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#### 1 Abstract

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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.

8 We show in this study that the synthesis of the E. coli pleiotropic PhoPQ two-9 component system is repressed by MicA, a  $\sigma$ E-dependent sRNA regulator of porin 10 biogenesis. MicA directly pairs with *phoPQ* mRNA in the translation initiation region of 11 *phoP* and presumably inhibits translation by competiting with ribosome binding. 12 Consequently, MicA down-regulates several members of the PhoPQ regulon. By linking PhoPQ to  $\sigma E$ , our findings suggest that major cellular processes such as Mg<sup>2+</sup> transport, 13 14 virulence, LPS modification or resistance to antimicrobial peptides are modulated in 15 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 Hfg, 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.

Interestingly, regulation of TCS by sRNAs is not restricted to EnvZ-OmpR. Other examples include regulation of *E. coli dpiBA*, encoding a TCS induced by  $\beta$ -lactams and 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 Salmonella. It is induced in response to low Mg<sup>2+</sup> and Ca<sup>2+</sup>, as well as in the presence of 6 antimicrobial peptides. Genes that are regulated by PhoPQ are involved in Mg<sup>2+</sup> transport, 7 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  $\sigma E$  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  $\sigma E$ , 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

#### 1 Results

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## 3 Hfq and MicA affect the expression of *ompT* in the absence of OmrA and OmrB 4 sRNAs

5 Expression of the outer membrane protease OmpT was previously shown to be 6 regulated by two redundant Hfq-binding sRNAs, OmrA and OmrB, via a direct 7 interaction between the conserved 5' end of OmrA/B and the early coding region of the 8 ompT mRNA (Fig. 1, Guillier and Gottesman, 2006; Guillier and Gottesman, 2008). 9 However, in a strain lacking OmrA/B, the expression of an *ompT-lacZ* translational 10 fusion is still 1.3-fold higher in an hfq mutant compared to a wild-type strain (Fig. 2A, 11 Table S1). One possible explanation for this observation is that, in addition to and 12 independently of OmrA/B, other sRNAs could control OmpT synthesis.

13  $\sigma E$  is an alternative sigma factor whose activity is induced under conditions of 14 envelope stress leading to the accumulation of unfolded OMP in the periplasm (Ruiz and 15 Silhavy, 2005).  $\sigma E$  activates the transcription of numerous genes, including genes for 16 periplasmic chaperones and proteases (Mutalik et al., 2009; Rhodius et al., 2006). It also 17 activates transcription of two Hfq-binding sRNAs, RybB and MicA, that down-regulate 18 the synthesis of major OMPs, such as OmpC and OmpA in E. coli, thereby reducing the 19 accumulation of unassembled periplasmic OMPs (Johansen et al., 2006; Papenfort et al., 20 2006; Rasmussen et al., 2005; Thompson et al., 2007; Udekwu et al., 2005; Udekwu and 21 Wagner, 2007). We reasoned that, since OmpT is a rather abundant OMP (Molloy et al., 22 2000), its expression could be repressed by  $\sigma$ E-dependent sRNAs.

1 The activation of  $\sigma E$  relies on a proteolytic cascade that leads to the degradation 2 of RseA, an anti-sigma factor that sequesters  $\sigma E$  at the inner membrane under non-3 inducing conditions (Fig.1, Ades, 2008). To test whether the expression of ompT was affected by  $\sigma E$ , we compared the activity of the *ompT-lacZ* fusion in *rseA*<sup>+</sup> and *rseA*<sup>-</sup> 4 5 strains. There is a decrease of about 1.2 fold in the  $\beta$ -galactosidase activity in the *rseA*<sup>-</sup> 6 strain (Fig. 2B and Table S1). Even though this effect is very modest, it was enough to 7 convince us to look for  $\sigma E$ -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  $\beta$ -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  $\beta$ -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<sup>-</sup> strain 20 (see above), as this effect is abolished in a *micA<sup>-</sup> rseA<sup>-</sup>* double mutant (Fig. 2B, Table S1). 21

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

1 MicA (previously SraD) is an Hfg-binding sRNA that was identified in several 2 searches for bacterial sRNAs (Argaman et al., 2001; Zhang et al., 2003). It was shown to 3 inhibit synthesis of two OMPs, OmpA and LamB, by pairing in the translation initiation 4 region of their mRNAs (Bossi and Figueroa-Bossi, 2007; Rasmussen et al., 2005; 5 Udekwu et al., 2005). However, no obvious complementarity was found between MicA 6 and the *ompT* mRNA region present in our fusion, suggesting that control of *ompT* by 7 MicA might be indirect. The translational *ompT-lacZ* fusion used in this study carries 220 8 nts of ompT upstream of the start codon and its transcription is driven by the ompT9 promoter. Therefore, this reporter fusion should be sensitive to regulation exerted either 10 at the level of transcription and/or translation initiation. 11 *ompT* transcription was shown to be activated by PhoP, the response regulator of 12 the PhoP-PhoQ TCS, probably in a direct manner (Eguchi et al., 2004; Zwir et al., 2005; 13 Fig. 1). Therefore, one possible explanation of our results is that MicA controls ompT14 through PhoPQ. To test this hypothesis, we measured the effect of MicA sRNA on the 15 expression of the *ompT-lacZ* fusion in a strain where *phoP* was inactivated by a 16 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 17 18 repression. The effect of MicA dropped from 3.3-fold in the wt strain to 1.2-fold in the 19 phoP mutant, whereas repression by OmrA was similar in the wt and mutant strains 20 (roughly 1.4-fold) (Fig. 3 and Table S1). These results clearly show that repression of

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ompT by MicA requires PhoP.

#### 23 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-phoO, 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 present, complementarity between MicA and the phoP translation initiation region is 8 9 poorer. This is the case in Yersinia pestis, Klebsiella pneumoniae and Photorhabdus 10 *luminescens* (data not shown).

In order to determine whether the predicted interaction in *E. coli* takes place *in vivo*, we constructed a translational *phoP-lacZ* reporter fusion. This fusion encompasses nts -36 (i.e. transcription start site of the phoPp1 promoter) to +30 of the *phoP* mRNA placed at the *lacZ* locus in frame with the 9<sup>th</sup> codon of *lacZ*. Transcription of this fusion is driven by a  $P_{BAD}$  promoter; only control at the post-transcriptional level is therefore expected to affect its expression.

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

1 activity of an *ompT-lacZ* fusion (Fig. 2C). A *phoP-lacZ* fusion carrying the compensatory 2 changes was no longer controlled by wt MicA, whereas control was partially restored by 3 MicAmut (repression of 1.1- and 2-fold respectively) (Fig. 4B and Table S1). As for the 4 wt phoP-lacZ fusion, overproduction of OmrA up-regulated expression of the mutant 5 fusion by 1.4-fold. The reason for the positive action of OmrA on these fusions, as well 6 as of MicAmut on the wt fusion, is not clear and we suspect that these effects are most 7 likely indirect, possibly through Hfq titration. Together, these results unambiguously 8 show that MicA base-pairs with *phoPO* mRNA around the *phoP* start codon. This leads 9 to down-regulation of *phoP* expression. *phoQ*, in an operon with *phoP*, is also down-10 regulated, as measured with a *phoPQ-lacZ* fusion (data not shown).

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#### 12 MicA sRNA links PhoP synthesis to $\sigma E$ activity

13 In the previous experiment, MicA was expressed from a multicopy plasmid and 14 its transcription was driven by an IPTG-inducible promoter. Because MicA is strongly 15 overexpressed in these experiments (see lane pMicA of Fig. 4C), we decided to look at 16 the effect of MicA induction on *phoP* expression, when MicA was transcribed from its 17 own promoter under more physiological conditions. In a first experiment,  $\sigma E$  was 18 activated using an *rseA*<sup>-</sup> strain. Consistent with a control of *phoPO* by MicA, this resulted in a 1.5-fold decrease in the expression of the P<sub>BAD</sub>-phoP-lacZ fusion. This effect was due 19 20 to MicA, since activity was no longer decreased in an *rseA<sup>-</sup> micA<sup>-</sup>* double mutant (Fig. 4D 21 and Table S1). We then used different envelope stresses to induce  $\sigma E$ . Surprisingly, we 22 found that several of them had no effect on *phoP* expression and so far, we do not have a 23 clear explanation for this. However, other stresses, such as addition of procaine for

1 instance, slightly decreased *phoP* expression in a reproducible manner (data not shown). 2 Procaine most likely induces  $\sigma E$  through EnvZ-OmpR activation, which is expected to 3 result in an elevated synthesis of OMPs. In agreement with this, Thompson and 4 Gottesman isolated a plasmid carrying *envZ-ompR* as a multicopy activator of the  $\sigma E$ -5 dependent *rybB-lacZ* fusion in a genetic screen (Thompson *et al.*, 2007). This plasmid is 6 referred here as pEnvZ-OmpR.

7 In the presence of pEnvZ-OmpR, MicA was produced to a much lower level than 8 in the *rseA* mutant (Fig. 4C, compare lanes pEnvZ-OmpR and *rseA*<sup>-</sup>). Nevertheless, this 9 was sufficient to observe a slight decrease in the expression of *phoP*: 1.4-fold at the best 10 (Fig. 4E and Table S1). Since this effect is modest, we repeated this experiment 7 times, 11 and results were similar. Shown in Fig. 4E is a representative experiment where  $\beta$ -12 galactosidase activity was measured on two duplicate samples. As expected, this decrease 13 is reproducibly dependent on MicA as it is not observed in the *micA* mutant strain. In 14 contrast, pEnvZ-OmpR activates the expression of the  $\sigma$ E-dependent *rybB-lacZ* fusion in a MicA-independent manner (induction of 5.4- and 5.8-fold respectively in  $micA^+$  and 15 16 *micA*<sup>-</sup> cells) (Fig. 4E and Table S1). Note that these experiments were performed in the 17 absence of *ompA*, a major target of MicA. However, in several experiments, a similar effect of pEnvZ-OmpR on *phoP* was observed in *ompA*<sup>+</sup> cells. 18

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#### 20 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).

1 One can therefore wonder what the effect of regulating a TCS will be on the expression 2 of its target-genes. To gain insight into this question, we looked at how MicA 3 overproduction affects the expression of different genes whose transcription is controlled 4 by PhoQ-PhoP, in addition to *ompT*. We focused on two targets of PhoPQ: MgrR, a 5 sRNA regulator of LPS modification (Moon and Gottesman, 2009), and yneM, that 6 encodes a small membrane protein (Hemm et al., 2008). Interestingly, MgrR and yneM are differentially regulated by PhoPQ in response to Mg<sup>2+</sup> levels, with MgrR being 7 expressed even at rather high  $Mg^{2+}$  (half-maximal expression occurs at  $[Mg^{2+}]_{50\%} \sim 5$ 8 mM) whereas *yneM* is expressed only at fairly low  $Mg^{2+}$  ([ $Mg^{2+}$ ]<sub>50%</sub> ~ 0.005 mM), which 9 10 is closer to what is observed for other PhoP targets (Moon and Gottesman, 2009, and 11 Moon and Gottesman, personal communication).

Despite their differential responses to  $Mg^{2+}$ , repression of *lacZ* fusions to these 12 13 two genes by MicA was similar (repression factors of 3.1 and 2.7 for mgrR-lacZ and 14 *yneM-lacZ* respectively), whereas, as expected, activity of these fusions was either not 15 affected or slightly increased by ectopic expression of either MicAmut or OmrA (Fig. 5A, 16 5B, and Table S1). It is interesting that MicA also repressed the activity of *ompT-lacZ* 17 and *phoP-lacZ* fusions to a similar extent (repression of 3.1 and 3.6-fold respectively, see 18 above). Therefore, as for *ompT*, MicA represses *mgrR* and *yneM* expression, presumably 19 through *phoPQ*. In agreement with this, we found that MicA no longer controls the 20 expression of an mgrR-lacZ fusion expressed from the PhoP-independent promoter  $P_{BAD}$ 21 (Fig. 5C and Table S1). It will be interesting to determine whether MicA also controls 22 other targets of this TCS that can be differentially regulated as a function of PhoPQ 23 stimulation (Miyashiro and Goulian, 2007).

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

3 Starting from the observation that ompT expression increases in an hfg 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 compared to the  $hfq^+$  strain, Fig. 6A and Table S1), clearly showing that, as OmrA/B, 10 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 verify this, we measured the activity of this fusion in  $hfq^+$  and  $hfq^-$  strains: hfq18 19 inactivation resulted in a 3.7-fold increase in  $\beta$ -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*<sup>-</sup> 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 this, the activity of ompT-lacZ in both  $hfq^+$  and  $hfq^-$  cells was determined in the absence 3 4 of a functional PhoP regulator. Despite a lower level of expression, the results were similar to those obtained in a  $phoP^+$  strain : the activity was increased by 1.3-fold in the 5 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).

11

#### 12 **Discussion**

13 We show here that PhoPQ synthesis is negatively regulated at the post-14 transcriptional level by MicA, a  $\sigma$ 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.

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

several negatively acting sRNAs (Chen *et al.*, 2004; Udekwu *et al.*, 2005 for instance).
This is likely to be accompanied by degradation of the mRNA, since a decrease in *phoP*mRNA level was observed by transcriptome analysis after MicA induction (Gogol and
Gross, manuscript in preparation). Again, this is true for many sRNAs acting by pairing
(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 *phoPO* 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 MicAmut 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 MicAmut on *lamB* expression has not been tested so far. 17

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

- CyaR and the TIR region of *phoP*, we found that overproduction of CyaR did not affect
   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 TCS regulators, such as PhoP and OmpR, without affecting the inducing signals, clearly 12 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.

More specifically, our results indicate that some conditions of cell envelope stress known to induce the  $\sigma E$  response will lead to repression of the PhoPQ regulon, which includes genes involved in magnesium transport, resistance to antimicrobial peptides, acid resistance and LPS modification. From a physiological standpoint, it is not surprising that resistance to antimicrobial peptides, acid resistance and LPS modification could be affected by cell envelope stress. Other members of the PhoPQ regulon are 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.

3 Interestingly, Moon and Gottesman recently identified *eptB* as a major target of 4 the PhoP-regulated sRNA MgrR. eptB is involved in LPS modification and is regulated 5 by  $\sigma E$ , most likely in an indirect manner (Moon and Gottesman, 2009). While results 6 from Moon and Gottesman demonstrate that there clearly is an effect of  $\sigma E$  on the 7 transcription initiation of *eptB* (K. Moon and S. Gottesman, unpublished results), it is 8 possible that down-regulation of MgrR by MicA (via PhoP) also contributes to the 9 observed  $\sigma E$  up-regulation of *eptB*. Irrespective of the mechanism of this regulation, this 10 provides another example of intertwined regulation of the  $\sigma E$  and PhoPQ regulons.

11

It is also worth noting that the extent of these controls is limited, as is most often 12 13 the case for regulation exerted by sRNAs. For instance, whereas the activity of the *ompT*-14 lacZ fusion used in this study was decreased by almost 30-fold when PhoP was 15 inactivated (Fig. 3), down-regulation of *phoPQ* by MicA resulted only in a 3.1-fold or 16 1.2-fold decrease in *ompT-lacZ* when MicA was overproduced from a plasmid or induced 17 from the chromosome using an *rseA*<sup>-</sup> mutant allele respectively (Fig. 2). Therefore, MicA 18 likely participates in fine-tuning the regulation of *phoPO* under conditions where the  $\sigma E$ 19 regulon is induced, such as envelope stress and to a lesser extent stationary phase. The 20 limited extent of this regulation is highlighted by the fact that induction of MicA from the 21 chromosome has only a modest effect on phoP expression (Fig. 4E). It is possible 22 however that other envelope stresses will have a stronger effect on MicA accumulation 23 and *phoP* control.

1	Our observation of a strong effect of an <i>hfq</i> mutant on the <i>phoP-lacZ</i> fusion, even
2	in the absence of MicA, raises the exciting possibility that sRNAs other than MicA will
3	regulate phoPQ. Two-component systems are widely used in bacteria as a way to sense
4	and adapt to the environment. More than 30 of them have been described in the E. coli
5	genome and it is tempting to speculate that, in addition to PhoPQ, DpiBA and OmpR-
6	EnvZ, many more will be subject to sRNA-mediated regulation. It will also be interesting
7	to elucidate how these controls affect the expression of the downstream genes and the
8	overall physiology of the cell.
9	
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11	We thank E. Gogol and C. Gross for sharing unpublished results. We are grateful to K.
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17	Institute, Center for Cancer Research.
18	
19	Experimental procedures
20	
21	Bacterial strains
22	With the exception of DH5 $\alpha$ that was used as the recipient strain for cloning
23	procedures, all strains used in this study are derivatives of MG1655. They were grown

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

4 Construction of  $\Delta micA::cm$ ,  $\Delta micA::tet$ ,  $\Delta omrAB::tet$  and  $\Delta ompA::kan$  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  $\Delta micA::cm$  and  $\Delta ompA::kan$  or NM1200 for  $\Delta micA::tet$ 9 and  $\Delta omrAB::tet$ , 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),  $\Delta omrAB::kan$  (Guillier and 12 Gottesman, 2006), phoP::TnCm, phoP::kan (Bougdour *et al.*, 2008) and  $\Delta rseA::kan$ 13 (Thompson *et al.*, 2007) were then moved by P1vir transduction as requested. Note that 14 in  $\Delta micA::cm$  and  $\Delta micA::tet$  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).

1 Recombinants were selected on LB plates without NaCl supplemented with 6% sucrose. 2 Chloramphenicol sensitive colonies were purified three times on the same medium and 3 the P<sub>BAD</sub>-phoP-lacZ construct was confirmed by sequencing. For the construction of the 4  $P_{BAD}$ -phoPmut-lacZ fusion, the PCR product was obtained as follows. First, a PCR 5 fragment was amplified from genomic DNA of strain MG1655 using primers phoP-lac 6 for and phoPmut (Table S2) and the Phusion polymerase. This reaction was then treated 7 with DpnI enzyme, purified and the resulting PCR product was used as a template in a 8 second PCR reaction, with primers phoP-lacZ for and phoP-lacZ rev and Phusion DNA 9 polymerase. Recombination in strain PM1205 and selection of recombinants was then 10 carried out as above. 11 To construct the  $P_{BAD}$ -mgrR-lacZ fusion (strain MG1484), a PCR product synthesized with the partially complementary primers 5'PBAD-mgrR and 3'mgrR-lacZ 12

was recombined in strain PM1205 as previously. Recombinants were selected on M9
plates containing 6% sucrose, 0.2% arabinose and 20µg/ml Xgal. Verification of
recombinants was as above.

16

17 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

1 digested with EcoRI and AatII (RybB) or AatII and HindIII (MicA) and ligated in the 2 pBRplac vector cut with the same enzymes. Plasmids were checked by sequencing. 3 *pMicAmut* : mutation was introduced in pMicA using the Quickchange Site-4 directed mutagenesis kit (Stratagene) with MicAmut for and MicAmut rev primers. After 5 sequencing, the AatII-HindIII fragment was cloned in pBRplac to avoid any unwanted 6 mutations. 7 The plasmid pEnvZ-OmpR (a.k.a pK4-55) was isolated from a genomic library 8 constructed in pHDB3 (Ulbrandt et al., 1997) as an activator of a rybB-lacZ fusion 9 (Thompson *et al.*, 2007). 10 11  $\beta$ -galactosidase assays Cells were grown overnight in LB medium - supplemented with ampicillin, 12 13 arabinose or IPTG when necessary, see below - and diluted 500-fold in fresh medium. 14 When the  $OD_{600}$  reached 0.4, the  $\beta$ -galactosidase activity was assayed as described in 15 (Miller, 1992) using 0.5 ml of culture, or 0.2 ml for experiment shown in Fig. 5A. 16 Media used in the different experiments are LB (Fig. 2A, 2B, 6A and 6C), LB + 17 ampicillin (Fig. 4E, rybB-lacZ fusion), LB + 0.02% arabinose (Fig. 4D and 6B), LB + 18 ampicillin + IPTG 100 µM (Fig. 2C, 3, 5A and 5B), LB + ampicillin + IPTG 100 µM + 19 0.02% arabinose (Fig. 4B), LB + ampicillin + 0.02% arabinose (Fig. 4E, P<sub>BAD</sub>-phoP-lacZ 20 fusion) or LB + ampicillin + IPTG 100  $\mu$ M + 0.004% arabinose (Fig. 5C). 21 Results are the average value of at least two (Fig. 2, 5A, 5B, 6B and 6C) or three 22 (Fig. 3, 4B, 4D and 6A) independent experiments, except for Fig. 4E and 5C where a 23 single representative experiment carried out on two duplicate samples is represented.

1

2 RNA extraction and Northern-Blot analysis

3	RNA was extracted from cells diluted 500-fold from an overnight culture and
4	grown to exponential phase (OD <sub>600</sub> ~ 0.4) in LB medium, supplemented if necessary. For
5	Fig. 2C, RNA was extracted at the same time as samples taken to assess the $\beta$ -
6	galactosidase activity. Extraction was done as previously described using 650 $\mu$ l of
7	culture (Guillier and Gottesman, 2006). A constant amount of total RNA (3 µg for Fig.
8	2C and 2.5 $\mu$ g for Fig. 4C) was then separated on an 8% acrylamide TBE-urea gel, and
9	transferred in TAE 1X to an Hybond-N+ membrane (Amersham). RNA was finally
10	detected using specific biotinylated probes (sequence in Table S2) and the Brighstar
11	Biodetect kit (Ambion). After boiling in SDS 0.5% for 15 minutes, the same membrane
12	could be hybridized with a different probe.
13	
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#### 1 Figure Legends

2

**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  $\sigma E$ response by down-regulating the level of several OMPs.

8

## 9 Fig. 2. Both Hfq and MicA affect *ompT* expression in the absence of OmrA and 10 OmrB.

11 (A) The  $\beta$ -galactosidase activity of an *ompT-lacZ* translational fusion is increased in an 12 *hfq* mutant (strain MG1194) compared to the  $hfq^+$  isogenic strain (strain MG1188) in the 13 absence of OmrA and OmrB sRNAs. (B) Activity of the same fusion is slightly decreased 14 in an *rseA<sup>-</sup>* strain, but not in an *rseA<sup>-</sup>* micA<sup>-</sup> double mutant. Strains used here are MG1173 15 (wt), MG1447 (rseA) and MG1461 (rseA) micA). (C) The activity of the fusion in a 16 strain lacking *omrA* and *omrB* chromosomal copies was measured after transformation of 17 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 18 19 repression factors. Levels of the different sRNAs in this experiment were analyzed by 20 Northern-Blot with SsrA used as a loading control.

21

#### 22 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).

26

#### 27 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

1 *phoP-lacZ* mRNA restore the control of a P<sub>BAD</sub>-*phoP-lacZ* construct. Strains used in this 2 experiment are MG1430 (P<sub>BAD</sub>-phoP-lacZ fusion) and MG1431 (P<sub>BAD</sub>-phoPmut-lacZ 3 fusion) and are deleted for the chromosomal micA gene. (C) Comparison of MicA levels 4 by Northern-Blot when overexpressed from a plasmid or induced from the chromosome 5 using EnvZ-OmpR overproduction or an *rseA*<sup>-</sup> allele. Blot was also probed for SsrA as a 6 loading control. RNA was extracted from strain MG1425 transformed with pBRplac or 7 pMicA, MG1490 transformed with pHDB3 or pEnvZ-OmpR, or from strain MG1425, 8 MG1459 or MG1460 as described in Experimental Procedures. (D, E) Activity of the 9  $P_{BAD}$ -phoP-lacZ fusion is decreased upon induction of micA chromosomal copy in a 10 MicA-dependent manner. Strains are MG1425 (wt), MG1459 (rseA<sup>-</sup>) and MG1460 (rseA<sup>-</sup>) 11 *micA*) on panel D. On panel E, they are MG1490 and MG1491 (P<sub>BAD</sub>-phoP-lacZ, micA<sup>+</sup>) 12 and micA<sup>-</sup> respectively), or MG1492 and MG1493 (rybB-lacZ, micA<sup>+</sup> and micA<sup>-</sup> 13 respectively), transformed with pHDB3 (-) or pEnvZ-OmpR (+).

14

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

16 The  $\beta$ -galactosidase activity of two PhoP-regulated fusions (Moon and Gottesman, 2009) 17 was measured in presence of plasmids overexpressing MicA, MicAmut or OmrA sRNAs. 18 Strains used in this experiment are KM112 (panel A) and KM194 (panel B). (C) A 19 similar experiment was performed with an *mgrR-lacZ* fusion under the control of a PhoP-20 independent promoter, P<sub>BAD</sub> (strain MG1484).

21

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

(A)  $\beta$ -galactosidase activity of *ompT-lacZ* was compared in  $hfq^+$  and  $hfq^-$  isogenic strains, 23 24 in the presence or absence of MicA and/or OmrA/B. Strains are MG1173 (wt), MG1196 25 (hfq<sup>-</sup>), MG1451(micA<sup>-</sup>), MG1455 (micA<sup>-</sup> hfq<sup>-</sup>), MG1188 (omrAB<sup>-</sup>), MG1194 (omrAB<sup>-</sup> hfq<sup>-</sup>) 26 ), MG1449 (*micA<sup>-</sup> omrAB<sup>-</sup>*) and MG1450 (*micA<sup>-</sup> omrAB<sup>-</sup> hfq<sup>-</sup>*). The same experiment was 27 done using the  $P_{BAD}$ -phoP-lacZ fusion in micA<sup>+</sup> and micA<sup>-</sup> strains (B), or the ompT-lacZ 28 fusion in a phoP::kan context in the absence of MicA or OmrA/B (C). Strains used in 29 panel B are MG1425, MG1453, MG1452 and MG1458 (wt, hfg<sup>-</sup>, micA<sup>-</sup> and hfg<sup>-</sup> micA<sup>-</sup> 30 respectively); and in panel C MG1446, MG1454, MG1456, MG1457, MG1465 and 31 MG1466 (wt, *hfq<sup>-</sup>*, *micA<sup>-</sup>*, *hfq<sup>-</sup>* micA<sup>-</sup>, *omrAB<sup>-</sup>* and *hfq<sup>-</sup>* omrAB<sup>-</sup> respectively).

2 Table 1. Strains and plasmids used in this study

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4 Table S1.  $\beta$ -galactosidase activities measured in this study. Shown in this table are the 5 average and the standard deviation of at least two (Fig. 2, 5A, 5B, 6B and 6C) or three 6 (Fig. 3, 4 and 6A) independent experiments. For Fig. 4E and 5C, a single representative 7 experiment done with two duplicate samples is shown. Measurements were done on cells 8 grown to exponential phase (A<sub>600</sub> of about 0.4) in rich medium (LB (Fig. 2A, 2B, 6A and 9 6C), LB + ampicillin (Fig. 4E, rybB-lacZ fusion), LB + 0.02% arabinose (Fig. 4D and 10 6B), LB + ampicillin + IPTG 100 µM (Fig. 2C, 3, 5A and 5B), LB + ampicillin + IPTG 11  $100 \,\mu\text{M} + 0.02\%$  arabinose (Fig. 4B), LB + ampicillin + 0.02\% arabinose (Fig. 4E, P<sub>BAD</sub>-12 *phoP-lacZ* fusion) or LB + ampicillin + IPTG 100  $\mu$ M + 0.004% arabinose (Fig. 5C)). 13

14 Table S2. Oligonucleotides used in this study

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MG1655Wild-type strainF. BlattnerDJ480MG1655 $\Delta lac X174$ D. JinDJ624DJ480 mal::lacl <sup>q</sup> D. JinNM300D1480 mini $\lambda$ tat <sup>R</sup> N. Maidalani	
DJ480MG1655 $\Delta lac X174$ D. JinDJ624DJ480 $mal::lacI^q$ D. JinNM300DI480 mini $\lambda tat^R$ N. Maidalani	
DJ624 DJ480 mal:: $lacI^q$ D. Jin NM300 D1480 mini 2 tat <sup>R</sup> N. Maidalani	
NM300 D1480 mini $1 \text{ tot}^R$ N Maidalani	
$1 \sqrt{1} \sqrt{1} \sqrt{1} \sqrt{1} \sqrt{1} \sqrt{1} \sqrt{1} 1$	
NM1200 MG1655 mini- $\lambda cm^R$ N. Majdalani	
PM1205 MG1655 mal::lacl <sup>q</sup> $\Delta araBAD araC^+$ lacl' :: $P_{BAD}$ -cat- Mandin and Gottesman, 2009	
$sacB-lacZ, mini-\lambda tet^R$	
MG1173 DJ624 $\lambda$ RS <i>ompT-lacZ</i> This study	
MG1188 DJ624 $\lambda$ RS <i>ompT-lacZ <math>\Delta</math>omrAB::kan</i> Guillier and Gottesman, 2008	
MG1194 MG1188 hfq::cm This study	
MG1196 MG1173 hfq::cm This study	
MG1423 MG1188 phoP::TnCm This study	
MG1425 MG1655 mal:: $lacI^{q} \Delta araBAD araC^{+} P_{BAD}-phoP-lacZ$ This study	
MG1430 MG1655 mal:: $lacI^{q} \Delta araBAD araC^{+} P_{BAD}-phoP-lacZ$ This study	
$\Delta micA::cm$	
MG1431 MG1655 mal:: $lacI^{4} \Delta araBAD araC^{+} P_{BAD}-phoPmut-lacZ$ This study	
AmicA::cm MG1446 MG1173 phoP::kan This study	
MG1447 MG1173 ArseA ···kan This study	
MG1449 MG1188 AmicAtet This study	
MG1450 MG1194 AmicAtet This study	
MG1450 MG1174 ZmicAtet This study	
MG1452 MG1425 AmicA::tet This study	
MG1453 MG1425 hfa::cm This study	
MG1454 MG1446 hfa::cm This study	
MG1455 MG1196 AmicAtet This study	
MG1456 MG1446 AmicAtet This study	
MG1457 MG1454 AmicAtet This study	
MG1458 MG1453 AmicAtet This study	
MG1459 MG1425 ArseA ··· kan This study	
MG1460 MG1459 AmicAtet This study	
MG1461 MG1447 AmicAtet This study	
MG1465 MG1446 AmmrABtet This study	
MG1466 MG1454 AomrABtet This study	
MG1484 MG1655 mal: $lacl^q$ AaraBAD araC <sup>+</sup> P <sub>BAD</sub> -mgrR-lacZ This study	
MG1490 MG1425 AcmpA::kan This study	
MG1491 MG1452 AompA::kan This study	
MG1492 KMT12000 AompA··kan This study	
MG1493 KMT12000 AompA::kan AmicA::tet This study	
KMT12000 DJ480 rvbB-lacZ Thompson and Gottesman. 2007	
KM112 MG1655 mal::lacI <sup>q</sup> $\Delta$ araBAD araC <sup>+</sup> mgrR-lacZ Moon and Gottesman, 2009	
KM194 MG1655 mal:: $lacI^q \Delta araBAD araC^+$ yneM-lacZ Moon and Gottesman, 2009	

Plasmid	Description	Construction or source
pBRplac	Modified P <sub>Llac0-1</sub> promoter in pBR322, Amp <sup>R</sup> , Tet <sup>R</sup>	Guillier and Gottesman, 2006
pMicA	<i>micA</i> gene under control of modified P <sub>LlacO-1</sub> , Amp <sup>R</sup>	This study
pMicAmut	<i>micAmut</i> gene under control of modified P <sub>LlacO-1</sub> , Amp <sup>R</sup>	This study
pOmrA	<i>omrA</i> gene under control of modified P <sub>LlacO-1</sub> , Amp <sup>R</sup> , Tet <sup>R</sup>	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 <sup>R</sup>	Ulbrandt et al., 1997
pEnvZ-	pHDB3 carrying the fragment 'pckA-envZ-ompR-greB-	Thompson and Gottesman, 2007
OmpR	yhgF', Amp <sup>R</sup> (Plasmid pK4-55 in Thompson and	
-	Gottesman, 2007)	
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2 <b>Table 1.</b>		

- 1
- 2 Table 1.



Fig. 1





Fig. 2

215x279mm (600 x 600 DPI)



#### Fig. 3

215x279mm (600 x 600 DPI)

1.17

<sub>1</sub>1.39



Fig. 4

215x283mm (600 x 600 DPI)



C. PBAD-mgrR-lacZ fusion



Fig. 5

215x279mm (600 x 600 DPI)



#### A. ompT-lacZ fusion in phoP<sup>+</sup>background

#### B. PBAD-phoP-lacZ fusion

C. ompT-lacZ fusion in phoP::kan background



Fig. 6

257x279mm (600 x 600 DPI)