompA::kan$</i>	This study
MG1493	KMT12000 <i>$\Delta ompA::kan$ $\Delta micA::tet$</i>	This study
KMT12000	DJ480 <i>rybB-lacZ</i>	Thompson and Gottesman, 2007
KM112	MG1655 <i>mal::lacI^q $\Delta araBAD$ araC⁺ mgrR-lacZ</i>	Moon and Gottesman, 2009
KM194	MG1655 <i>mal::lacI^q $\Delta araBAD$ araC⁺ yneM-lacZ</i>	Moon and Gottesman, 2009

Plasmid	Description	Construction or source
pBRplac	Modified P _{LlacO-1} promoter in pBR322, Amp ^R , Tet ^R	Guillier and Gottesman, 2006
pMicA	<i>micA</i> gene under control of modified P _{LlacO-1} , Amp ^R	This study
pMicAmut	<i>micAmut</i> gene under control of modified P _{LlacO-1} , Amp ^R	This study
pOmrA	<i>omrA</i> gene under control of modified P _{LlacO-1} , Amp ^R , Tet ^R	Guillier and Gottesman, 2006
pRybB	<i>rybB</i> gene under control of modified P _{LlacO-1} , Amp ^R , Tet ^R	This study
pHDB3	Empty vector for the genomic library, derivative of pBR322, Amp ^R	Ulbrandt <i>et al.</i> , 1997
pEnvZ-OmpR	pHDB3 carrying the fragment ' <i>pckA-envZ-ompR-greB-yhgF</i> ', Amp ^R (Plasmid pK4-55 in Thompson and Gottesman, 2007)	Thompson and Gottesman, 2007

1

2 **Table 1.**

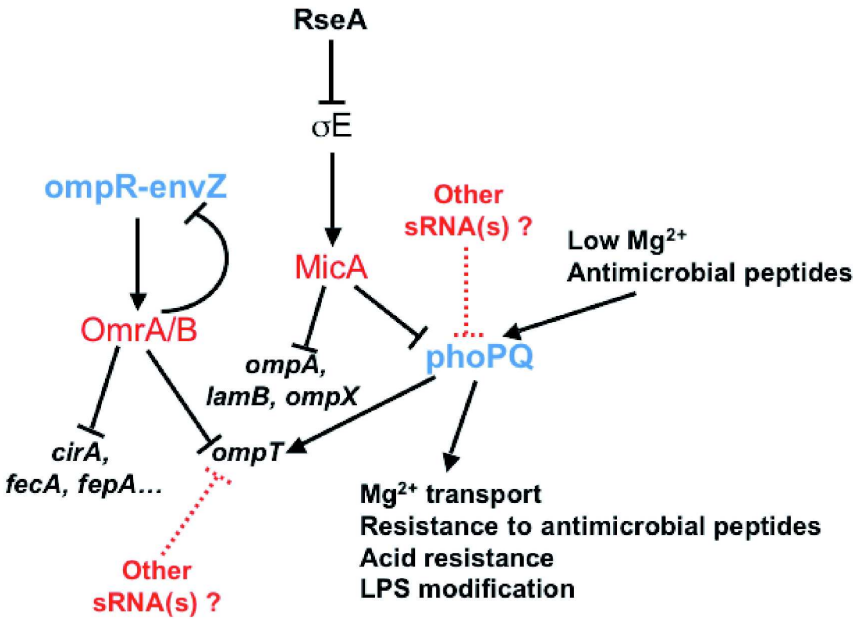


Fig. 1

254x190mm (600 x 600 DPI)

review

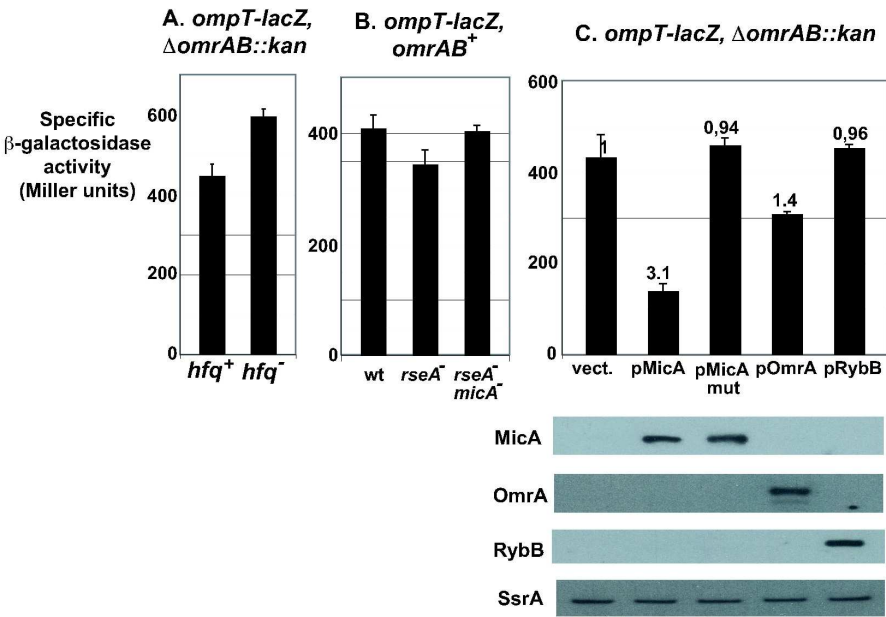


Fig. 2

215x279mm (600 x 600 DPI)

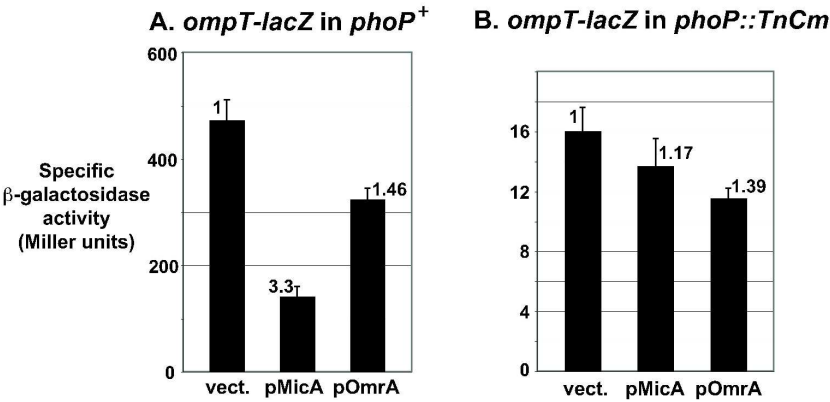


Fig. 3

215x279mm (600 x 600 DPI)

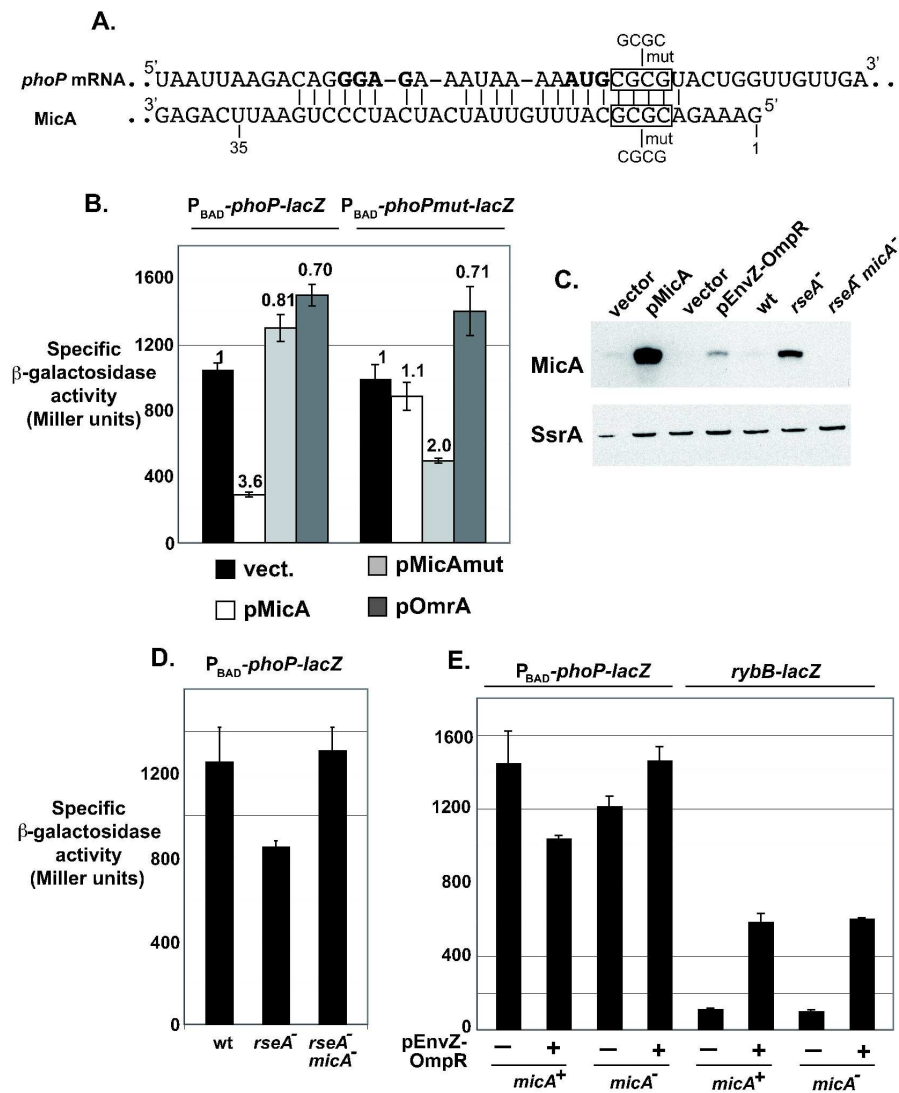


Fig. 4

215x283mm (600 x 600 DPI)

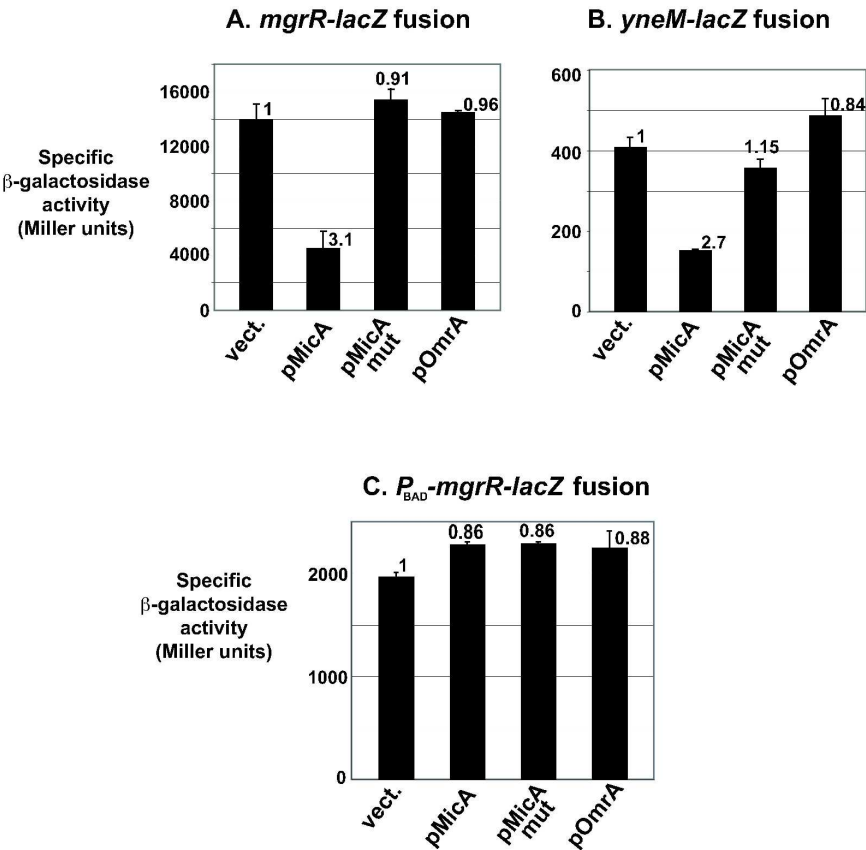


Fig. 5

215x279mm (600 x 600 DPI)

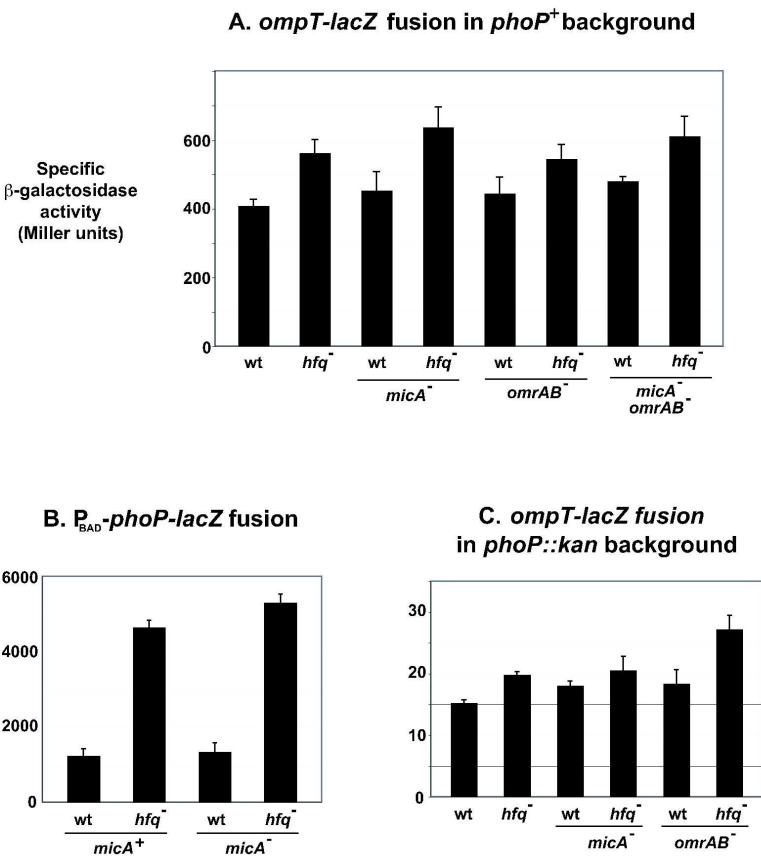


Fig. 6

257x279mm (600 x 600 DPI)