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Review Article

The *Escherichia coli* multiple antibiotic resistance activator protein represses transcription of the *lac* operon

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In *Escherichia coli*, the *marRAB* operon is a determinant for antibiotic resistance. Such phenotypes require the encoded transcription factor MarA that activates efflux pump expression. To better understand all genes controlled by MarA, we recently mapped binding of the regulator across the *E. coli* genome. As expected, many MarA targets were adjacent to genes encoding stress response systems. Surprisingly, one MarA-binding site overlapped the *lac* operon regulatory region. Here, we show that MarA specifically targets this locus and can block transcription of the *lac* genes. Repression is mediated by binding of MarA to a site overlapping the *lac*P1 promoter −35 element. Control of the *lac* operon by MarA does not impact antibiotic resistance.

Introduction

The *Escherichia coli* multiple antibiotic resistance (*mar*) locus provides resistance to tetracyclines, quinolones, β-lactams and a range of phenolic compounds [1–3]. These resistance phenotypes require *marA* that encodes a transcriptional activator [4,5]. A key role of MarA is to activate expression of the *acrAB-tolC* encoded efflux pump [3,6,7]. Hence, toxic molecules are removed from cells as a consequence of MarA production. Like all members of the AraC/XylS family, MarA binds asymmetric DNA sequences using two helix-turn-helix DNA-binding motifs [5]. The consensus DNA sequence for MarA binding is called the 'marbox'. Such sequences usually occur upstream of promoters and stimulate transcription [8]. Depending on the position and orientation of the marbox, promoters activated by MarA are classified into two groups. Class I promoters usually contain a marbox in the reverse orientation ∼60 base pairs upstream of the transcription start site [8]. At class II promoters the marbox is in the forward orientation that overlaps the promoter −35 element [8]. A few promoters, repressed by MarA, have a marbox overlapping the −35 element in the reverse orientation [9].

Recently, we mapped the binding of MarA to sites across the *E. coli* genome [10]. We identified over 30 loci interacting with MarA. In most cases, these were regulatory regions of known stress response genes. However, we were surprised that MarA also bound upstream of the *lac* operon. Regulated by the cAMP receptor protein (CRP) and *lac* repressor, the operon is required for lactose metabolism [11]. Hence, on binding allolactose, the *lac* repressor undergoes a conformational change and disassociates from its DNA target sites (*lac* operators). If this is concomitant with glucose starvation, CRP activates transcription of the *lac* operon from the *lac*P1 promoter [11]. Given that there is no obvious link between the *lac* locus and antibiotic resistance, we sought to understand the role of MarA. We show that the *lac* operon is subject to repression by MarA and that this requires a reverse orientation marbox overlapping the *lac*P1 −35 element. This regulation does not impact the ability of *E. coli* to resist antimicrobial compounds.

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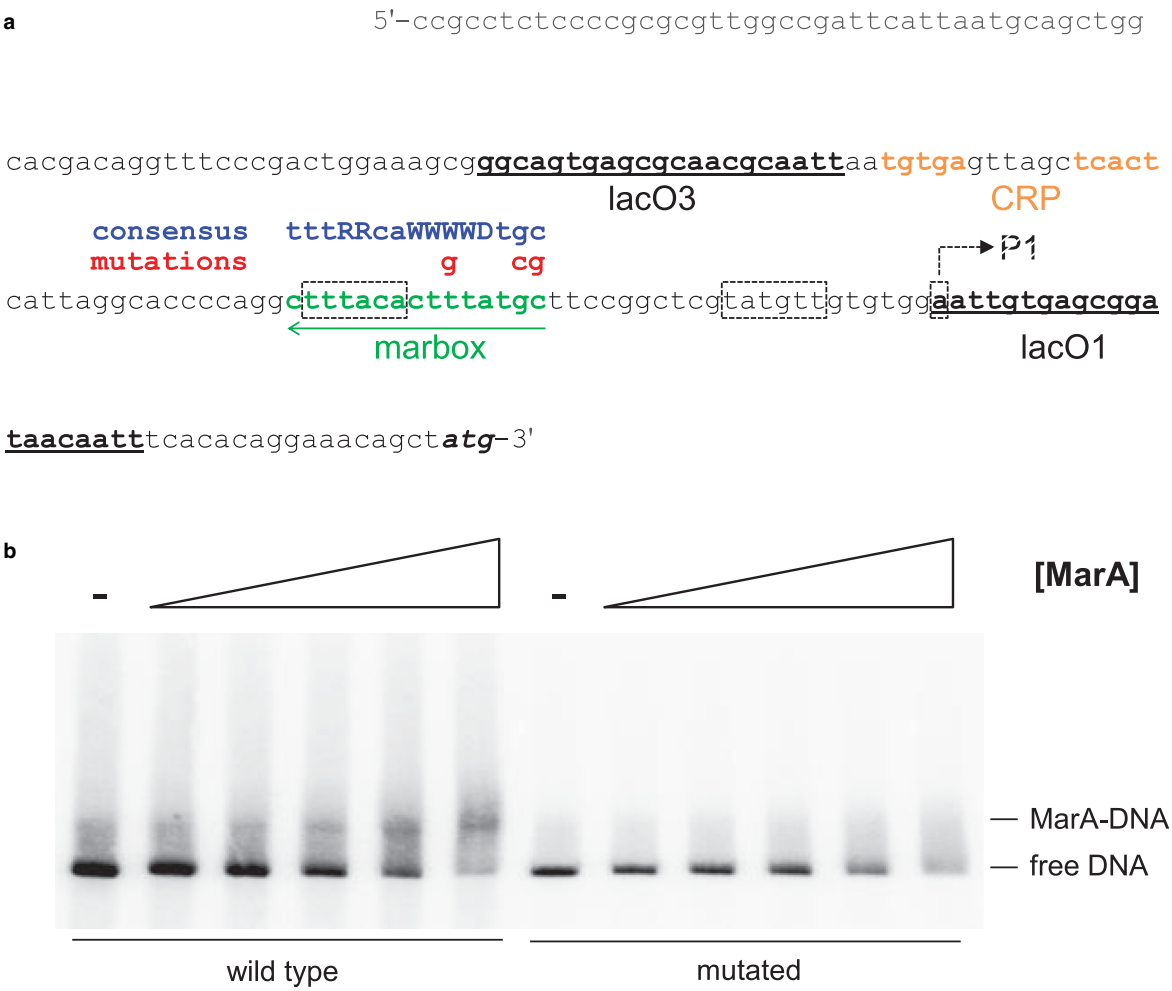

Figure 1. Binding of MarA to the *lacZYA* intergenic region.

(**a**) Sequence of 202 bp the *lac* operon regulatory DNA fragment used in this work. The *lac*P1 promoter −10 and −35 elements are boxed and the corresponding transcription start site is indicated by a bent arrow. The promoter is activated by binding of CRP to its target highlighted in orange. Binding sites for the *lac* repressor, *lac*O3, and *lac*O1 are in bold type face and underlined, while the *lacZ* start codon is bold and in italics. The proposed marbox is highlighted green and orientation is indicated by a green arrow. Mutations made to the marbox are shown in red and the consensus marbox sequence is in blue: R = a or g, W = a or T, D = a, g or t. Oligonucleotide *lac*P-F (5′-atgctagaattcaccgcctctccccgcgcgtt-3′) was used with *lac*P-R (5′-tcgatcaagcttcatagctgtttcctgtgtgaaat-3′) or *lac*PM-R (5′-tcgatcaagcttcatagctgtttcctgtgtgaaattgttat ccgctcacaattccacacaacatacgagccggaacgatacagtgtaaagcctggggtgc-3′) to generate DNA fragments, encoding variants of *lac* operon regulatory region, flanked by *Eco*RI and *Hin*dIII restriction sites. The DNA fragments were cloned in plasmid pRW50 [18] or pSR [19] as required. (**b**) Binding of MarA to the *lac* operon intergenic region *in vitro* requires the predicted marbox. The results of EMSAs, using the wild-type or mutated 202 bp DNA fragment, are shown. Where present, MarA was used at concentrations of 0.05, 0.1, 0.2, 0.4, or 0.6 μM. The position to which the free DNA fragment, or DNA in complex with MarA, migrates during electrophoresis is indicated. Assays were done as described previously [20,21]. MarA protein was purified as described by Kettles [22]. Briefly, DNA fragments were generated by PCR amplification from an *E. coli* genomic DNA template. Following purification, PCR products were cut with *Hin*dIII and *Eco*RI prior to being end-labelled with [γ^{-32P}]-ATP and polynucleotide kinase. The DNA fragments were incubated with MarA in buffer containing 20 mM Tris pH 7,10 mM $MgCl_2$, 100 mM EDTA, 120 mM KCl. Reactions were analysed by electrophoresis through a 5% polyacrylamide gel. Raw gel images are shown in Figure S1.

Mara binds specifically to a marbox at the *lac* operon regulatory region

The *E. coli lac* operon encodes genes required for the metabolism of lactose [11]. Transcription of the operon is blocked by the *lac* repressor protein due to binding at the O1 and O3 operator sites. This repression is relieved in the presence of allolactose (a metabolic precursor of lactose) or non-hydrolysable β-galactosides such as isopropyl β-D-1-thiogalactopyranoside (IPTG) [11]. Transcription of the operon is driven from the *lac*P1 promoter. This is activated in response to glucose starvation by CRP [11]. In previous work, we used chromatin immunoprecipitation, coupled with DNA sequencing, to map the biding of MarA across the *E. coli* chromosome [10]. To our surprise, MarA bound the intergenic region adjacent to the *lac* operon. The sequence of the region is shown in Figure 1a. The *lac* operators, *lac*P1 promoter, and predicted marbox, are highlighted. Our first goal was to determine if MarA indeed bound the inferred site. To facilitate this, we generated a 202 bp DNA fragment encompassing the sequencing shown in Figure 1a. We also made derivatives of the DNA with mutations in key positions of the predicted MarA target site. The mutations, designed to avoid altering the promoter −35 element, are shown in red above the wild-type DNA sequence in Figure 1a. The consensus marbox sequence is also shown for comparison (blue). We tested the binding of MarA to the DNA fragments using electrophoretic mobility shift assays (EMSAs). The results are shown in Figure 1b. Mutation of the marbox reduced MarA binding to the DNA fragment.

Mutation of the marbox does not alter *lacZYA* promoter activity

We reasoned that mutations we had made could inadvertently impact *lac*P1 promoter activity. To test this, we cloned the 202 bp DNA fragment, or the mutated derivative, upstream of the λ*oop* terminator in plasmid pSR. Purified plasmid derivatives where then used as templates for *in vitro* transcription. The results of the

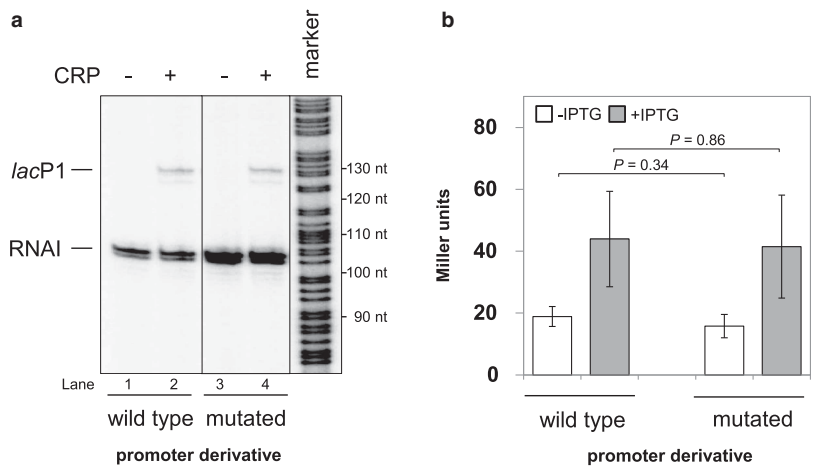

Figure 2. Mutation of the marbox upstream of *lacZYA* does not basal *lac*P1 activity.

(**a**) Activation of the *lac*P1 promoter by CRP. The figure shows results of *in vitro* transcription assays using the wild-type or mutated 202 bp *lac* regulatory DNA fragment cloned in plasmid pSR [19]. RNA polymerase was used at a concentration of 0.4 μM and CRP at a concentration of 1 μM. The transcripts generated from *lac*P1 are labelled and the RNAI transcript is generated from the plasmid replication origin. Preparations of σ^{70} were made as described by Grainger et al. [23]. The RNA polymerase core enzyme was purchased from NEB. The RNA polymerase holoenzyme was generated by incubating the core enzyme with a 4-fold excess of σ-factor at room temperature for 20 min prior to use. CRP was purified according to the protocol of Savery et al. [24]. The *in vitro* transcription experiments were done using a procedure similar to that described in prior work [21] using the system of Kolb et al. [19]. Labelled RNA products were analysed on a denaturing polyacrylamide gel. Raw gel images are shown in Figure S1. (**b**) The graph shows levels of β-galactosidase activity measured in lysates of the *lac*– *E. coli* strain T7 Express (NEB) carrying different promoter::*lacZ* fusions in plasmid pRW50 [18]. Cultures in LB media were grown to exponential phase in the presence or absence of 1 mM IPTG as indicated. When the culture reached mid-exponential phase, the cells were lysed and β-galactosidase levels were measured by the Miller method [25]. Activities are the average of three or more independent experiments. Error bars indicate the standard deviation of three independent experimental replicates. *P* was determined using a two-tailed Student's *t*-test.

Biochemical Society Transactions (2019) **47** 671–677
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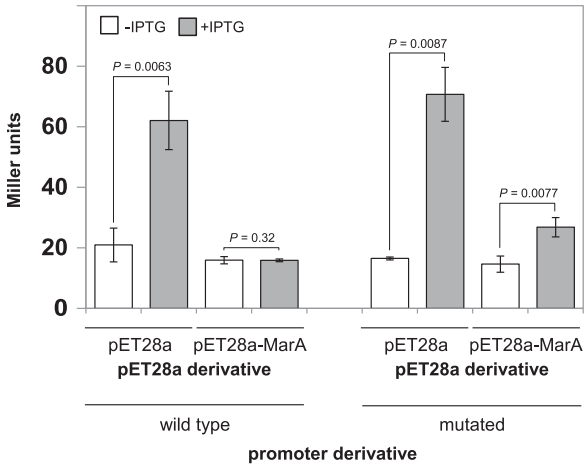

Figure 3. Expression of MarA inhibits induction of *the* lac operon in a marbox-dependent manner.
The graph shows levels of β-galactosidase activity measured in lysates of *E. coli* strain T7 Express. The cells contained either empty pET28a or pET28a-MarA, in combination with different promoter::*lacZ* fusions in plasmid pRW50 [18,23]. Cultures in LB media were grown to exponential phase in the presence or absence of 1 mM IPTG. Error bars indicate the standard deviation of three independent experimental replicates. *P* was determined using a two-tailed Student's *t*-test.

experiment are shown in Figure 2a. The smaller RNAI transcript is derived from the plasmid replication origin and serves as a control. In the absence of CRP, transcription from *lac*P1 was undetectable (lane 1). This was unchanged upon mutation of the marbox (compare lanes 1 and 3). Addition of CRP stimulated transcription (lane 2). The *lac*P1 promoter generated a transcript of 129 nt and 126 nt in length. This is consistent with previous reports regarding alternative transcription start sites at *lac*P1 [11]. We did not detect transcription from any of the ambiguous 'promoter-like' sequences in the *lac* regulatory region [11]. Importantly, activation of *lac*P1 was not impaired by the marbox mutations (lane 4). In parallel experiments, we also cloned the regulatory DNA fragments in plasmid pRW50 to generate a promoter::*lacZ* fusions. In subsequent β-galactosidase assays, the two promoter derivatives had indistinguishable properties (Figure 2b). Hence, the wild-type and mutated versions of the *lac* regulatory region differ only with respect to their ability to bind MarA.

Repression of *lacZYA* expression by MarA requires the marbox

The identified marbox overlaps the *lac*P1 −35 hexamer in the reverse orientation (Figure 1a). Hence, both the position and orientation of the marbox suggest repression by MarA at this locus [9]. To check this, we utilised

Table 1 MICs of different antibiotics in the presence and absence of lac operon expression

	MG1655		JCB387	
Drug*	**−IPTG**	**+IPTG†**	**−IPTG**	**+IPTG†**
Ampicillin	5	5	5	5
Chloramphenicol	4	6	3	5
Doxycycline	2	2	1.5	2
Kanamycin	3	6	6	6
Tetracycline	2.5	2.5	1.5	1.5
Ciprofloxacin	0.024	0.024	0.020	0.016

*The MIC value determined for each drug is in mg/l.
†IPTG was added to a final concentration of 1 mM. Values shown are the average of two independent experiments.

plasmid pET28a and a derivative encoding MarA. The plasmids were used to co-transform *lac*− *E. coli* T7 express with the pRW50 derivatives described above. The various permutations are indicated below the graph in Figure 3. Note that, for these strains, the addition of IPTG has the potential to simultaneously induce transcription from *lac*P1 and expression of MarA from pET28a. We monitored the effects of IPTG addition by measuring β-galactosidase activity in lysates of the various cultures. The data for the wild-type 202 bp DNA fragment are shown in Figure 3 (first four bars). Strikingly, high β-galactosidase activity could only be detected in the presence of IPTG and the absence of pET28a encoded MarA. The most likely explanation is that MarA, expressed from pET28a-MarA, binds the marbox and represses *lac*P1. Consistent with this, in the context of the mutated marbox, IPTG significantly enhanced β-galactosidase activity in both the presence and absence of MarA (Figure 3, final four bars). Hence, the effect of MarA on *lac*P1 transcription requires the marbox. We note that IPTG induction of *lac*P1, overlapping the mutated marbox, in the presence of MarA, was only partial (Figure 3, final two bars). This is most likely due to residual MarA binding. Indeed, weak binding to the mutated marbox was evident at high MarA concentrations in our EMSA assays (Figure 1b).

Expression of the *lacZYA* operon does not alter the minimum inhibitory concentration of common antibiotics

Regulation by MarA hints that expression of the *lac* operon might have unexpected effects on the ability of *E. coli* to grow in the presence of antibiotics. For example, it is conceivable that the *lac* permease, encoded by *lacY*, could inadvertently allow harmful compounds to enter the cell in some circumstances. To investigate possible effects, we determined the minimum inhibitory concentration (MIC) of common antibiotics in the presence and absence of IPTG. Experiments were done with *E. coli* strain MG1655 (*lac*+) or JCB387 (*lac*−) [12,13] as described by Sharma et al. [10] according to the protocol of Wiegand et al. [14]. Results were only accepted if the observed MIC for the control ATCC *E. coli* 25922 strain [15] was within one doubling dilution of the expected result. The results are shown in Table 1. Addition of IPTG did not alter the MIC for any of the antibiotics tested in either strain.

Perspectives

The position and orientation of the marbox overlapping *lac*P1 resemble that observed at other MarA repressed promoters [9]. However, there are notable differences. For example, assuming a reverse marbox consensus of 5′-tttRRcaWWWWDtgc-3′, the *purA* marbox extends from promoter position -36 to −50, while the *hdeAB* marbox extends from −31 to −45 [9]. Thus, in both cases, only partial overlap exists between the marbox and promoter −35 hexamer. In the case of *lac*P1, the marbox extends from -23 to −37 and completely overlaps the −35 element. We suggest that MarA can repress transcription from multiple positions at target promoters. This

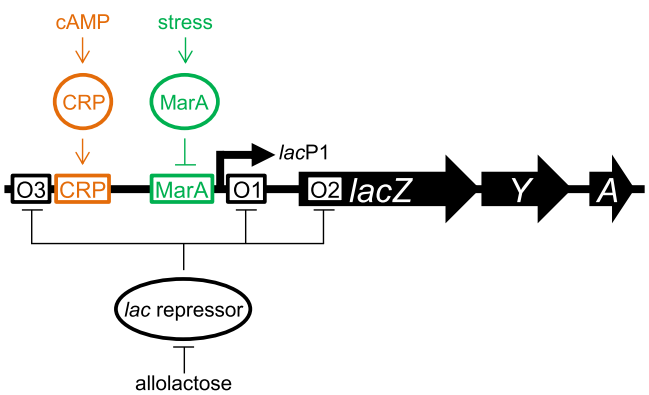

Figure 4. Schematic representation of *lac* operon regulation by the *lac* repressor, CRP, and MarA.

The *lac*P1 promoter is shown as a bent arrow. Genes within the *lac* operon are block arrows labelled *lacZ*, *Y*, and *A*. Binding sites for transcription factors are shown as boxes and labelled accordingly. The cyclic AMP receptor protein (CRP) activates *lac*P1 in response to elevated cAMP levels (orange pathway). In the absence of allolactose, the *lac* repressor blocks operon expression (black pathway). The multiple antibiotic resistance activator protein (MarA) can also repress *lac*P1 activity (green pathway).

Biochemical Society Transactions (2019) **47** 671–677
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contrasts with transcription activation by MarA that requires precise positioning of the regulator [8]. Overall, our observations suggest reduced expression of the *lac* operon could be beneficial in conditions where *marA* is expressed. For instance, this may represent a mechanism to prevent the expression of metabolic enzymes when growth is inhibited. However, it is not clear why the *lac* operon should be singled out for such regulation. Alternatively, the *lac* locus marbox may be an evolutionary relic awaiting resolution. Regardless of the underlying reasons, it is clear many gene regulatory proteins bind unexpected targets [16,17]. The interaction we have identified here is a further example of this phenomenon. In summary, MarA is an unexpected regulator of the *lac* operon and acts in addition to the known factors CRP and *lac* repressor (Figure 4).

Abbreviations

CRP, cAMP receptor protein; EMSAs, electrophoretic mobility shift assays; IPTG, isopropyl β-D-1-thiogalactopyranoside; MIC, minimum inhibitory concentration.

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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