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The 216 bp *marB* gene of the *marRAB* operon in *Escherichia coli* encodes a periplasmic protein which reduces the transcription rate of *marA*

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Abstract

The *marRAB* operon is conserved in seven genera of enteric bacteria (*Escherichia, Shigella, Klebsiella, Enterobacter, Salmonella, Cronobacter,* and *Citrobacter*). MarA is a transcriptional regulator affecting many genes involved in resistance to stresses, and MarR is an autorepressor of the operon, but a role for the *marB* gene has been unclear. A recent work reported that deletion of *marB* causes resistance to certain stresses and increases the amount of *marA* transcript. We show here that the small (216 bp) *marB* gene encodes a protein, not an sRNA, since two different stop codons within the predicted open reading frame of *marB* prevented plasmid-borne *marB* from complementing Δ*marB::Kan*. The Δ*marB::Kan* mutation did not increase the stability of the *marA* transcript, suggesting that MarB does not destabilize the *marA* transcript but rather reduces its rate of transcription. Placing the putative signal sequence of MarB upstream of signal-sequence-less alkaline phosphatase guided the phosphatase to its normal periplasmic location. We conclude that MarB is a small periplasmic protein that represses the *marRAB* promoter by an indirect mechanism, possibly involving a signal to one of the cytoplasmic regulators of that promoter.

Keywords

transcript stability; small protein; regulation

Introduction

Regulation of the expression of the *marRAB* (multiple antibiotic resistance) operon in *Escherichia coli* includes both autorepression by MarR and autoactivation by MarA (Martin *et al*., 1995; Seone & Levy 1995; Alekshum & Levy, 1997). This operon is expressed from a promoter upstream of *marR* that contains the *marO* operator targeted by MarR. MarR is a member of a more widely–recognized regulatory “MarR” family (Sulavik *et al*., 1995) and is inactivated by binding to salicylate, menadione, plumbagin, and 2,4-dinitrophenol (Alekshun & Levy, 1999). MarA is known to directly regulate about forty genes, mostly as an activator (Martin & Rosner, 2002). It mediates multidrug resistance in *E. coli* by up-regulating expression of the AcrAB-TolC multidrug efflux pump (Li & Nikaido, 2004) and of MicF (Cohen *et al*., 1988), a small inhibitory RNA that down-regulates translation of the

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outer membrane porin OmpF. The genes of the MarA regulon are involved in a wide spectrum of activities including oxidative stress, resistances to antibiotics, solvents, bile salts, and to other hazardous substances, as well as functions in energy metabolism and nutrient transport (Alekshun & Levy, 1997).

Although the *marRAB* operon is conserved in seven genera of enteric bacteria (*Escherichia, Shigella, Klebsiella, Enterobacter, Salmonella, Cronobacter,* and *Citrobacter*), the role of the small *marB* gene has been unclear. Nichols *et al.* (2011) found that deletion of *marB* in *E. coli* increases the amount of *marA* transcript and increases the resistance to a number of drugs and stresses in a manner correlating with the effects of a deletion of *marR*. Lee & Mitchell (2012) reported that a *marB* deletion increases expression of *inaA,* known to be directly upregulated by the MarA protein. To better understand the role of the *marB* gene, we investigated whether the gene product MarB acts as a protein or an sRNA, how MarB decreases the level of *marA* transcript, and in which compartment of the cell MarB is located.

Materials and Methods

Strains and plasmids

The bacterial strains and plasmids used in this study are described in Table 1. The Δ*marB* deletion parental strain LV22 was obtained from strain JW1525 by removal of the kanamycin resistance gene, using plasmid pCP20 as previously described (Datsenko & Wanner, 2000). The complementation plasmid pMB102-marB was constructed by replacing *marA* of pMB102 (between HindIII/BamHI sites) with *marB* with its native Shine-Dalgarno motif. We used primers MarB-pMB102-F (5′-TTACCCAAGCTTAACAGCTAGTTGAAAACGTGAC-3′ with HindIII restriction site underlined) and MarB-pMB102-R (5′-AATCGCGGATCCGATGTCGGGGCCAGAACA-3′ with BamHI restriction site underlined) to create the 303 bp *marB* amplicon used in this construction. The DNA sequence of all constructs was confirmed (Tufts University Core Facility).

Gene expression and transcript half-life

Reverse transcription followed by real-time quantitative PCR (RT-qPCR) was used to determine the expression levels of *marA* in the wild type (BW25113), Δ*marB::Kan* (JW1525), and Δ*marB* (LV22) strains, and of *marR* in the wild type and Δ*marB::Kan* strains. *gapA*, which encodes glyceraldehyde-3-phosphate dehydrogenase, was used as an endogenous reference gene according to the method of Viveiros *et al.* (2007). The specific primers for RT-qPCR of *marA* were: marA–F: CATAGCATTTTGGACTGGAT and marA–R: TACTTTCCTTCAGCTTTTGC (Viveiros *et al.*, 2007), yielding a 187 bp fragment from the middle of *marA*. The primers for *gapA* and *marR* are described elsewhere (Viveros *et al.*, 2007). These primers were also employed to generate the PCR DNA products used to perform qPCR standard plots for each gene.

Cultures of the different strains were inoculated from −80°C stocks into LB broth, grown at 37°C overnight, diluted 1:1000 into fresh LB, and grown for 2 to 3 h to an optical density at 600 nm (OD_{600}) of about 0.3 (Beckman DU530 spectrophotometer). Then, total RNA in the cultures was stabilized using RNAprotect bacterial reagent (Qiagen), bacteria were digested with lysozyme-proteinase K, and total RNA was isolated using an RNeasy minikit (Qiagen) and On-Column DNase I digestion (Qiagen) according to the manufacturer's specifications. RNAs were then treated with RQ1 RNase-free DNase (Promega) to remove any remaining DNA, repurified with the RNeasy minikit, and RNA purity and concentration were determined using a NanoDrop ND-1000 spectrophotometer. Reverse transcription was

performed using the SuperScript III first-strand synthesis system (Invitrogen) with the specific primers aforementioned and 200 ng of RNA ("RT-plus" reactions). Reactions without reverse transcriptase ("RT-minus" reactions) were used as controls to confirm the lack of contaminating DNA in the RNA samples. The cDNAs obtained from the RT-plus and RT-minus reactions were quantified after 45 cycles using a Mx3000P detection system (Stratagene) with 25 μl reaction mixtures of primers (300 nM each), cDNA (4 μl of a 1:10 dilution of the RT-plus or RT-minus reaction mixture), and 2X QuantiTect SYBR Green qPCR master mix from Qiagen (12.5 μl). Absolute transcript numbers were calculated using standard plots obtained by qPCR of serial dilutions of gene-specific, gel-purified PCR products of known concentrations.

For the *marA* mRNA half-life experiments in the parental strain and Δ*marB::Kan* mutant, overnight cultures of each strain were diluted and grown as before. RNAs were then extracted before (0 min) and 0.5, 1, 2, 3, 4, 6, and 8 min after addition of rifampin (500 μg ml^{-1} final concentration) to stop RNA synthesis. The number of *marA* transcripts was determined by RT-qPCR in three independent experiments for each strain as described above and the values were averaged. The slope (−k) of a linear regression fit of the curve for a plot of ln[average *marA*] vs time in rifampin was determined using Microsoft Excel software and the half-life (= ln2/k) was calculated.

Stop codons in MarB

Two different stop codons were placed singly in *marB* by a single base pair change at codons 23 and 42 of the 72-codon MarB open reading frame in the pMB102-marB complementation plasmid. To make these mutations we used the megaprimer method (Colosimo *et al.*, 1999) with pMB102-marB as template; a first round of PCR was done to create the first stop codon, named 42-STOP, with an external forward primer MarB-pMB102-F (mentioned above) and a mutated reverse primer MarB-mut42-R (5′-CAAACGGTGGGTGTTCCT**A**CGATGGGGGAACAACC-3′ with the mutation **A**), generating a 194 bp mutant megaprimer. In the case of the second stop codon, named 23-STOP, we used the same MarB-pMB102-F forward primer mentioned above and a different mutated reverse primer MarB-mut23-R (5′-CAACTGGCTGCGTGGTTT**A**TTCCGCAACGCCCTGCGC-3′ with the mutation **A**), generating a 137 bp mutant megaprimer. In each case, a second PCR was carried out using each mutant megaprimer plus forward (MarB-pMB102-F) and reverse (MarB-pMB102-R) external primers, both described above. The 303 bp *marB* amplicon thus generated was cloned between the HindIII and BamHI sites of pMB102. The mutations were verified by sequencing and the mutated plasmids were introduced into wild type BW25113 and its Δ*marB::kan* derivative by electroporation.

Fusion of *marB* to the gene for alkaline phosphatase (*phoA*)

PhoA localizes to the periplasm because of its native amino-terminal signal sequence. The following three regions were cloned at the single PstI site of plasmid pCH58 (Hoffman & Wright, 1985; Hoffman, 1986) upstream of *phoA* lacking its native signal sequence: 1) the entire *marB* gene; 2) the isolated putative signal sequence of *marB*; and 3) the known native signal sequence of *phoA* (as a control). In pCH58, upstream of the PstI site is the *bla* (β-lactamase) promoter followed by the first 4 codons of Bla (insufficient for localization), while downstream of the PstI site is a modified *phoA* gene missing both its entire signal sequence and the following 13 codons. The *phoA* sequences of pCH58 had been derived from a PstI-BamHI fragment from pCH48 (Hoffman, 1986), while the *bla*/Bla sequences came from the PstI-BamHI small fragment of pKT218 (Talmadge *et al.*, 1981).

Cloning details are as follows. In each primer, the PstI site is underlined. The entire *marB* gene lacking its stop codon was cloned between (and in frame with) Bla and PhoA using a wild type chromosomal template amplified by forward primer marB-PstI-F (5′-AAAGCTGCAGCTATGAAACCACTTTCATCCGCAA-3′) and reverse primer marB-PstI-R-new (5′-AAAGCTGCAGACATAGCGTGTTGATTATAATAG-3′). The resulting plasmid was named pCH58-marB. The *marB* signal sequence alone was inserted using the marB-PstI-F primer described above and reverse primer marB-SS-PstI-R (5′-AAAGCTGCAGCTTCCGCAACGCCCTGCGCGGAA-3′), creating plasmid pCH58-marB-SS. In a similar fashion, the native *phoA* signal sequence was inserted using the following primer pair: PhoA-PstI-F (5′-AAAGCTGCAGCTGTGAAACAAAGCACTATTGC-3′) and PhoA-PstI-R-new (5′-AAAGCTGCAGCCCGGTTTTCCAGAACAGG-3′), creating plasmid pCH58-SS, which was used a positive control. Transformants were selected on LB plates supplemented with 20 μg tetracycline ml^{-1}. Plasmids containing the sequences cloned in the forward orientation were found/verified using DNA sequencing.

Alkaline phosphatase activity was measured on cells grown in LB broth by the production of *p*-nitrophenol from *p*-nitrophenyl phosphate as described by Manoil *et al.* (2000). Units of alkaline phosphatase activity were defined as 1000 X [change in A_{420} per OD_{600} Unit per minute]. The number of OD_{600} Units was found by multiplying the OD_{600} by the volume of culture assayed (ml).

Statistical analysis

At least three determinations were made for each experiment; we report the mean and the standard error of the mean (SEM). The statistical significance of differences between two means was determined by unpaired Student's *t* test (two independent samples with equal variance, with two-tailed distribution), using Microsoft Excel software.

Results and Discussion

MarB reduces the expression of *marA* and *marR*

A recent work reported that deletion of the *marB* gene of *E. coli* enhances transcription of *marA* (Nichols *et al.*, 2011). To confirm this unexpected finding, we studied the expression level of *marA* by RT-qPCR in wild type, Δ*marB::Kan*, and Δ*marB* strains (BW25113, JW1525, and LV22 respectively). We found that the Δ*marB::Kan* strain had 3.8 fold more *marA* transcript than did the wild type strain; while the Δ*marB* strain had 2.5 fold more (Fig. 1a); the difference between Δ*marB::Kan* and Δ*marB* was statistically insignificant ($P = 0.32$). These results confirmed that a *marB* deletion increased the level of *marA*.

Complementation of the Δ*marB::Kan* mutation with *marB* on a plasmid (pMB102-marB) reduced transcription of *marA* to wild type levels (Fig. 1b), showing that the effect of Δ*marB::Kan* was due to the loss of *marB* and not to the insertion of *Kan* per se. In the wild type strain, the number of *marR* transcripts per ng total RNA (5790+/−566) was very similar to that of *marA* (5541+/−1152), consistent with cotranscription of both genes in the *marRAB* operon regulated by the known *mar* promoter. The number of *marR* transcripts was enhanced 2.2-fold by the Δ*marB::Kan* mutation (up to 12700 +/− 1520). The difference between the *marR* result and the 3.8-fold increase seen for the *marA* transcript was of borderline statistical significance ($P = 0.056$), suggesting that both *marR* and *marA* were affected similarly by Δ*marB::Kan*.

Δ*marB::Kan* did not increase the stability of the *marA* transcript

There are two ways in which Δ*marB::Kan* might enhance the amount of *marA* and *marR* transcripts: 1) increase transcript stability and 2) increase the rate of transcription. We compared the stabilities of the *marA* mRNA transcripts of the parental strain and the Δ*marB::kan* mutant. We found no differences between their half-lives (2.6 min vs 2.3 min; Fig. 2). Therefore, Δ*marB::Kan* presumably caused an increase in the rate of *marA* transcription, probably from the *marRAB* promoter.

MarB is a protein

Since the *marB* gene has a consensus ribosomal binding site (Cohen *et al.*, 1993) and the open reading frame has a clear periplasmic signal sequence, it has been assumed that the small *marB* gene (216 bp) encodes a protein rather than a small RNA (Misra *et al.,* 2005). We investigated whether this assumption was valid. If MarB acted as a protein, it would be inactivated by a single bp change that created a stop codon within the open reading frame. We therefore converted the CAG codon for residue Glu42 of MarB in pMB102-marB to TAG (42-STOP). A second stop codon was also made in a second *marB* plasmid: CAA for residue Glu23 was converted to TAA (23-STOP). Neither of these minute changes was predicted by MFOLD (MFOLD, 2003) to influence the folding of *marB* mRNA. As before, we measured the level of *marA* transcript in the wild type harboring vector pJPBH and in the Δ*marB::Kan* strain harboring pMB102-marB-42-STOP, pMB102-marB-23-STOP, or the vector control pJPBH. We found that each stop codon prevented complementation by *marB*: the number of *marA* transcripts did not decrease (Fig. 3), though they had decreased with wild type *marB* (Fig. 1b). These results proved that MarB indeed acts as a protein.

Localization of MarB to the periplasm

If MarB protein was in the periplasm, as predicted (Misra *et al.*, 2005), this would put constraints on how it might function. For example, a previous suggestion that MarB might interact with and inhibit the cytoplasmic MarA/RNA polymerase complex (Nichols *et al.*, 2011) would not be possible. It was important therefore to test for the location of MarB. Efforts to localize MarB using a MarB-6H fusion protein constructed via the expression vector pET21b (Novagen) failed: no polyhistidine fusion protein was detectable at any IPTG level in any cellular fraction by Western blotting using anti-6H.

Therefore, we took an approach involving *phoA*, the gene for the periplasmic enzyme alkaline phosphatase (PhoA). PhoA is active only when it has been brought to the periplasm by an amino terminal signal sequence, and this fact has been used to identify signal sequences (Hoffman & Wright, 1985; Varga & Kaplan 1989, Okamoto *et al.*, 1991). We fused the entire *marB* gene, including its predicted signal sequence, to a downstream modified *phoA* gene missing its own native signal sequence, to find whether PhoA activity would result in cells. The vector was plasmid pCH58, in which transcription occurs from the *bla* (β-lactamase) promoter; the 4 amino-terminal codons of the Bla signal sequence were fused in frame to modified PhoA (see Materials and Methods). The *marB* gene, cloned at the PstI site, was in frame both with the 4 Bla codons (upstream) and the modified PhoA (downstream), generating a Bla-MarB-PhoA fusion protein in pCH58-marB. We also cloned just the putative 21-codon MarB signal sequence itself, without the rest of MarB, creating pCH58-marB-SS. Finally, as a control, the native 21-codon signal sequence of PhoA was similarly placed, creating pCH58-SS.

The results in Table 2 show that wild type strain BW25113 carrying the plasmid with the entire *marB* sequence produced a much higher PhoA activity (390 units, pCH58-marB) than did the strain itself (4.3 units) or the strain with vector pCH58 (22 units). As anticipated, the 4 residues of the signal of periplasmic Bla protein in vector pCH58 were not enough to

locate PhoA to the periplasm. The construct with the MarB signal sequence alone produced the same level (410 units, pCH58-marB-SS) as did the construct with the entire *marB* sequence, showing that the rest of the MarB sequence had no effect. Therefore the MarB signal sequence alone was sufficient to export PhoA to the periplasm, showing that MarB is indeed a periplasmic protein.

The 4-fold higher activity seen for the control plasmid containing the native PhoA signal sequence (1600 units, pCH58-SS) when compared to the MarB signal could reflect a more efficient export of PhoA by the native PhoA signal sequence or a greater number of native PhoA molecules in the cell.

Possible mode of action of MarB

We found that negative regulation of *marA* expression by MarB appears to be at the level of transcript formation rather than transcript stability. Nichols *et al.* (2011) reported, although without presentation of data, that MarB does not act via MarR and does not affect the degradation of MarA via Lon protease. Taken together these results suggest that MarB may decrease the function of the canonical *marRAB* promoter by altering the activity of one of its non-MarR direct regulators (MarA, SoxS, Rob, Fis, Crp, or FruR (Martin & Rosner, 1997; Martin *et al.*, 1999; Shimada *et al.*, 2011; Zheng *et al.*, 2004) or of an indirect regulator such as PAP I (Ruiz & Levy, 2010). This possibility awaits further study.

As a periplasmic protein, MarB obviously cannot directly contact any of these cytoplasmic proteins that regulate the known promoter of *marRAB*. Instead, MarB presumably exerts its control indirectly, perhaps via a chemical signal which alters (either directly or indirectly) the activity of one of the cytoplasmic regulators of the operon. The small MarB protein (predicted to contain 51 amino acids once processed) may function as a homomultimer or by interacting with another protein or component of the periplasm or the membrane (see Hobbs *et al.*, 2011) to respond to a stress and/or to create a signal.

MarB has no homology with any protein of known function. Among the seven bacterial genera having a *marRAB* operon, the open reading frame for MarB is more poorly conserved (41–55% identity) than are those of MarR (82–98% identity) and MarA (90–98% identity). Nevertheless, a periplasmic signal is predicted for all of the diverse MarBs using the SignalP 4.1 server (Petersen *et al.*, 2011), and a conserved multi-charged sequence motif (G)SDKSD (starting about 16 residues before the end of the protein) would be present in all of the mature MarBs (Punta *et al.,* 2012). A β strand of 5–6 residues starting at the 14th residue is predicted for mature MarB proteins of all genera using Jpred 3 (Cole *et al.*, 2008). The predicted isoelectric points of the mature proteins range from 4.2 to 5.4, indicating that all MarBs are acidic. These observations suggest that MarB, like the other two more highly conserved proteins in the operon, likely has a similar function in all seven genera.

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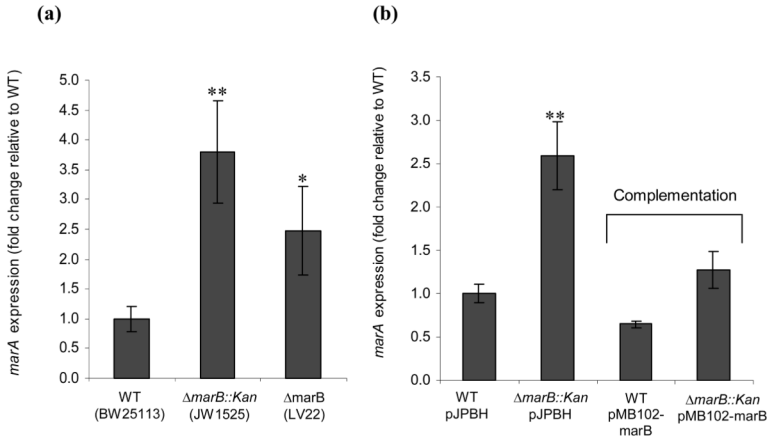

Fig. 1.

marA gene expression **(a)** in wild type and Δ*marB* mutant strains and **(b)** when complemented by wild type *marB*. Shown are relative amounts of *marA* gene transcript in wild type (WT) and Δ*marB::kan* mutant hosts carrying no plasmid **(a)** or carrying either complementing plasmid pMB102-marB or control vector pJPBH **(b).** Fold 1 means no change in *marA* expression in a mutant compared to the level for the parental strain. The results in each chart are normalized to the first sample and are presented as the average +/− the standard error of the mean (Fig. 1*a*, n=5; Fig. 1*b*, n=3). Statistically significant differences for a mutant compared to the level for the parental strain are shown as * ($P < 0.05$) or ** ($P < 0.01$).

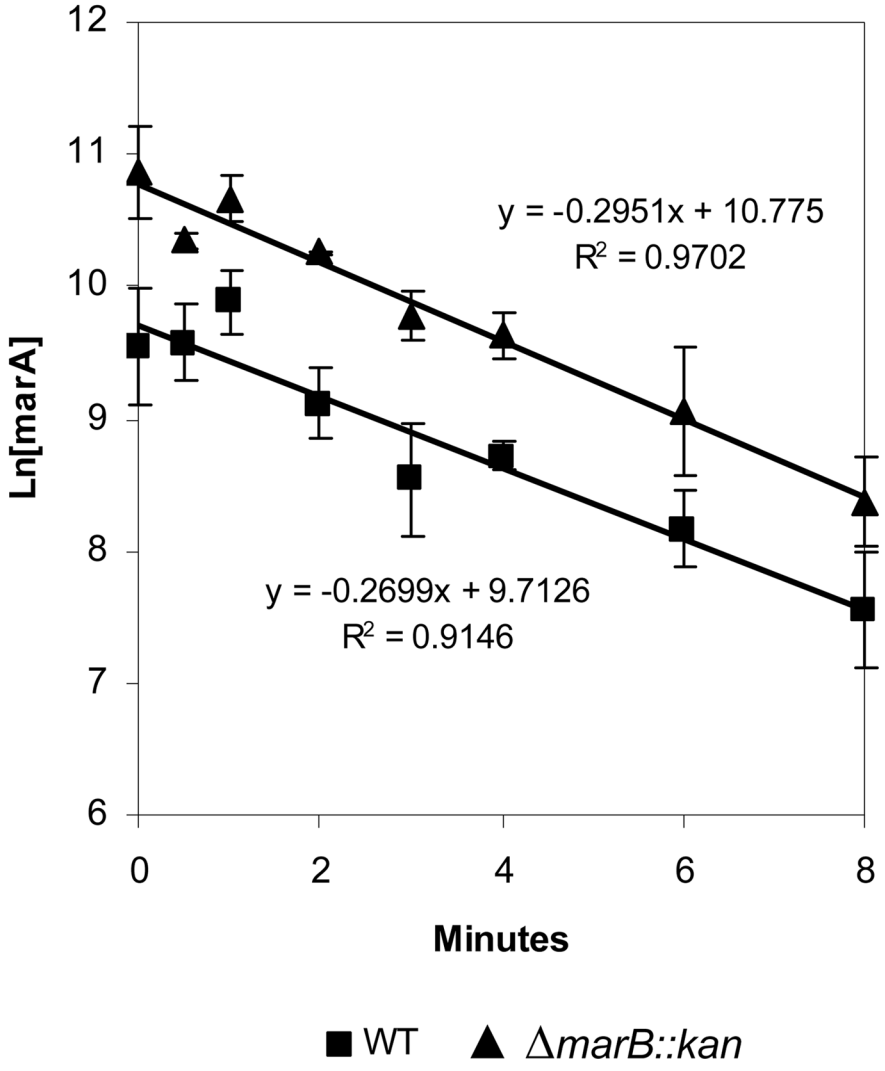

Fig. 2.

Deletion of *marB* had no effect on stability of *marA* mRNA. The half-life of *marA* mRNA was compared in wild type (WT) and Δ*marB::kan* strains using rifampin as detailed in Materials and Methods. Error bars show SEM. No difference was found: the *marA* half-life was 2.6 min for the wild type strain and 2.3 min for its Δ*marB::kan* derivative. The correlation coefficient (R^2) describes how well the data fit the linear equations shown and can range from 0 to 1, the latter being a perfect fit. A correlation coefficient greater than 0.8 is generally described as strong. The absolute numbers of *marA* transcripts per ng of total RNA before addition of rifampin (time zero) were 17000 for the wild type strain BW25113 (■), and 64500 for the Δ*marB::kan* mutant (▲).

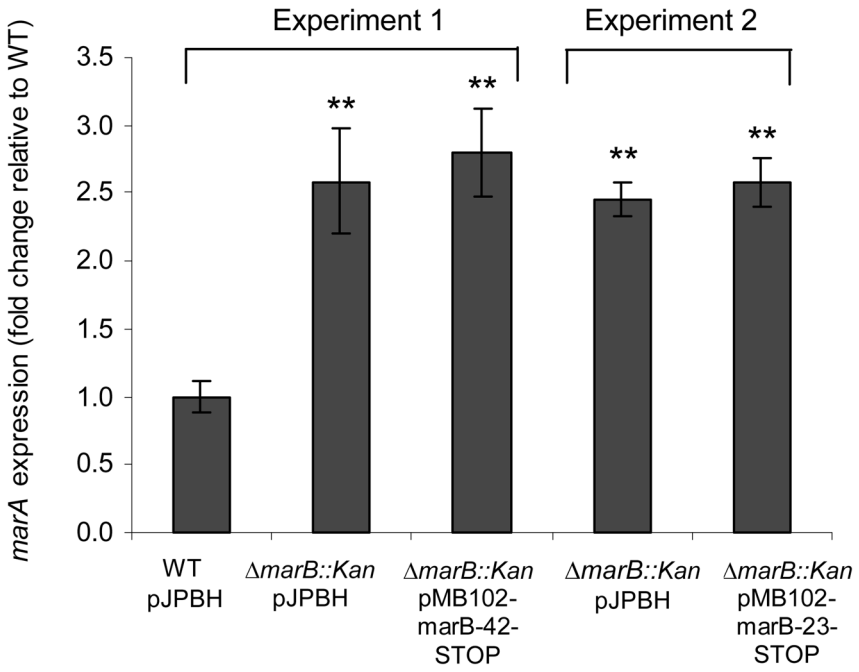

Fig. 3.

Lack of complementation by MarB mutants containing an internal stop codon. Shown are relative amounts of *marA* gene transcript in the WT strain bearing control vector pJPBH (set to 1.0) and in the Δ*marB::kan* strain carrying the control vector or carrying pMB102-marB-(STOP) with stop codons at residue 42 or residue 23 within MarB. Two separate experiments are shown. Complementation by the wild type *marB* can be seen in Fig. 1b, where the same WT/pJPBH control was used. Results (n=3 for each condition) are presented as in Fig. 1. The absolute number of *marA* transcripts per ng of total RNA for WT/pJPBH was 12600.

Table 1

Strains and plasmids used in this study

Strain/Plasmid	Genotype or relevant characteristic	Reference/source
E. coli K-12 strains		
BW25113	Wild type; F^- λ^- Δ*(araD-araB)567* Δ*lacZ4787*(::*rrnB-3*) *rph-1* Δ*(rhaD-rhaB)568 hsdR514*	CGSC (Keio) (Baba *et al.*, 2006)
JW1525	BW25113 Δ*marB::Kan*	CGSC (Keio) (Baba *et al.*, 2006)
LV22	BW25113 Δ*marB*	This study
plasmids		
pJPBH (control)	*ori colE1 lacI,* Amp^r	(Barbosa & Levy, 2002)
pMB102	*ori colE1 lacI lacZp::marA*; Amp^r	(Pomposiello *et al.*, 2001)
pMB102-marB	*ori colE1 lacI lacZp::marB*; Amp^r	This study
pMB102-marB-42-STOP	*ori colE1 lacI lacZp::marB*-42-stop codon; Amp^r	This study
pMB102-marB-23-STOP	*ori colE1 lacI lacZp::marB*-23-stop codon; Amp^r	This study
pCH58	*ori pMB1, phoA fusion* vector, Tet^r	(Hoffman & Wright, 1985)
pCH58-marB	*ori pMB1, marB-phoA* fusion, Tet^r	This study
pCH58-marB-SS	*ori pMB1, marB* signal sequence-*phoA* fusion, Tet^r	This study
pCH58-SS	*ori pMB1, phoA* signal sequence-*phoA* fusion, Tet^r	This study
pCP20	*oriR101*, plasmid for excision of *kan* and *cat* markers by FLP-mediated site-specific recombination; Amp^r Chl^r	(Datsenko & Wanner, 2000)

Table 2

PhoA enzymatic activities in wild type strain BW25113 carrying plasmids containing the indicated *phoA* gene fusions

Plasmid	Promoter	Signal sequence	PhoA activity units[‡] (SEM)
—	—	—	4.3 (1.3)
pCH58	*bla*	—	22 (6.7)
pCH58-marB[*]	*bla*	*marB*	390 (40)
pCH58-marB-SS[#]	*bla*	*marB*	410 (19)
pCH58-SS	*bla*	*phoA*	1600 (250)

[*] pCH58-marB contains full *marB* (except stop codon) cloned upstream of *phoA*.

[#] pCH58-marB-SS contains only *marB* signal sequence cloned upstream of *phoA*.

[‡] These values are the averages of at least 6 independent experiments, with SEM in parentheses.