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1   **Regulation of the AcrAB-TolC efflux pump in Enterobacteriaceae**

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1 **Abstract**

2 Bacterial multidrug efflux systems are a major mechanism of antimicrobial resistance

3 and are fundamental to the physiology of Gram-negative bacteria. The Resistance-

4 Nodulation-Division (RND) family of efflux pumps are the most clinically significant as

5 they are associated with multi-drug resistance. Expression of efflux systems is

6 subject to multiple levels of regulation, involving local and global transcriptional

7 regulation as well as post-transcriptional and post-translational regulation. The best-

8 characterised RND system is AcrAB-TolC, which is present in Enterobacteriaceae.

9 This review describes the current knowledge and new data about the regulation of

10 the *acrAB* and *tolC genes* in *Escherichia coli* and *Salmonella enterica*.

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1 **Introduction**

2 Bacterial multidrug efflux systems are a major and common mechanism of

3 intrinsic antimicrobial resistance employed by bacteria. Efflux systems are able to

4 extrude a variety of structurally diverse antimicrobials and some metabolites [10].

5 Many efflux pumps are fundamental to the physiology of some species of Gram-

6 negative bacteria, and are required for virulence and formation of biofilms [10]. The

7 most clinically significant efflux pumps in Gram-negative bacteria are members of the

8 Resistance-Nodulation-Division (RND) family as they recognise a broad range of

9 substrates and are associated with multi-drug resistance (MDR) [10]. These pumps

10 exist as a tripartite system, spanning both the inner and outer membrane [10, 5] .

11 The best characterised RND system is AcrAB-TolC, and is present in

12 Enterobacteriaceae including *Escherichia coli, Salmonella* species and *Klebsiella*

13 *pneumoniae*. Pumps homologous to AcrAB-TolC exist in other species of Gram-

14 negative bacteria, such as MexAB-OprM, MexCD-OprJ and MexXY-OprM in

15 *Pseudomonas spp.*, CmeABC in *Campylobacter spp.*, MtrCDE in *Neisseria spp.* and

16 AdeABC in *Acinetobacter baumannii* [19, 25, 26, 28, 30, 36, 48, 27].

17 Expression of efflux systems is subject to multiple levels of regulation,

18 involving local and global transcriptional regulation as well as post-transcriptional

19 and post-translational regulation. As RND MDR efflux systems show high levels of

20 homology at the gene and protein level, AcrAB-TolC has been studied most

21 extensively as the prototype of this class of pumps [27] . In *E. coli,* expression of the

22 *acrAB* and *tolC* genes is primarily controlled by MarA, whereas in *Salmonella*

23 *enterica* these genes are controlled by RamA. This review will focus on the current

24 state of knowledge of the regulation of the *acrAB* and *tolC genes* in *E. coli* and *S.*

enterica, respectively. We also describe other factors that have been recently discovered.

Local transcription regulation

The mechanism of RND MDR efflux pump regulation is broadly similar in different species; there is local repression of pump genes as well as global transcription factor regulation. In *E coli, Salmonella spp.* and *Klebsiella spp.*, the local repressor AcrR acts as a modulator to prevent the over-expression of *acrAB* [29, 47, 72]. AcrR has been extensively studied in *E. coli.* It is part of the TetR family of transcriptional repressors and when induced it represses *acrAB* [64]. *acrR* is located upstream of the *acrAB* operon, is transcribed divergently and can repress its' own synthesis(Fig.1) [29]. Clinical and veterinary isolates of *E. coli* and *Salmonella* have been identified with mutations in *acrR* that lead to loss of repression and subsequent over expression of AcrAB-TolC and MDR [47, 72]. Transcription of *acrR* is increased under general stress conditions, including 4% ethanol, 0.5M NaCl and the onset of stationary phase in Luria-Bertani (LB) medium [29]. In the absence of functional AcrR, these stress conditions were shown to induce *acrAB*, indicating that AcrR does not act in isolation [29, 72].

Other proteins that locally regulate *acrAB* and *tolC* include AcrS/EnvR, the histone-like nucleoid structuring protein (H-NS), and Suppressor of Division Inhibition (Sdi)A [27]. AcrS/EnvR is a repressor of the *acrEF* efflux pump genes; it can also repress *acrAB* in *E. coli* [21]. AcrS/EnvR may repress *acrAB* in response to increased activity in *acrEF*, allowing cross-regulation of RND efflux pumps [21]. In *S. enterica* and *E. coli*, H-NS regulates expression of genes by responding to environmental signals such as pH, osmolarity and temperature; it also down regulates expression of *acrEF* but not *acrAB* [14, 43, 44]. SdiA, a LuxR protein

1 present in *E. coli and S. enterica,* can positively regulate *acrAB* [42, 49]. These

2 regulators are thought to play a minor role in regulation of *acrAB* and *tolC* as deletion

3 of these genes results in little effect in efflux via AcrAB [27].

4 **Mar regulation of *acrAB-tolC* in *E. coli***

5 The multiple antibiotic resistance operon, Mar, in *E. coli* was discovered by a

6 transposon insertion in *marA.* It is expressed as two separate transcriptional units

7 controlled by a common region of DNA, *marO* [13]. *marR, marA* and *marB* genes are

8 involved in multiple antibiotic resistance, however, the *marC* gene, a putative

9 transmembrane protein, is not [37].

10 MarR is a protein that blocks its own transcription in the absence of any

11 environmental signal [4]. The MarR family of proteins have a unique ability to sense

12 phenolic compounds. MarR recognises and binds palindromic DNA sequences as

13 dimers and the DNA binding domain contains a helix-turn-helix (HTH) motif that

14 favours this activity [3, 62, 65]. Under normal conditions, MarR represses the

15 *marRAB* operon by binding to two palindromic sequences within the operator DNA

16 sequence *marO* that contains its promoter [33]. Transcription of *marRAB* will only

17 occur when repression by MarR is disrupted. This can be due to the presence of

18 certain ligands (e.g. phenolic compounds such as sodium salicylate), antibiotics,

19 oxidative stress or mutation of *marR*, *marO* or the MarR binding site [12].

20 De-repression of the Mar operon leads to expression of *marA* that encodes a

21 global transcriptional activator, MarA, a member of the AraC/XylS family of proteins.

22 This family has a unique feature: a 100 amino acid sequence that forms a domain

23 that contains two helix-turn-helix (HTH) motifs that bind DNA [35]. MarR always

24 represses *marA*. MarA undergoes positive feedback as it binds to a DNA sequence

upstream of the repression site of MarR known as the marbox. This represses *marR*

allowing *marA* to be activated (Fig. 1). The marbox is usually 20 bp in length, highly

degenerate and asymmetric [32]. There are approximately 10,000 copies of the

marbox in the *E. coli* chromosome but most are inactive [32]. Structural studies show

that MarA utilises the two HTH motifs containing the recognition helices 3 and 6 that

bind two major grooves on the DNA as a monomer and bends the DNA by 35° (Fig.

2) [16, 50]. Expression of MarA leads to activation of many genes in its regulon

including *acrAB* and *tolC* giving increased drug efflux and multiple drug

resistance[46]. In the absence of any environment signal or mutation, MarR binds

the operator sites and repression of the Mar operon resumes (Fig. 1).

MarB is transcribed as a part of the second transcription unit of the *mar* locus.

marB is located downstream of *marA* and has its own ribosome binding site [13].

MarB increases the level of MarA by an unknown mechanism, however, its' predicted

periplasmic signal sequence suggests that MarB can act post-transcriptionally [68].

The complete *mar* regulon (also known as *mar/sox/rob* regulon) is poorly

defined. This is partly because the MarA homologues, SoxS and Rob, which also

belong to the AraC family, recognise the same DNA sequence as MarA and regulate

transcription similarly [14, 35]. Transcription of *marRAB* and *acrAB-tolC* can also be

driven by factors other than MarR and MarA. The MarA homologues SoxS and Rob

also bind the same DNA sequence as MarA and are known to activate transcription

of *marRAB* and *acrAB-tolC* [39, 40]. The activation of *marRAB* increases when

another factor known as Factor for Inversion Stimulation (FIS) binds upstream of the

marbox. This activation was only seen in the presence of MarA, RobandSoxS [34].

The expression of MarA, SoxS and Rob is influenced by specific environmental

stimuli, but together ensure appropriate efflux pump regulation via a variety of stress signals.

Like MarA, SoxS is a small protein, of 107 amino acids, formed of a single domain containing two HTH motifs connected by a 27 Å rigid helix [31, 32]. SoxS proteins share 41% and 67% identity and similarity with MarA respectively[35]. Unlike most AraC family proteins, MarA and SoxS have no ligand sensing or dimerisation domain. In the absence of any stress signals the repressor SoxR binds to the *soxS* promoter and represses the transcription of SoxS [20]. Oxidative stress activates *soxS* and SoxS can repress its own expression. MarA and Rob have also been shown to repress the level of SoxS [11, 45].

Rob is a 289 amino acid protein [35]. It is formed of two domains, of which the N-terminal domain shares 51% and 71% identity and similarity with MarA [2, 35]. The N- terminal domain has been shown to have two HTH motifs similar to MarA. Both MarA HTH motifs interact with the DNA major groove, but Rob interacts with DNA major groove utilizing only one HTH motif [23]. The other motif is responsible for binding to the DNA backbone and so the DNA remains unbent [23]. Rob differs from MarA and SoxS by virtue of its second domain; a C-terminal domain that may be involved in ligand binding [23]. Similar to MarA and SoxS, Rob is constitutively expressed and is abundant. Rob binds with higher affinity than MarA or SoxS to the marbox, however, most of the Rob proteins are inactive due to post-transcriptional sequestration [17]. This prevents activation and in turn prevents activation of other promoters regulated by Rob [17]. Sequestrated Rob clumps are not formed when compounds such as 2,2′-dipyridyl activate Rob [60]. When activated, Rob regulates many promoters by binding to the marbox in response to antibiotics and organic solvents [22]. MarA, SoxS and Rob can repress the transcription of Rob [11, 38, 61].

Over-expression of these transcription factors has been found in antibiotic resistant veterinary and human isolates of *E. coli.* Antibiotic resistance in these isolates is often multi-factorial with resistant isolates harbouring multiple mutations, however, the most resistant isolates typically show increased efflux [15]. This is not caused by consistent over-expression of a single transcription regulator, but rather a combination where some isolates show over-expression of MarA and/or SoxS [71]. This correlates with laboratory mutants (Table 1) and re-enforces the evidence that in *E. coli* several global regulators control production of AcrAB-TolC.

Until recently, it was thought that *mar* mutations caused MDR solely by over-expressing the AcrAB-TolC efflux pump, however, the *mar* system regulates other genes as well as *acrAB* and some are involved in resistance to certain classes of drugs. By carrying out ChIP-Seq experiments in ETEC H10407 (which carries a MarR mutation), Sharma *et al.*, [63], identified 33 target genes that are regulated by MarA. In addition to the *acrAB* and *tolC* genes, MarA targets genes that play a role in transport, DNA damage repair, and transcription regulation. These include *xseA* which when deleted conferred increased susceptibility to ciprofloxacin, and *mlaFEDCB* which when deleted conferred increased susceptibility to tetracycline [63]. The authors inferred that with AcrAB-TolC, MarA mediates drug resistance.

Ram regulation in *Salmonella*

RamA is an AraC/XylS transcription activator that is a homologue of MarA and regulates expression of the genes encoding the AcrAB-TolC MDR efflux pump [1]. It has a similar structure to MarA and binds to DNA via a similar mechanism [53]. Like MarA in *E. coli*, RamA regulates expression of *acrAB* and *tolC* in *S. enterica* [55] and other Enterobacteriaceae, including *Klebsiella pneumoniae, Enterobacter aerogenes*

and *Enterobacter cloacae* [55]. MarA, SoxS and Rob are also found in these species and they too play a role in expression of *acrAB*. However, RamA functions as the primary regulator [42]. RamA is not found in *E. coli* or *Shigella species* [55].

Like MarA, RamA activates *acrAB* and *tolC* by directly binding to a degenerate nucleotide sequence upstream of the *acrAB* and *tolC* loci known as the rambox [42]. When RamA is constitutively expressed at high levels bacteria are MDR [56]. When *ramA* is highly expressed, there is a concomitant over-expression of *acrAB*, and when *ramA* is inactivated the expression of *acrAB* is reduced [6]. Overproduction of AcrB and increased expression of the AcrAB-TolC efflux pump confers MDR [6]. When *acrB* is inactivated, there is a fourfold increase in levels of *ramA* suggesting a feedback mechanism [6]. In the absence of *ramA* it is also difficult to select MDR mutants suggesting that *ramA* is required for MDR [57].

The *ramR* gene is located upstream of *ramA*; RamR is a repressor of *ramA* transcription (Fig. 1). Point mutations and insertions in *ramR* and the region between *ramA* and *ramR* have been identified in MDR clinical and veterinary isolates of *S.* Typhimurium and other *S. enterica* serovars[1, 55]. These mutations and insertions prevent RamR from binding to the *ramA* promoter causing increased expression of RamA and subsequent increase in expression of AcrAB-TolC and MDR [1, 54, 55, 75]. The RamR binding site is 28 bp in length and covers the essential features of the promoter region of *ramA*, including the -10 conserved region, the transcriptional start site of *ramA* and two 7 bp inverted repeats[8]. By determining the crystal structure of RamR Yamasaki *et al*., identified substrates of the RamR protein which include crystal violet, ethidium bromide and rhodamine 6G [74]. All the compounds

tested were found to interact with various amino acid residues of RamR, reduce DNA-binding affinity, and induce over-expression of *ramA* [74].

Expression of *ramA* is under multilevel control, with several factors stimulating expression of this transcription factor. RamA is synthesized *de novo* in response to inducers [1]. Various environmental stimuli have been shown to influence efflux by AcrAB by increasing expression of *ramA*. Baucheron *et al* demonstrated that bile activates *acrAB* through de-repression of *ramA* [9]. Nikaido *et al* demonstrated increased expression of *ramA* in response to the bacterial metabolite indole as it enhanced the promotor activity of *ramA* [42]. Bailey *et al* demonstrated that phenothiazides and serotonin-uptake inhibitors such as amitriptyline, induced *ramA* expression and were associated with a phenotype of efflux inhibition [6]. Chlorpromazine induced the expression of *ramA* in wild-type *Salmonella* and there was a concomitant decrease in the level of *acrB* transcript [6]. Lawler *et al* further demonstrated that exposure of *Salmonella* to several biocides, and certain antibiotics such as chloramphenicol, ciprofloxacin, rifampicin, cloxacillin and cefamandole also increased *ramA* expression [24].

Interestingly, not all antibiotics which are exported via the AcrAB-TolC efflux pump directly increase *ramA* expression, and over-expression of *ramA* is greatest when part of the AcrAB-TolC pump is inactivated leading to lack of the tripartite pump [24]. Seventeen antibiotics known to be exported via the AcrAB-TolC efflux pump were tested by Lawler *et al*, and only five significantly increased expression of *ramA* [24]. This may suggest, that *ramA* expression is not always triggered by direct action of specific chemical compounds to RamR, but also that the up-regulation of *ramA* may occur in response to other stimuli [24].

Environmental factors involved in regulation of *acrAB-tolC*

The induction of *acrAB-tolC* in the presence of environmental factors such as bile, fatty acids or cationic peptides, is clinically relevant due to Enterobacteraciae encountering these compounds inside the gastro intestinal tract of the host. In *E. coli* binding of bile, fatty acids and cationic peptides to Rob produces conformational changes and so it induces *acrAB* [58, 70]. In *Salmonella spp.* induction of *acrAB* by bile is dependent on RamA; this occurs by bile inhibiting the binding of RamR to the promoter region of *ramA* [9]. Induction of *acrAB*, in both *E. coli* and *Salmonella spp.*, can also occur in situations where the cell is under oxidative stress. This is SoxRS dependent and requires the oxidation of the iron–sulphur clusters in SoxR, which in turn induces production of SoxS resulting in the induction of *acrAB* [73]. Induction of *acrAB* can also be triggered by salicylate, a phenolic phytohormone implicated in plant defence against bacterial pathogens. Salicylate binds to MarR, causing conformational changes which leads to disassociation of MarR from the *marRAB* promoter. As a result, expression of *marA* is de-repressed, which induces expression of *acrAB* [65].

Other factors involved in regulation of *acrAB-tolC* (post-transcription and post-translation)

The Lon protease is an ATP-dependent protease belonging to the AAA (ATPases associated with a variety of cellular activities) super-family and can be found in both *Salmonella* and *E. coli* [41, 52]. In *E. coli*, Lon has been shown to play a role in regulation of MarA and SoxS at a post-translational level by proteolytic degradation (Fig. 1) [18]. Levy *et al*, demonstrated that mutations in Lon leads to

increased AcrAB efflux and MDR as MarA is not degraded [41]. The impact upon MDR was greatest when combined with a *marR* mutation [41].

Lon performs similar functions within *Salmonella* as *E. coli*, with additional roles in Salmonella Pathogenicity Island (SPI)-1 gene expression and heme biosynthesis [66, 69]. The Lon protease also proteolytically degrades RamA (Fig. 1) [52]. Lon recognises the N-terminal region of RamA, and by binding to this site can degrade the protein [52]. In this way levels of RamA can be re-set to basal levels when the protein is no longer required or there is no longer an inducing stimulus.

Ricci *et al.*, [51] recently showed that the global regulator Carbon Storage Regulator A (CsrA) is involved in the regulation of AcrAB (Fig. 1). CsrA is an RNA binding protein that acts as a global regulator of diverse genes. CsrA binds directly to the 5' end of the *acrAB* transcript. This in turn alters RNA secondary structure preventing the formation of a repressive RNA structure that impedes binding of the ribosome, thus allowing for more efficient translation of the AcrAB proteins [51].

There are subtle differences between regulation of AcrAB-TolC in different species of Enterobacteriaceae

A range of local and global regulators with complex interactions tightly regulates the AcrAB-TolC pump. In *E. coli*, MarA, Sox and Rob have such a tightly woven mechanism of interaction that their target site is termed the *mar/sox/rob* regulon. Each regulator responds to environmental signals as well as each other allowing precise control of the AcrAB-TolC efflux pump and other target genes.

In *Salmonella*, RamA is the predominant regulator, but MarA, SoxS and Rob also influence AcrAB-TolC and cause over-expression and MDR. Mutations causing

over expression of SoxS lead to increased resistance to fluoroquinolones, chloramphenicol and tetracycline, but confers less MDR when compared to mutations in *ramA* or *ramR* (Table 1) [75]. Mutations in *rob* induces *mgtA* transcription which is involved in Mg^{2+} transport; this can confer tolerance to cyclohexane [7].

In addition to Mar, Sox, Rob, there are other factors in some other Enterobacteriacae species not found in *E. coli* or *Salmonella*. For instance, in *Klebsiella pneumoniae* there is *romA* and *rarA*, which are further regulators of the AcrAB pump. RomA is a second transcription factor that can act independently of RamA. It is in the *romA-ramA* locus regulated by RamR [59]. A further factor in *K. pneumoniae*, RarA, can also be induced independently of the other regulators and when over-expressed can also cause increased expression of *acrAB* and MDR [67].

Concluding remarks

RND MDR efflux pumps such as AcrAB-TolC play a vital role in Gram-negative bacteria, and consequently are under complex regulation. This allows for temporary activation of pumps under certain conditions and a rapid return to basal states. This implies that over-expression is not beneficial to the bacterium; possibly because of high-energy requirements. Constitutive de-repression conferring MDR to clinically useful drugs is due to evolutionary pressure of drug exposure. In order to fully understand the mechanisms involved it is essential that the full repertoire of regulatory factors, including those that are species specific, are identified. This knowledge will help identify those factors that could be targets for drug discovery and which could be inhibited as a mechanism to down-regulate MDR efflux and sensitise bacteria to antibiotics susceptible to efflux.

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1 **Legend to Figures**

2 **Figure 1.**Transcriptional regulation of the *acrAB* (red and blue) and *tolC* (yellow)
3 regulon by *E. coli marRAB* (green) and *S. enterica ramRA* (purple) genes. Post
4 transcriptional regulation of *acrAB* by CsrA (grey) and post translational regulation of
5 RamA by Lon protease (orange).

6 **Figure 2:** Structure and binding site of MarA: A) MarA (purple) binds the major
7 grooves of DNA (black) by its DNA binding domain (generated via Rasmol by using
8 PDB 1B10). B) The 20 bp consensus sequence bound by MarA, which is highly
9 degenerate and asymmetric. Adapted from Rhee et al.,1998[24]. Similar binding may
10 be observed in the case where RamA binds DNA.

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