MarA, SoxS and Rob of *Escherichia coli* – Global Regulators of Multidrug Resistance, Virulence and Stress Response

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Abstract: Bacteria have a great capacity for adjusting their metabolism in response to environmental changes by linking extracellular stimuli to the regulation of genes by transcription factors. By working in a co-operative manner, transcription factors provide a rapid response to external threats, allowing the bacteria to survive. This review will focus on transcription factors MarA, SoxS and Rob in *Escherichia coli*, three members of the AraC family of proteins. These homologous proteins exemplify the ability to respond to multiple threats such as oxidative stress, drugs and toxic compounds, acidic pH, and host antimicrobial peptides. MarA, SoxS and Rob recognize similar DNA sequences in the promoter region of more than 40 regulatory target genes. As their regulons overlap, a finely tuned adaptive response allows *E. coli* to survive in the presence of different assaults in a co-ordinated manner. These regulators are well conserved amongst *Enterobacteriaceae* and due to their broad involvement in bacterial adaptation in the host, have recently been explored as targets to develop new anti-virulence agents. The regulators are also being examined for their roles in novel technologies such as biofuel production.

Keywords: Antibiotic resistance, AraC family regulators, *Escherichia coli*, *Enterobacteriaceae*, global regulators, drug development.

1. INTRODUCTION

Bacteria are found in every possible habitat on the planet and adaptation to changing external factors is essential for their survival and growth. This ability to adapt resides in multiple regulatory networks that control gene expression in a co-ordinated manner in response to environmental stimuli. Signals are transmitted to transcriptional regulators that interact with target DNA in the promoter region of a specific open reading frame (ORF), activating or repressing the expression of the target gene [1]. In certain cases, transcription regulators control genes and operons that belong to different metabolic pathways. These transcription factors are known as global regulators. By working in a co-operative manner on several promoters, global regulators provide flexible and finely tuned responses to external signals [1].

This review will focus on MarA, SoxS and Rob of *Escherichia coli*. These homologous regulators are excellent examples of global regulators that are part of multiple regulatory mechanisms necessary for the adaptive response. MarA, SoxS and Rob, which are all members of the AraC family of proteins, respond to many stimuli, including changing pH, the presence of antibiotics, oxidative stressors and organic solvents, all

of which threaten survival. Several studies have shown that these three transcriptional regulators (TRs) are closely related and bind to a degenerate consensus sequence creating considerable overlap in the genes they control [2-7]. Because the genes of the regulons are often under the control of all three TRs to various degrees, a network of pathways is formed that allows small adjustments in response to different extracellular threats [6, 7]. One of their principal roles is to mediate multidrug resistance by up-regulating expression of the AcrAB-TolC multidrug efflux pump [8] and of MicF [9], a small inhibitory RNA that down-regulates the outer membrane porin OmpF. In addition to their contribution to drug resistance, MarA, SoxS and Rob have roles at the host-pathogen interface [10]. Furthermore, a recent study has identified a number of attractive candidates for MarA/SoxS/Rob-controlled loci found to play a role in persistence of *E. coli* in a mouse model of kidney infection [11].

With this great ability to adapt to environmental threats, it is not surprising that bacteria become resistant to all classes of antimicrobial drugs. Antimicrobial drug development has focused on targeting essential proteins that prevent growth of the micro-organism or which are bacteriocidal [12]. However, exposure of bacteria to antibiotics, especially at levels below the minimal inhibitory concentration (MIC), allows selection of adaptive responses and mutations so that micro-organisms can grow in the presence of the drug. Recent studies have brought

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forward the concept of targeting virulence factors instead of essential growth determinants [13, 14]. By targeting virulence, the fitness of a bacterium within the host is impaired, allowing in theory, the host immune system to combat the infection. If new compounds could target non-essential processes, selective pressure would be reduced and the development of bacteria with antibiotic resistance phenotypes decreased. Due to their broad involvement in bacterial adaptation within the host, the three TRs and homologous regulators in different bacterial genera have been used as targets to develop new anti-virulence agents. Furthermore, in recent years, a novel application of the TRs has been their use in increasing tolerance to organic solvents during the production of biofuels.

2. MarA AND THE *marRAB* OPERON

The *mar* locus was first identified by a Tn5 transposon insertion that reversed a multiple drug resistance (MDR) phenotype of *E. coli* isolates selected by growth on subinhibitory levels of tetracycline or chloramphenicol [15]. Later, genetic analysis showed that the *mar* locus consisted of two divergently transcribed units: *marC* and the *marRAB* operon [16]. However, MarC, a putative integral inner membrane protein, does not contribute to the MDR phenotype [17]. MarA, encoded by the second gene in the *marRAB* operon, is a member of the AraC family of transcription regulators [16, 18, 19]. The direct function of this 127 residue protein in antibiotic resistance was first identified through genetic screening [20]. *marB*, located just downstream of *marA* in the operon, is of unknown function, although it has been shown to somehow increase the level of MarA messenger [21, 22]. MarB has a predicted periplasmic signal sequence, suggesting that it acts post-transcriptionally [22]. Interestingly, it has recently been reported to be involved in the transcriptional regulation of *inaA*, a MarA regulon gene (see section 5.4.2).

In the absence of a signal, the transcription of the whole operon is repressed by MarR, encoded by the first gene of the *marRAB* operon [16, 23]. The MarR regulator forms dimers and the DNA-binding domain of the protein consists of a winged helix-turn-helix motif [24], which promotes binding to two specific palindromic sites within the promoter region *marO*, named site 1 and site 2 (Figure **1A**) [25]. The MarR-*marO* interaction is highly specific with an apparent Kd of ~ 10^{-9}M [25]. Binding of MarR to the *marO* promoter region is relieved when a ligand, such as the phenolic

sodium salicylate, the naphthoquinones menadione and plumbagin, 2,4-dinitrophenol, or the metabolite 2,3-dihydroxybenzoate, interacts with MarR, inactivating it and allowing transcription of the *marRAB* operon [26-29]. Once de-repression occurs, MarA activates or represses the transcription of the genes in its regulon [6, 7]. MarA also activates its own transcription by binding as a monomer to the 20-bp *marbox* located upstream of the -35 hexamer in the promoter region of the *marRAB* operon [30]. Transcription of *marRAB* leads to the production of two transcripts of 1.1 and 0.9 kb length [16, 31]. The source of the 0.9kb transcript could be an internal promoter located within *marR* or a result of processing the 1.1 kb transcript. In the absence of a molecular signal, MarR binds again to the operator and repression of transcription resumes. Additional regulation is provided by the Lon protease that rapidly degrades MarA [32]; the half-life of MarA is extremely short (~3 min), which ensures that the response cascade is rapidly removed once the stress signal disappears. Thus, in the absence of signal, MarA levels are extremely low [32]. Activation of *marRAB* has been shown to increase in the presence of Fis (Factor for inversion stimulation), a small DNA binding and bending protein [33]. Fis binds to the *marO* promoter region at a DNA sequence located upstream of the *marbox*. Binding of Fis stimulates the transcription of the *marRAB* operon only in the presence of MarA, SoxS or Rob, acting as an auxiliary protein [33]. MarA, which lacks the effector domain characteristic of other AraC-like proteins, was co-crystallized with the *marbox* found in *marO* [18]. The tridimensional structure showed that MarA contains two helix-turn-helix (HTH) motifs, of which recognition helices 3 and 6 bind two successive major grooves in the DNA, consequently bending the DNA [18]. Alanine-scanning mutagenesis has identified the amino acid residues within the recognition helices that are involved in DNA binding [34]. The structural requirements for *marbox* function in transcriptional regulation are discussed below (Section 5.1).

Several transcription factors, other than MarR and MarA, have been shown to regulate the transcription of *marRAB*. Overexpression of homologous regulator, SoxS, activates transcription of the operon [35]. Martin and colleagues also demonstrated that purified SoxS was able to bind the *marbox* located within *marO* [30]. Basal levels of Rob were also shown to be responsible for basal levels of *marRAB* transcription [2, 36]. Later, *in vitro* studies demonstrated that Rob also binds to the *marbox* as a monomer [37]. However, cross talk

between the *mar* and *sox* systems is limited under physiological conditions. For example, SoxS, when expressed, is not expected to activate *marRAB* transcription in the absence of salicylate, when MarR still represses the promoter [38]. In the case of the *rob* and *mar* systems, the situation is different as salicylate is able to activate *marRAB* through Rob [38]. The mechanism by which Rob is activated by salicylate is still unknown. Another regulator, EmrR, has been reported to repress the *marRAB* operon upon overexpression from a multicopy plasmid [39, 40]. EmrR, a MarR homolog also known as MprA, is the transcriptional repressor of the EmrAB operon that codes for an efflux pump [41, 42]. Whether or not there are environmental conditions where levels of EmrR are high enough to repress *marRAB* has not been demonstrated. Additionally, two regulators of the carbon metabolism, CRP and Cra, have been shown to control *marRAB* transcription. The activity of these TRs varies with glucose concentration: CRP (cyclic AMP [cAMP] receptor protein) is activated after binding to cAMP, which is synthesized in the absence of glucose [43], while Cra (catabolite repressor activator) is inactivated upon interaction with inducers such as D-fructose-1-phosphate or D-fructose-1,6-biphosphate, both generated in the presence of glucose [44]. These regulators have opposing effects on *marRAB* transcription: CRP positively regulates *marRAB* transcription [45], whereas Cra represses *marRAB* [46].

3. SoxS AND SoxR

Early on in the characterization of the *marRAB* operon, *soxR* and *soxS* loci were identified as an oxidative response system [47-49]. *soxR*, which encodes a regulator of the MerR family, is divergently transcribed from *soxS* (Figure **1B**). The SoxR promoter is embedded within the *soxS* structural gene [47, 48]. In the absence of an oxidative stress signal, the SoxR homodimer binds to the promoter region of *soxS* and prevents enhanced transcription [49, 50]. SoxR repression, combined with the action of the Lon protease and to a lesser extent, the FtsH protease, leads to very low levels of SoxS protein in the absence of an induction signal [32]. When the SoxR [2Fe-2S] cluster is oxidized, SoxR becomes an activator of *soxS* transcription [51-55]. This activation has long been considered to be the result of oxidization by the superoxide anion generated by compounds such as the napthoquinones, xenobiotics and paraquat [7, 56, 57] or by nitric oxide generated for example by macrophages [58]. However, it has recently been shown that superoxide is not the oxidizer of SoxR;

rather the redox cycling drugs themselves oxidize SoxR as they in turn are reduced [59]. Once synthesized, SoxS is able to repress its own transcription [60]. MarA and Rob have also been shown to repress *soxS* expression when ectopically overexpressed [38].

The SoxS protein is closely related to MarA (41 % identity, 67 % similarity), although slightly shorter in length (107 amino acids). Like MarA, SoxS lacks the dimerization domain of AraC-like proteins [19, 61] and binds to a 20-bp sequence (*soxbox*) as a monomer [3, 4, 62]. The SoxS regulon was found to have a remarkable degree of overlap with the MarA regulon [6, 7]. However, because of the difference in affinities of MarA and SoxS for different promoters and also because of their different methods of activation, SoxS is the first responder to oxidative stress and MarA is the first to react to antibiotic assault. The regulons of both MarA and SoxS are discussed below (Section 5).

4. Rob

Rob was first identified by its ability to bind to the right border of *oriC* DNA [63] but it appears to have no function in replication and the physiological meaning of its binding to the *oriC* sequence is not known [2]. Rob's function in resistance to antibiotics, organic solvents and superoxide-generating agents was first shown by overexpression of Rob through a plasmid [2, 36]. Later, Rob was found to activate transcription of multiple antibiotic and superoxide resistance genes by interacting as a monomer with the highly degenerate sequence recognized by MarA and SoxS [37, 64]. Unlike MarA and SoxS that are synthesized *de novo* in the presence of the activating signal, Rob is constitutively expressed (Figure **1C**). However, while abundant in the cell, Rob remains in an inactive state [37, 63] due to its sequestration in intracellular foci [65]. A recent study suggests that a fraction of Rob is in an active form even in the absence of inducer [38]. Transcription of Rob has been shown to be repressed by MarA, SoxS [66-68] and by Rob itself [38].

Rob is a 289 amino acid protein of the AraC family of regulators. Its N-terminal DNA-binding domain has 51 % identity and 71 % similarity with MarA [19, 69]. Rob differs from MarA and SoxS by the presence of a C-terminal domain [70]. The crystal structure of Rob in complex with the *robbox* of the *micF* promoter revealed two HTH motifs within the N-terminal domain, which, not surprisingly, is similar to that of MarA [70]. However, unlike MarA, only one HTH motif interacts
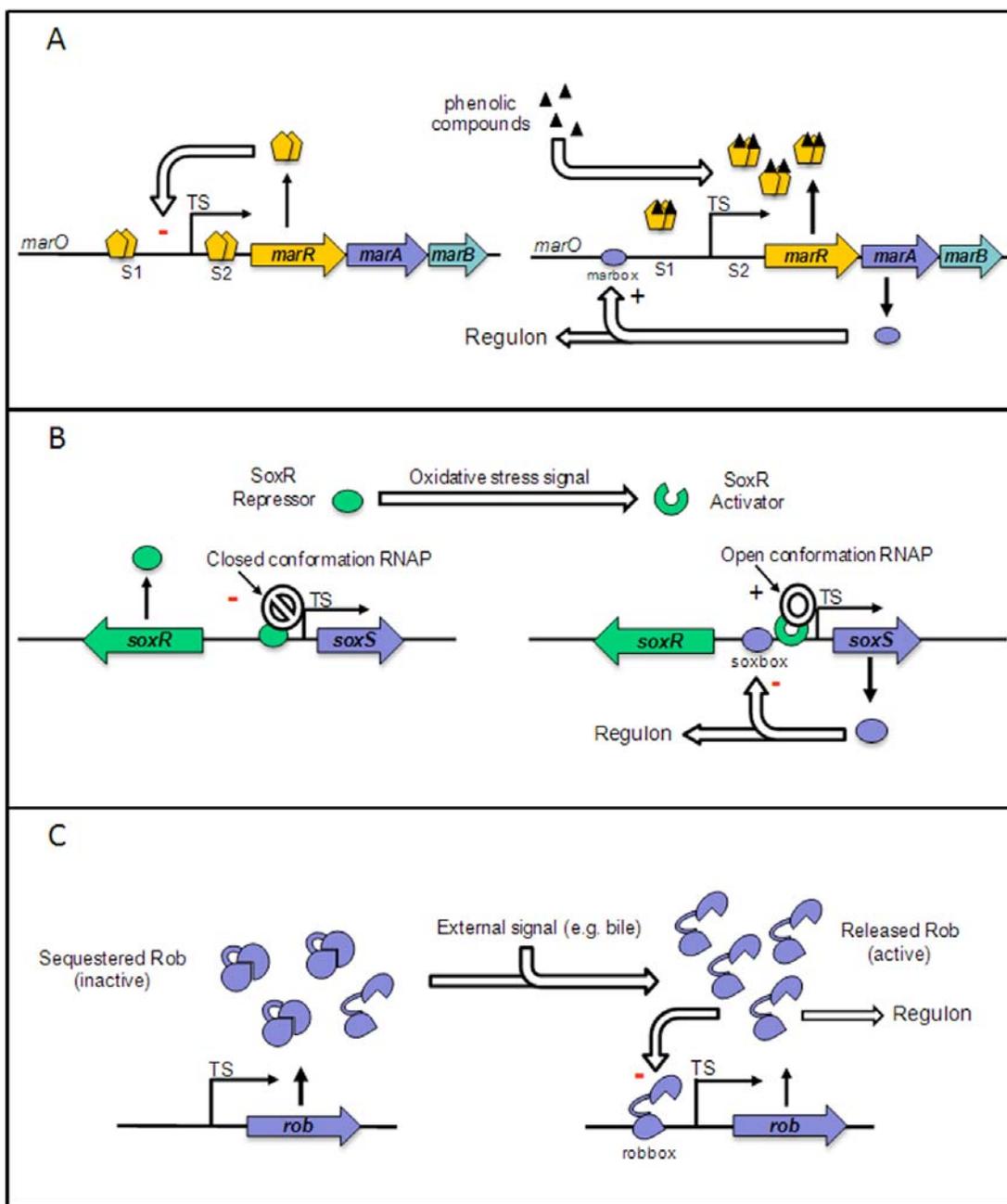
Figure 1: Schematic representation of *marRAB* (A)*, soxRS* (B) and *rob* (C) loci. Respective mechanism of transcription activation are described in the text. TS, transcription start; S1, site 1; S2, site 2; *marO*, promoter region of the *marRAB* operon.

with the major groove. The other HTH motif interacts with the backbone of the DNA instead of lying in the adjacent major groove [70]. Consequently, the DNA appears unbent [70]. For a long period of time, the function of Rob's C-terminal domain was unknown, until several studies showed its role in sequestration and release from sequestration, and consequent activation upon interaction with bile salts or dipyridyl [71-73] (Figure **1C**). The C-terminus also acts to protect the protein from degradation by proteases Lon and, to a lesser extent, ClpYQ [71].

5. THE REGULONS AND PHENOTYPES ASSOCIATED IN *E. coli*

The DNA-binding domain of MarA, SoxS and Rob is exceptionally similar in terms of primary sequence homology, DNA sequence recognition and transcriptional activation properties. However, the expression (MarA and SoxS) or activity (Rob) of the three TRs is stimulated by specific signals, suggesting that the three TRs diverge to generate an adequate response. Although each system can function independently, they are closely interlinked and their

physiological responses are comparable. In this section, we will discuss in detail the characteristics of a functional MarA/SoxS/Rob DNA-binding site and the genes that belong to their regulons.

5.1. Structural Requirements for *marbox* Function and Discrimination between the Regulon Promoters

The DNA binding site recognized by MarA, SoxS and Rob consists of an asymmetric and degenerate sequence that we will call the *marbox* for simplicity. The consensus sequence is illustrated in Figure **2A**. Martin and colleagues defined two classes of activated promoters, class I and II, that are distinct by the orientation and position of the *marbox* relative to the -35 and -10 recognition sequences of the RNA polymerase (RNAP) [5, 74]. In most class I promoters, the regulator binds upstream of the -35 hexamer to a *marbox* oriented backward (B). Among the class I promoters, *zwf* and *map* promoters are exceptions for which the *marbox* orientation is forward (F) [5, 74]. In
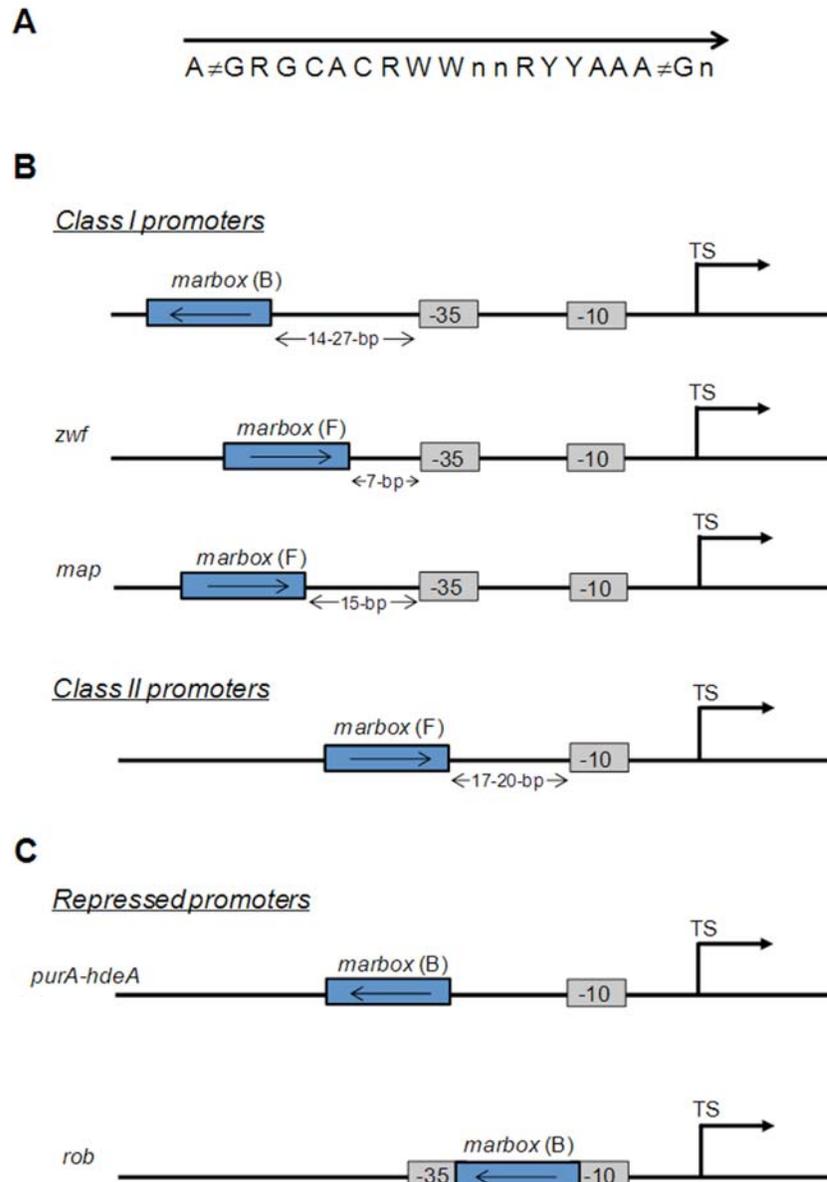

Figure 2: Sequence and structural characteristics of the regulon promoters. A. Consensus sequence for the MarA, SoxS and Rob DNA binding sites. R, A /G; Y, C/T; W, A/T; n, any bases. **B**. Schematic representation of class I and II promoters activated by MarA, SoxS and Rob proteins. For class II promoters, the *marbox* overlaps -35 hexamer sequence recognized by the RNA polymerase, which is not indicated. **C**. Schematic representation of promoters repressed by MarA, SoxS and Rob proteins. For *purA* and *hdeA* promoters, the marbox overlaps -35 hexamer sequence recognized by the RNA polymerase, which is not indicated. The marbox is represented by a blue box. The -10 and -35 sequences are represented by grey boxes. TS, transcription start site.

class II promoters, the regulator binding site is oriented in the forward direction (F) and overlaps with the -35 hexamer [5, 75]. The different classes of promoters are summarized in Figure **2B**.

The different orientations and positions of the *marbox* relative to the -35 and -10 signals of the RNAP imply two configurations of the ternary complex Regulator-RNAP-DNA. Transcriptional activation at class I promoters was shown to require interaction of the regulator with the C-terminal domain of the RNAP α-subunit (RNAP α-CTD), while activation at class II promoters has been shown to involve interaction with RNAP α-CTD and with region 4 of the sigma factor 70 [4, 5, 37, 76-79]. NMR studies have identified the specific residues of RNAP α-CTD that interact with MarA, and it is considered likely that MarA, SoxS and Rob bind similar residues [80]. Two pathways to the formation of the ternary complex Regulator-RNAP-DNA have been put forward. In the RNA capture pathway, the regulator binds the cognate DNA first and then recruits RNAP [81]. In the DNA-scanning pathway, the regulator binds first to RNAP and this binary complex scans the DNA for binding sites [75]. The latter is favored for the three TRs because MarA, SoxS and Rob interact with RNAP in solution [4, 37, 75, 76] and the ternary complex (Regulator-RNAP-DNA) is more stable than the binary complex (Regulator-RNAP) [75]. It has been also suggested that binding of the regulator to RNAP increases the kinetics of the polymerase, resulting in the need for a lower number of binary complexes to produce the same amount of transcripts [82].

Although different environmental signals induce the expression of MarA, SoxS and Rob, these three homologous regulators activate a common group of promoters, which results in MDR, oxidative stress response and organic solvent tolerance. Nevertheless, these regulators do not activate each of the regulon promoters to the same extent [2, 83]. For instance, compared to SoxS, MarA displayed lower binding affinity and lower transcription activity at promoters of genes involved in superoxide defense [83]. Small differences in the *marbox* sequences are the main factors responsible for discrimination [83]. An example given by Martin and Rosner shows that in MarA, steric hindrance occurs between the side chain of glutamic acid at position 89 (Glu-89) and the phosphate located between position 12 and 13 of the *marboxes* of class I promoters [84]. The steric hindrance lowers the affinity of the regulator for this DNA sequence. A thymine at position 12 in the *marbox* leads to higher affinity,

suggesting that it accommodates the side chain of Glu-89. Affinity was also increased by changing Glu-89 to an alanine [84]. This phenomenon implies that, with different affinities for DNA, the binding of each regulator to a specific promoter is also dependent on protein concentration [85]. For example, high concentrations of MarA are necessary to bind promoters such as the *acrAB* promoter. This is potentially because lower concentrations are necessary to activate other responses involved in antibiotic resistance such as *micF* transcription that turns down translation of the outer membrane porin *ompF* (see below). Moreover, high expression of AcrAB-TolC pump is costly in terms of the cell's energy resources [85].

The three TRs also act as transcription repressors. MarA was shown to directly repress *purA*, *hdeA* and *rob* transcription [67, 86]. The marboxes lie in backward orientation within all three repressed promoters. The *marboxes* of *hdeA* and *purA* promoters overlap the -35 recognition sequence of the RNAP [86], while in the *rob* promoter, it lies between the -10 and -35 sites [67]. It has been first reported that MarA represses transcription of *rob via* a RNAP-DNA-MarA ternary complex [67]. However, the same research group later demonstrated that non-specific binding of RNAP to DNA was responsible for the ternary complexes, and that repression appears to occur by steric hindrance of RNAP [68].

5.2. Multidrug Resistance and Organic Solvent Tolerance

One of the principal phenotypes of expression of MarA, SoxS and of activation of Rob is MDR. During the initial discovery of the *marA* locus, spontaneous drug resistant mutants were found to be less susceptible to several other antibiotics; this susceptibility could be further decreased by stepwise growth on increasing antibiotic concentrations [15, 31, 87]. Transposon insertion in *marR* led to a 3-8-fold increase in the minimum inhibitory concentration (MIC) of several antibiotics [16]. Additionally, expression of MarA from a multicopy plasmid led to increases in MIC of 4-5 fold [20]. Deletion or mutation of *marA*, however, resulted only in small decreases in MIC of just under 2 fold or less [26]. Therefore, increasing expression of MarA leads to a greater effect than deleting it. Reduced antibiotic susceptibility by SoxRS was first described in the early 1990's [48, 88] after treatment of *E. coli* with oxidizing agents. Control was found to occur through SoxR activating SoxS transcription (Figure **1B**).

Decreased antibiotic susceptibility due to higher Rob expression was identified in 1995 when its N-terminal sequence similarity with the MarA and SoxS sequences was noted [2]. The mechanism by which the three TRs cause decreased antibiotic susceptibility is through activation of similar genes.

5.2.1. The AcrAB-TolC Efflux Pump

All three TRs regulate transcription of the efflux pump AcrAB-TolC, a member of the resistance-nodulation-division (RND) family of proteins. *acrA* and *acrB* are part of the same operon and are regulated through the *acrA* promoter. AcrB provides a channel between the cytoplasm and the periplasm and interacts with TolC *via* the adaptor protein AcrA. TolC provides a continuation of the AcrB channel from the periplasm to the extracellular space. Compounds may also enter the pump from the periplasm *via* channels in AcrB. The pump is one of the most important in antibiotic resistance and, as the interior of the channel is hydrophobic, in efflux of a diverse array of lipophilic compounds. An excellent review is given by Nikaido and Takatsuka [89].

Several studies [8, 64, 90, 91] have shown that MDR and organic solvent tolerance (see below) phenotypes of the three TRs are in large part due to this pump. For the *marRAB* operon, elevated transcription of *acrAB* occurs in *marR* mutants and deletion of *acrAB* results in loss of MDR in such mutants, leading to a 2-3 fold decrease in MICs compared with the isogenic wild type strain [8, 92]. The MICs of the *marR* mutants were further elevated in the absence of *acrR*, the main repressor of *acrAB,* suggesting that constitutively expressed *marA* raises the MICs again, through a derepressed *acrAB* operon [93]. More directly, microarray data of MarA induced by salicylate or constitutively expressed in a *marR* mutant showed 2-3 and 3-4 fold increases in *acrA* and *tolC* transcript levels respectively [6]. Furthermore, MarA has been shown to bind the *tolC* promoter *in vitro* [94] and substrate accumulation in strains with mutations in *tolC* led to the upregulation of *marRAB,* suggesting a regulatory feedback loop [95]. Interestingly, 10-fold variations in the levels of TolC in either direction from wild type levels do not appear to affect MICs, suggesting that, of the TolC protein produced, only a small fraction is operational [96].

Microarray studies showed that SoxS increases the transcription of *acrA* by 2 fold upon exposure of cells to paraquat [7] and directly binds to the promoter

sequence of *acrA* and *tolC* [74, 85, 97]. Rob also depends largely on the pump for decreasing antibiotic susceptibility [64] and has been shown to activate the transcription of an *acrAp-lacZ* promoter fusion in the presence of bile salts, independently of *marA* and *soxS*, and to bind to the *acrA* promoter [73].

There are several layers of control for the pump as well as the *acrA* promoter. TolC has 4 promoters that are bound by different regulators. MarA, SoxS and Rob bind the third and fourth promoters, while other transcriptional regulators bind the first and second [97]. Recently it has been shown that a small 49 residue membrane protein, AcrZ, also regulates the pump as *acrZ* deletion changes the substrate specificity of the pump. Furthermore, AcrZ co-purifies with the pump and is also regulated by all three TRs [98].

While AcrAB is the only pump known to be regulated by these TRs, it is has been shown that pumps from different families can work synergistically and have a greater than additive effect against certain antibiotics [99]. AcrAB upregulation by the TRs and upregulation of other pumps by alternative pathways would then decrease antibiotic susceptibility more substantially.

AcrAB-TolC Efflux of other Substrates than Antibiotics

The AcrAB-TolC efflux pump is also important for the efflux of other components such as triclosan and pine oil [100]. Overexpression of the *mar* operon resulted in reduced triclosan susceptibility, presumably by upregulation of the *acrAB/tolC* genes. In a few cases, MDR strains with triclosan resistance have been identified with overexpression of *marA* or *soxS* [101]. With respect to pine oil, deletion of *acrAB* made strains hypersusceptible suggesting active pine oil efflux; increased susceptibility was dependent on MarA as deletion of SoxS or Rob only caused increased susceptibility if the *marA* locus was removed. Strains overexpressing MarA and showing decreased pine oil susceptibility also exhibited MDR [100].

Upregulation of the AcrAB-TolC pump by MarA (SoxS and Rob) may also have other consequences as AcrAB-TolC has been shown to efflux mammalian steroidal hormones, which bacteria are exposed to in the urinary, vaginal and gastrointestinal tract [102]. AcrAB-TolC has also been demonstrated to partner with the YojI protein (previously thought to be an ATP binding cassette type pump) to efflux microcin J25. Without *yojI* or *tolC*, *E. coli* become very susceptible to microcin J25 [103]. Microcins are small bacteriocins

(peptides) of 10kDa or less, produced by bacteria as antimicrobials to outcompete other bacteria. Cationic antimicrobial peptides (CAMPs) on the other hand are larger amphipathic peptides that are part of the host defense against invading bacteria. CAMPs can upregulate MarA with the assistance of Rob, thus decreasing susceptibility to CAMPs *via* AcrAB [104]. Efflux of steroidal hormones, CAMPs, peptides such as microcin J25 and enterobactin could all affect how bacteria survive in human hosts either as commensals or as pathogens.

AcrAB-TolC in Organic Solvent Tolerance

Organic solvents are present in different environments where *E. coli* can be found, such as soil and waste water, and present a challenge to bacterial growth. Organic solvents disrupt the membrane and inactivate bacterial proteins leading to cell lysis and death [105].

Loss of *marA* or *marORAB* causes *E. coli* to grow less well in n-hexane, while over-expression of *marORAB* results in better growth in the presence of n-hexane and growth in cyclo-hexane where previously no growth occurred [90, 106]. The organic solvent susceptibility in the *marORAB* deletion can be complemented by expression of the *marCORAB* operon, *soxS* or *rob* but none of the TRs can complement organic solvent sensitivity due to AcrB deletion. That the different regulators may depend on each other with respect to AcrAB-TolC expression was separately demonstrated by Nakajima and colleagues [36], who showed that the over-expression of Rob that leads to organic solvent tolerance is partially dependent on the presence of SoxS.

It is not known how organic solvents activate the transcription of *marA* or *soxS* or how they activate Rob. As for antibiotics, increases in organic solvent tolerance mediated by the three TRs could allow adaptation to higher levels of solvents by mutation. Since the same regulon genes are involved, it seems likely that increasing organic solvent tolerance would also decrease antibiotic susceptibility and might also increase the number of drug resistant bacteria in the environment.

Recently, genetic manipulation of TRs has been applied to biofuel production where organic solvent tolerance is a necessity. Oh and co-workers [107] showed that *marR* deletion in *E. coli,* together with deletion of *fadR* (which changes the composition of the inner membrane to make it less permeable to organic solvents) caused an increase in growth and cell viability with organic solvents, and also decreased organic solvent accumulation within the bacteria. In a similar vein, Watanabe and Doukyu [108] identified mutations in AcrR and MarR that maximize the tolerance of the bacteria to organic solvents. However, in a different biofuel study [109], overexpression of the TRs (MarA, SoxS and Rob) in strains that overproduce free fatty acids (FFAs) that are potential precursors for high density biofuels did not lead to an increased tolerance for FFAs.

In the general theme of pollution, Rob and SoxS have also been found to mediate resistance to some heavy metals in *E. coli* [36], though whether or not this is dependent on AcrA-TolC is unknown as currently there is no known efflux of metals by this pump in *E. coli.* Resistance to some heavy metals could occur through the superoxide dismutases, one of which, *sodA*, is a member of all three TR regulons (Section 5.3.2).

5.2.2. Outer Membrane Permeability

The outer membrane of Gram negative bacteria is a physical barrier to hydrophobic and hydrophilic compounds and many toxic molecules [110]. The major outer membrane proteins F and C of *E. coli* (OmpF/C) form pores in the outer membrane and facilitate diffusion of nutrients and excretion of toxic products [110]. Since OmpF and OmpC are central to the accumulation of antibiotics such as penicillin, cephalosporin and tetracycline, decreased OmpF/C levels results in reduced sensitivity to antibiotics [111-113]. The environment-dependent regulation of these porins is complex and involves a network of transcription regulators and small non-coding RNAs [114-118].

micF, a small inhibitory RNA of 93 nucleotides [119], regulates OmpF expression at the post-transcriptional level by binding the 5' untranslated region of the mRNA, such that it shields the promoter region, the Shine-Dalgarno sequence and the first AUG codon [120]. Transcription of *micF* is controlled by several global regulators [121-123], and by the EnvZ/OmpR two component system [124, 125]. MicF levels are known to increase with osmolarity *via* the OmpR regulator, and at higher temperatures, although the mechanism by which this occurs is not clear [126]. Oxidative and toxic compounds also activate *micF*, leading to an adaptive response that protects bacteria from toxic compounds by decreasing membrane

permeability. Cohen and co-workers first found that strains with a MDR phenotype due to mutations in the *mar locus* had decreased levels of OmpF in their membranes and that this phenomenon required intact *micF* [9, 127]. MarA was later shown to bind to sequences adjacent to and slightly overlapping the -35 position of the *micF* promoter [34]. SoxS is also able to promote *micF* transcription *in vivo* [7] and purified SoxS has been shown to activate *in vitro* transcription of *micF*, indicating direct binding to the promoter [3, 4]. Moreover, Rob up-regulates *micF* [2, 37, 70, 128] and it is likely that this would also be part of the mechanism of bile tolerance by *E. coli* [128]. During salicylate induction, both MarA and Rob are responsible for the MicF-dependent decrease in OmpF levels [38]. Also, these two regulators have been shown to regulate OmpF translation through an unknown MicF-independent pathway [38].

YedS porin has also been shown to be regulated by MarA in a carbapenem resistant clinical isolate of *E. coli* [129]. The study revealed that a mutation found in *marR* led to increased expression of MarA, which resulted in activation of *micF* transcription. While OmpF and OmpC were absent in this isolate due to mutation in the corresponding genes, YedS, which shows high homology with OmpF, appeared to be functional and translation of its messenger was repressed by MicF.

5.3. Oxidative Stress

Oxidative stress is generated by oxidizing chemicals (e.g. paraquat, plumbagin, menadione), antibacterial compounds including certain antibiotics, host defense mechanisms and normal metabolic processes resulting in the formation of the reactive oxygen species (ROS), superoxide, hydrogen peroxide and the hydroxyl radical. These molecules are able to damage RNA, DNA, proteins and lipids. Superoxide dismutases convert superoxides to hydrogen peroxide and water. Catalases/peroxidases convert hydrogen peroxide to water and oxygen. There is no enzyme available to convert hydroxyl radicals to a safe compound and this ROS is therefore highly toxic.

Of the three TRs, SoxS is the first responder to oxidative stress. There is some controversy as to the mechanism of SoxR activation and as mentioned in section 3, it is in fact redox-cycling drugs themselves that oxidize SoxR rather than superoxide. The reduced redox-cycling drugs then donate the electron acquired from SoxR to a respiratory molecule such as a quinone, becoming reoxidised and able to continue to

oxidize further SoxR proteins [59]. It is has also been shown that high oxygen concentrations activate SoxR in the absence redox-cycling drugs [130] potentially through the decrease of NADPH. This molecule has been suggested as a source of electrons for SoxR reducing enzymes (*rsxABCDGE* operon and *rseC*) [130-133].

There is also evidence that under anaerobic conditions, SoxR can be activated by redox cycling drugs if nitrate is present [59]. In this case, nitrate acts as an electron acceptor to reoxidise reduced redox-cycling drugs. Additionally, SoxR is activated by nitric oxide (NO) [134]. NO nitrosylates the iron sulfur cluster forming dinitrosyl–iron clusters and this occurs more efficiently in the absence of oxygen. SoxR is not a response factor of NO under aerobic conditions [135]. We speculate that SoxR could be a defense mechanism against NO produced by macrophages during an infection [136] under anaerobic conditions such as in the gut.

The genes regulated by SoxS, MarA and Rob that are involved in the respiratory pathway and in oxidative stress response are shown in Table **1**. Several of the genes are involved in maintaining the pool of NADH and NADPH that is depleted during oxidative stress. These genes are part of the respiratory pathways: glycolysis, the Krebs cycle (also called the citric acid cycle or tricarboxylic acid (TCA) cycle) and the pentose phosphate pathway (Table **1**, Figure **3**). Others contribute to lowering levels of superoxide and are oxidative stress response genes. Other proteins of the regulons, such as AcrAB and OmpF, act to keep out redox-cycling drugs (discussed in section 5). Here, we present an overview of these genes that are regulated by MarA, SoxS and Rob.

5.3.1. Respiratory Enzymes

Figure **3** shows a simplified representation of the respiratory pathways: glycolysis, the Krebs cycle and the pentose phosphate pathway (PPP). Highlighted are the enzymes regulated by one or more of the three TRs. For some, the evidence is based only on microarray studies and for others, more experiments have been performed. Because of their connection within the respiratory pathway, all will be mentioned, if only briefly.

zwf and fpr

zwf is an important enzyme in the PPP that codes for glucose-6-phosphate dehydrognease (G6PDH) (Figure **3**). It converts glucose-6-phosphate to

Table 1:   Respiratory and oxidative stress genes regulated by MarA/SoxS/Rob[a]

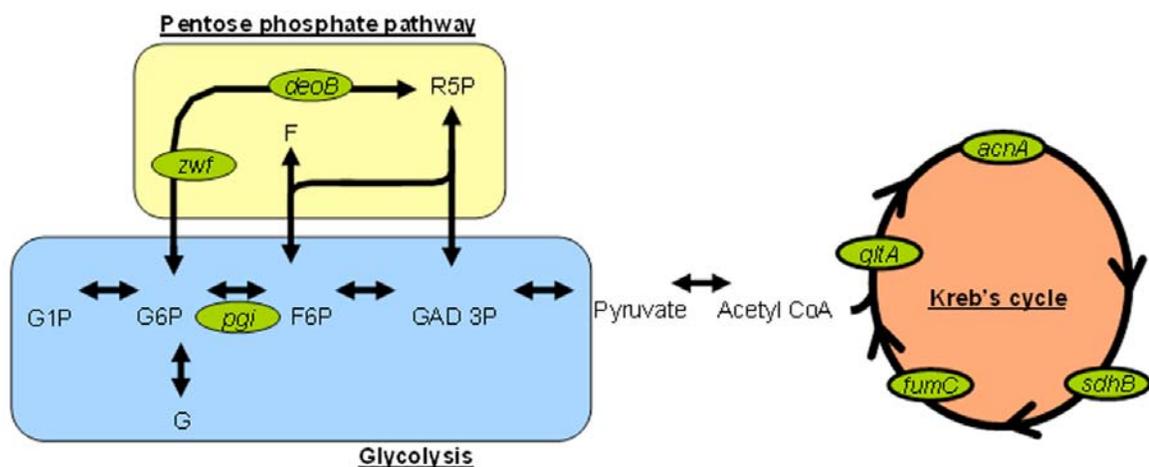| Gene	Protein encoded	Pathway[b]	Reaction		Regulated by
			From	To	
acnA	Aconitase A	Krebs	Citrate	Isocitrate	MarA, SoxS, Rob
deoB	Deoxytibouratase, phosphopentomutase	PPP	2 deoxy-D-ribose 6-P	2 deoxy-D-ribose 1-P	MarA, SoxS
			D-ribose 5-P	D-ribose 1-P	
fumC	Fumarase C	Krebs	Fumarate	S-Malate	MarA, SoxS, Rob
fpr	Ferredoxin NADP$^+$ reductase		2 reduced ferredoxin + NADP$^+$ + H$^+$	2 oxidized ferredoxin + NADPH	MarA, SoxS, Rob
gltA	Citrate synthase	Krebs	Oxaloacetate	Citrate	MarA, SoxS
pgi	Glucose-6-phosphate isomerase	Glycolysis	β-D-glucose 6-P	D-fructose 6-P	MarA, SoxS
sdhB	Subunit of Succinate dehydrogenase iron-sulfur protein	Krebs	Succinate	Fumarate	SoxS
sodA	Mn Superoxide Dismutase		Superoxide	Oxygen + hydrogen peroxide	MarA, SoxS, Rob
zwf	Glucose-6-phoshate dehydrogenase	PPP	Glucose-6-P	6-P-D-gluconolactone	MarA, SoxS, Rob

[a]References in text.
[b]PPP, pentose phosphate pathway; Krebs, Krebs Citric Acid Cycle.

Figure 3: MarA, SoxS and Rob regulated enzymes in simplified respiratory pathway. Enzymes shown are regulated by one or more of MarA, SoxS and Rob (see Table **1**). *acnA* – aconitase A, *sucD* - succinyl CoA synthase, *sdhB* – succinyl dehydrogenase B, *fumC* – fumarase C, *gltA – citrate synthase, pgi* – glucose-6-phosphate isomerase , *zwf* – glucose-6-phosphate dehydrognease, *deoB* – phosphopentomutase. G – glucose, F – fructose, P – phosphate, R – ribulose, GAD – glyceraldehyde.

6-phosphogluconolactone and concurrently converts NADP$^+$ to NADPH; it is the major source for NADPH in *E. coli* [137]. During oxidative stress, levels of G6PDH rapidly increase as a result of SoxS induction increasing NADPH that can then be used to reduce oxidized molecules and proteins [137]. However, increasing levels of G6PDH lead to a decrease in SoxS. Giro and colleagues [137] attribute this to the increased levels of NADPH used by SoxR reducing enzymes (*rsxABCDGE* operon and *rseC*) [131, 132] leading to SoxR reduction and SoxS repression. In

effect, this provides a negative feedback loop between *zwf/*G6PDH and SoxS.

Ferredoxin NADP$^+$ reductase (Fpr) on the other hand promotes the conversion of NADPH to NADP$^+$ by reducing ferredoxin and flavodoxin; the latter is also a member of the SoxS regulon [7, 138]. Ferredoxin and flavodoxin act to reduce the iron sulfur clusters of various enzymes (e.g. ribonucleotide reductase, methionine synthase and pyruvate/formate lyase) oxidized during oxidative stress conditions, restoring

their function [137, 139]. Giro and colleagues [137] found that following oxidative stress induction, a 30 minute lag occurred before Fpr levels increased. At this time, oxidative damage would already have occurred and therefore Fpr may be used to repair enzymes damaged by oxidation after the fact rather than as an immediate response to limit damage. A balance between SoxR reduction/oxidation, G6DPH and Fpr expression must be in place as the levels of each varies with the redox state of the bacteria.

Further complexity occurs in this balance as *fpr* and *zwf* are also part of the MarA and Rob regulons, though their influence is weaker [85, 139] and there are other global transcriptional regulators such as OxyR and Fur that respond to oxidative stress; the latter also being a SoxS regulon member and involved in iron regulation [56, 140].

Pgi and DeoB

In the same theme as *zwf* regulation, glucose-6-phosphate isomerase (Pgi) is at the start of the glycolysis pathway and deoxyribouratase (DeoB – a phosphopentomutase) is part of the PPP (Figure **3**). Both have been shown by microarray to be regulated by MarA and SoxS [6, 7, 138]. As noted above, NADPH is produced by the PPP and glucose-6-phosphate is the starting point for this pathway and the second step of the glycolysis pathway. If *zwf* is triggered to initiate the PPP, then we speculate that *deoB* is also upregulated to ensure completion of the pathway and the replenishment of NADPH levels depleted under oxidizing condition. How SoxS and MarA balance this is not known but it is likely that it is influenced by the oxidizing state of the cytosol and the resulting effect on SoxR and other regulators that control these enzymes.

FumC, GltA, AcnA and SdhB

Fumarase C (FumC), Citrate synthase (GltA), aconitase A (AcnA) and succinate dehydrogenase B (SdhB) are all enzymes or subunits of enzymes of the Krebs cycle (Figure **3**, Table **1**). With the exception of FumC, only microarray evidence exists demonstrating regulation by MarA or SoxS [2, 6, 7, 138]. FumC and AcnA are additionally regulated by Rob [37, 74].

FumC is one of three fumarases in *E. coli.* In comparison with FumA and FumB, FumC is thermostable and stable under oxidative attack, perhaps because it does not contain a Fe-S cluster. Fumarase interconverts fumarate to L-malate in the Krebs cycle. It was first identified as a SoxS regulon

member by Liochev and Fridovich in 1992 [141]. It is intimately connected with *zwf* as strains where zwf gene is deleted show increased expression of *fumC*.

5.3.2. Oxidative Stress Response Genes

SodA and YggX

SodA and YggX are two of the most important components of the oxidative stress response. Superoxide dismutase A (SodA) converts superoxide ions to hydrogen peroxide [142]. It is induced by all three TRs and by heat shock, DNA binding drugs, ethanol, high salt, and heavy metals [143] (references therein), and by high oxygen conditions [130] *via* SoxS. Since the hydroxyl radical is highly lethal and not convertible, SodA (and SodB and SodC) acts in concert with catalase/peroxidases KatG, KatE and AhpC to convert superoxide molecules to hydrogen peroxide and the latter to water. In particular, SodA captures superoxide molecules being produced by the electron transport chain. From microarray data, *katE* and *ahpC* are also part of the SoxS regulon but no further studies have been done [7].

SoxS binds directly to the *yggX* promoter to produce the transcript for an 11kDa protein. MarA and Rob have no effect on *yggX* transcription [144]. Deletion of *yggX* almost abolished the ability of *E. coli* to protect against the effects of paraquat and it is suggested that YggX is involved in repair of Fe-S clusters [59].

NfsA (MdaA) and NfsB (NfnB)

NfsA and NfsB are oxygen insensitive flavodoxin monocleotide (FMN) binding nitroreductases with broad specificity for electron acceptors and use NAD(P)H as the electron donor [145, 146]. They are involved in resistance to nitrofurans such as nitrofurantoin [147]. NfsA is the major component while NfsB plays a more minor role [146]. Studies have shown that that *nfsA* and *nfsB* were upregulated by the constitutive or induced expression of MarA and that MarA, SoxS and Rob bind directly to the *nfsA* and *nfsB* promoters but with different affinities [74, 148]. Another study also showed that paraquat was able to upregulate the expression of *nfsA* in a *soxR* (and therefore presumably in a *soxS*) dependent manner [149]. Mutations in *nfsA* and *nfsB* have been found in clinical strains highly resistant to nitrofurantoin [147]. Nitrofurans are a second line of treatment for infections such as UTIs, where resistance to commonly used antibiotics is an increasing problem [147]. These strains were not analyzed for expression of the TRs, so it is not known if they were responsible for the resistance.

An interesting aspect of these two proteins is that together with NemA, they are also part of 2,4,6-trinitrotoluene (TNT) degradation. Though no-longer produced in the United States, TNT is a major soil contaminant at some industrials sites where explosives were manufactured. NemA, NfsA and NfsB enable *E. coli* to extract nitrogen from TNT [150]. If organic solvents that might typically be expected to be part of soil contamination at such industrial sites are also present, MarA might contribute to TNT breakdown through upregulation of *nfsA* and *nfsB*. It is not known if TNT increases expression of *marA*.

5.4. Acid Tolerance

5.4.1. HdeAB

HdeA and B are chaperone proteins that prevent denaturation of proteins at low pH [151]. Their expression levels also vary according to the bacterial growth phase and the operon is regulated by a host of different factors. MarA has been shown to act as a repressor of *hdeA* in microarray studies [6, 7]. It directly binds to the *hdeA* promoter to repress transcription *in vitro* [86]. *In vivo*, MarA enhances the repressor activity of H-NS on *hdeAB* during exponential and stationary phases at neutral and acidic pH and inhibits the activity of the *hdeAB* activator GadE during stationary phase, particularly at an acidic pH [152]. MarA also affects other *hdeAB* regulators such as RpoS, Lrp, GadX and GadW, though the effect was not as large as detected for H-NS and GadE. Many of the regulators of *hdeAB* themselves affect the transcription of other proteins and together with the regulation by MarA, results in a highly complex regulatory network, able to respond to the many different conditions that the cell might encounter.

5.4.2. InaA

Weak acids induce *inaA* expression [153] in a Rob, MarA and SoxS-dependent manner [7, 154, 155]. Although little is known about the protein product of *inaA*, it has recently been used as a marker for compounds toxic to micro-organisms present in lignocelluolose hydrolysate. Lignocellulose is a potential feedstock for biofuel production. Lee and coworkers [156] showed through qPCR and microarray studies of *E. coli* that *marR*, *marA*, *marB* and *inaA* were upregulated and occasionally downregulated in response to chemicals present as byproducts of lignocellulose breakdown. Using this information, Lee and Mitchell [157] fused the *inaA* promoter to the *luxCDABE* reporter system to detect toxic compounds

present in lignocellulose hydrolysate; deletion of *marA* prevented this response which is in agreement with MarA acting as an *inaA* activator. Interestingly the authors found that deletion of *marB*, about which very little is known, increased the reporter signal. Use of such a reporter system to detect the buildup of toxic compounds during the conversion of lignocellulose to biofuel would be useful in adapting strains to be resistant to the toxins and to identify their need for removal, to enhance the efficiency of the process.

5.5. Metabolism

5.5.1. PurA

PurA is an adenylsuccinate synthase and is necessary for *de novo* purine synthesis. Deletion of *purA* in *E. coli* has also been shown to decrease the ability of the bacteria to invade human brain microvascular endothelial cells that make up the blood-brain barrier, which *E. coli* is able to cross to cause new born meningitis [158]. Microarray studies show a 2-fold reduction of *purA* levels with constitutive MarA expression [6] and MarA is able to directly bind and repress the *purA* promoter, resulting in a 56% decrease in transcription *in vitro* compared with the wild type strain [86]. In the study of urinary tract infections, mutation of *purA* caused a decrease to 1/5 of the WT growth rate and was strongly outcompeted by the WT strain in human urine. The effect on UTI was not tested for this mutant [159]. However, it was shown that deletion of purA in *Salmonella typhimurium* decreased bacterial persistence in mice infected orally [160, 161].

5.5.2. map

Methionine aminopeptidase (*map*) is an essential metallooligopeptidase that catalyzes the removal of the N-terminal methione [162]. It is considered as an antibacterial target candidate since its inhibition or loss causes decrease in viability or death respectively [163]. Activation through binding to the *map* promoter has been shown through *lacZ* fusions during treatment by paraquat and dipyridyl implicating SoxS and Rob in transcriptional activation [74]. Sodium salicylate, and therefore by implication MarA, had a smaller and perhaps insignificant effect. Activation of *map* by SoxS has also been identified in two different microarray studies [7, 138]. The consequences of this regulation are unknown.

5.6. Virulence and Biofilm

With their extensive regulons the three TRs might be expected to have a role in virulence during

infections and we have touched on this point in some of the preceding sections through the functions of the regulon members. An example of their involvement during infections comes from a triple deletion mutant (*marA-soxS-rob*) failing to persist in an *E. coli* mouse model of ascending pylenophritis [10]. Warner *et al.* have further used microarrays to demonstrate how the zinc transport system *znuABC* is part of the SoxS operon and necessary for colonization of the mouse kidneys in this mouse model [11]. It is likely that the decreased susceptibility to CAMPs mediated by MarA and Rob also plays a role in virulence [104].

Biofilms are an aspect of virulence and their formation is a great problem during infections, as they are more resistant to antibiotics than planktonically growing bacteria. Upregulation of MarA by salicylate or *via* mutation in *marR* in a UPEC strain was shown to down-regulate the expression of *fimB* and therefore results in decreased biofilm formation. This could have implications for treatment of UPEC infections, where biofilms are problematic [164]. However, caution should be used as an earlier study showed that overexpression of the *mar* operon only protected non-pathogenic *E. coli* against very low levels of ciprofloxacin [165]. Additionally, Duo *et al.* [166] showed that overexpression of Rob caused increased resistance of biofilms against the aminoglycoside, tobramycin[166]. No involvement of SoxS in biofilms has been demonstrated.

5.7. Overlap with other Global Regulators

MarA also regulates proteins that have been shown to be involved in other stress adaptation mechanisms. For example, many of the genes that are regulated as a function of entry into stationary phase, hypertonic tension, acid shock and cold shock also appear in the regulon of MarA [167-169]. The genes in the regulon of RpoS are also involved in response to these stress factors [169] and many of the genes found in the regulon of RpoS are also found in the regulon of SoxS and MarA. This has been directly confirmed in the acid response where MarA and RpoS work antagonistically in the regulation of HdeA and B expression [152]. Paraquat induces not only the expression of *soxS* but also *oxyR*, *fur* and several other regulatory genes. Mapping out the different conditions when each transcriptional regulator is active is beyond the scope of this review but it is apparent that the network of response genes is able to minutely adjust the bacterial responses to altering conditions.

6. HOMOLOGS IN ENTEROBACTERIACEAE

Cohen and colleagues [170] identified 9 different genera including 3 *Enterobacter spp.*, *Klebsiella*, *Shigella*, *Citrobacter*, *Salmonella spp.*, and *E. coli,* in which they experimentally identified *marA* related sequences. Analysis of the *marRAB* operon with BLAST and FastA also identifies *marA* in these as well as in *Cronobacter*. The percentage amino acid identities of the encoded proteins with the *E. coli* counterparts are shown in Table **2**. Many of these Gram negative micro-organisms are pathogenic and, over recent years in the case of *Salmonella*, MarA, SoxS/R and Rob-like proteins have been shown to be involved in pathogenesis (see below). Dietrich confirmed that the regulators are confined to the *Enterobacteriaceae* [171].

6.1. MarA, SoxS and Rob in *Salmonella, Shigella, Klebsiella, Citrobacter, Enterobacter* and *Yersinia*

During food poisoning, *Salmonella enterica* causes intestinal inflammation with diarrhea. *Salmonella* is similar to *E. coli* in that it has a complete *marRAB* operon, *soxRS* loci and a *rob* gene. Table **2** shows the high identity that these proteins have with their *E. coli* counterparts. There have been several studies on MarA, SoxS and Rob in *Salmonella* and their involvement with regulating expression of the AcrAB efflux pump [172-175]. Most of the studies show that *marRAB* and *soxS* are induced by sodium salicylate and paraquat respectively and that this leads to increased MICs *via* the *Salmonella* AcrAB pump [173-176].

The situation changes with respect to bile induction. In *Salmonella*, bile can induce *marRAB* but does not induce or activate Rob, as it does in *E. coli*. In *Salmonella*, bile induction of *marRAB* leads to a decrease in susceptibility to antibiotics but this is not thought to be a major pathway for decreased susceptibility. Some of AcrAB upregulation through bile occurs independently of MarR and MarA, and it is likely that AcrAB still forms part of the major mechanism of antibiotic and bile resistance [172].

MarA, SoxS and Rob behave in a very similar way to their counterparts in *E. coli* in response to the oxidative stress compound sodium hypochlorite (NaOCl). Expression of MarA and SoxS are increased and deletion mutants cause a 20-fold increase in sensitivity to the compound [177]. All phagocytic cells, with the exception of macrophages, produce NaOCl *via*

Table 2:   MarA, SoxS and Rob Homologs in *Enterobacteriacea*[a]

Species	Gene	% Identity with *E. coli* gene[b]
Salmonella enterica subsp. Enterica serovar Typhimurium str. LT2	MarA	95(120/126)
	MarR	92 (133/144)
	MarB	46 (31/71)
	SoxS	95 (102/107)
	SoxR	96 (146/152)
	Rob	93(268/289)
	RamA	45 (45/101 MarA) 45 (45/100 SoxS)
Shigella flexneri 2a str. 2457T	MarA	100 (127/127)
	MarR	99 (142/144)
	MarB	97 (70/72)
	SoxS	99 (106/107)
	SoxR	100 (154/154)
	Rob	100 (289/289)
	RamA	not present
Klebsiella pneumoniae 342	MarA	93(115/124)
	MarR	83 (120/144)
	MarB	44 (70/72)
	SoxS	88 (95/107)
	SoxR	89 (136/152)
	Rob	81 (236/290)
	RamA	47 (47/104 MarA) 49 (49/100 SoxS)
Citrobacter koseri ATCC BAA-895	MarA	96 (122/127)
	MarR	93 (134/144)
	MarB	51 (33/65)
	SoxS	95 (102/107)
	SoxR	95 (145/152)
	Rob	90 (261/289)
	RamA (hypothetical)[a]	45 (47/104 MarA) 47 (47/100 SoxS)
Enterobacter cloacae subsp. *cloacae* ATCC 13047	MarA	94 (116/124)
	MarR	86 (124/144)
	MarB	48 (28/58)
	SoxS	90 (96/107)
	SoxR	92 (142/152)
	Rob	83 (240/289)
	RamA	49 (51/104) MarA 46 (46/100 SoxS)
Cronobacter sakazakii ATCC BAA894	MarA	90 (111/124)
	MarR	88 (126/144)
	MarB	-
	SoxS	88 (91/104)
	SoxR	87 (132/152)
	Rob	78 (224/287)
	RamA	not present

(Table 2). Continued.

Species	Gene	% Identity with *E. coli* gene[b]	
		% MarA identity	% Rob Identity
Yersinia pestis KIM	MarA47	47 (43/91)	32 (84/259)
	MarA48	48 (49/101)	67 (195/289)
	LcrF	24 (23/96)	28 (26/92)

[a]MarA, MarR, MarB, SoxS, SoxR and Rob sequences used were those of *E. coli* K12, accession number NP_416047.
[b]RamA sequences identified using BLAST search with *K. pneumonia* 342 RamA protein.

the myeloperoxidase enzyme. This could be one mechanism by which MarA and SoxS favor the survival of *Salmonella* during infections. Studies have shown that MarA was important for the colonization of chickens by *Salmonella* and played a role in antibiotic resistance development to tetracycline [178]. In contrast, deletion of *marA* did not affect the oral inoculation LD_{50} values of *Salmonella* in mice [176]. Additionally, in WT strains, MarA and SoxS co-operatively induce the expression of *ompW* upon exposure of the bacteria to menadione [179]. OmpW is a minor immunogenic membrane porin that has been implicated in paraquat resistance, osmoprotection and hydrogen peroxide, and hypochlorous acid influx [179 and references therein]. OmpW has not been shown to be regulated by the TRs in *E. coli,* but other porins are under their control, suggesting a similar theme for both species (see section 5.2.2).

Outside of the hospital, *Klebsiella pneumoniae* causes some form of respiratory disease such as pneumonia, bronchitis or bronchopneumonia. In a hospital setting, it often colonizes the lungs of patients causing inflammation and hemorrhage, particularly in immunocompromised patients. *K. pneumoniae* can also colonize the urinary tract, lower biliary tract and surgical wound sites. Infections are difficult to treat and MDR is increasingly seen. *Klebsiella*, like *Salmonella*, has all three TRs and they bear high identity to their *E. coli* counterparts (Table **2**). Together with AcrR, MarA, SoxS and Rob, are thought to be able to regulate AcrAB in *Klebsiella* [180-182]. Bratu *et al.* showed correlation between MarA/SoxS expression and antibiotic susceptibilities (levofloxacin and tigecycline) in clinical isolates [183].

Enterobacter cloacae is not a primary pathogen, but has been known to cause UTI and respiratory tract infections. It has *marRAB*, *soxS* and *rob* with high identity to the *E. coli* homologs (Table **2**). *E. cloacae* Rob was shown to cause 2-32 fold increases in antibiotic MICs when plasmid borne. It also downregulated OmpF expression, similarly to in *E. coli*

[184]. Additionally, Perez *et al*. [185] found that Rob and SoxS were able to increase expression of *acrAB* to different extents in the presence of sodium decanoate (a bile salt) and paraquat in a situation similar to that in *E. coli*.

Yersinia pestis and *pseudotuberculosis* are also *Enterobacteriaceae* and are the causative agents of plague and stomach infections respectively [186]. They have two proteins with 47 and 48 % identity to MarA (named MarA47 and MarA48 respectively). MarA48 has 67% overall identity and 54% identity with the C-terminal domain (regulatory domain, residues 122-289) of *E. coli* Rob (Table **2**). Studies showed that they were involved in colonization in a mouse pneumonic plague model [187]. Mar47 also increased transcriptional levels of *acrAB*, and was shown to be involved in antibiotic susceptibility [188]. No MarR exists in *Yersiniae spp.* so it is uncertain how MarA48 and MarA47 themselves are regulated in response to external stimuli [187, 188].

Little literature is available for *Citrobacter* and *Shigella flexneri,* other than sequence data identifying the presence of *marA*, *soxS* and *rob* (Table **2**). In *Shigella flexneri*, there is little evidence of their role in the antibiotic resistance of this pathogen [189, 190].

6.2. RamA in *Salmonella*, *Klebsiella*, Citrobacter and Enterobacter

Salmonella has a second MarA/SoxS-like protein called RamA. This protein was first identified in *Klebsiella pneumoniae* as being responsible for an MDR phenotype [191] and having 47% and 49% identity with *E. coli* MarA and *E. coli* SoxS (Table **2**). While not present in *E. coli*, RamA is present in a wide range of Enterobacteriaceae. *ramA* is regulated by transcriptional repressor RamR, located just upstream of *ramA.* Mutation or inactivation of RamR results in upregulation of *ramA* and an MDR phenotype [192-194]. RamA mediates its effects through AcrAB-TolC. Paraquat, bile and indole (an intercellular signaling

molecule) upregulate expression of RamA and therefore AcrAB, but bile does not alter the transcription of RamA indicating post transcriptional or post translational regulation [195]. Recently, an alternative induction mechanism has been proposed; many antibiotics that are substrates for AcrAB do not cause increased expression of RamA. However, if AcrAB is inhibited or disrupted and the strain subjected to these antibiotics, RamA expression increases. It is suggested that internal metabolites or stress signals resulting from the absence of the pump or a decrease in efflux, rather than the antibiotics themselves, causes the increased *ramA* transcription [196]. This effect is similar to the mechanism proposed for MarA, SoxS and Rob activation in *E. coli* in a *tolC* strain (see section 5.2.1).

In *Salmonella*, RamA also plays a role in nitric oxide metabolism *via* the flavohemoglobin, Hmp. NO produced at nanomolar concentrations can act as a signaling molecule whereas higher concentrations damage non-target enzymes [197]. Inactivation of RamA decreases expression of *hmp* and increases susceptibility to NO [198]. In *E. coli*, the gene *hmpA* is also upregulated by SoxS and is important in NO metabolism [56, 135]. In terms of virulence, while RamA appears to be involved in multiple signaling pathways, there is so far little evidence that it is important for the *in vivo* virulence properties of *Salmonella* [199].

RamA is also found in *Klebsiella* and is 63% identical to the *Salmonella* RamA. Additionally, it is also regulated by repressor RamR and mutations in this gene have been found in clinical strains with decreased susceptibility to tigecycline [182, 200]. Interestingly, a tigecycline resistant strain has also been identified with increased RamA levels, but with no mutation in RamR suggesting an alternative method of RamA regulation [182]. Unlike *Salmonella*, *Klebsiella* also has a gene named RomA, which lies in between RamA and RamR but the regulation of RomA by RamR is uncertain. It appears that RomA and RamA are independently regulated as their expression levels do not correlate [182]. Other studies show that RamR mutations, like SoxR and MarR mutations, also lead to decreased susceptibility to various antibiotics including ciprofloxacin, chloramphenicol, cefuroxime and trimethoprim/sulfmethexazole as well as tigecycline and this may also be mediated by cumulative effects of a decrease in porin OmpK35 and an increase in AcrAB [181, 201, 202].

Enterobacter cloacae has also been found to have a *ramA* gene regulated by an upstream *ramR* that can be activated to induce AcrAB expression in the presence of sodium salicylate [185]. Interestingly, the authors found that MarA was not affected and it was RamA that induced the increase in MIC through AcrAB. A subsequent study found that decreased susceptibility to tigecycline in clinical isolates was almost always due to RamR mutations causing an increase in RamA and therefore AcrAB expression [203].

In *Citrobacter,* only sequence data identifying the presence of RamA, RomA and RamR are published [182]. A homolog of RamA is not present in *Shigella* or in *Cronobacter*

6.3. LcrF in *Yersiniae spp*

A second transcription factor, LcrF, is present in *Yersinia spp (Y. pestis, Y. pseudotuberculosi and Y. enterolitica)*. Although LcrF has a much lower identity with MarA, SoxS and Rob (Table **2**), it falls into the AraC family of transcription factors. LcrF regulates the transcription of the type three secretion system that protrudes, like a needle, from the surface of the bacteria to make contact with immune system cells such as neutrophils and macrophages; the effector proteins known as Yersinia Outer Proteins (YOPs) can then be injected into the eukaryotic cell [204, 205]. The YOPs interfere with cellular processes in these cells, preventing their normal function such as the secretion of immune signaling molecules, the cytokines. This allows the bacteria to evade the immune system and continues the infection. Deletion of *lcrF* results in an absence of disease [206, 207]. LcrF has therefore been pursued as a drug target (see below).

7. DEVELOPMENT OF NOVEL ANTI-VIRULENCE AGENTS

Since antibiotics target bacterial processes essential for the growth of the micro-organism, these molecules exert a strong selective pressure on resistance development, based on the ability of the bacteria to survive in the presence of the drug. With a few exceptions, most antibiotics developed in the past decades are improved derivatives of existing chemical classes [208, 209]. It is reasonable to expect that these drugs will eventually be subject to a pre-existing resistance mechanism. Undoubtedly, fighting bacterial resistance will require a more innovative research approach.

As an alternative to traditional antimicrobials, research targeting pathogen virulence rather than

growth has been reported in the literature [13, 14]. Paratek Pharmaceutical Inc. used this approach to develop inhibitors of MarA, SoxS and Rob that can be used as anti-virulence compounds since studies have shown that these regulators controlled the virulence of *E. coli* in an animal model of ascending pyelonephritis infection [10]. Using the structures of MarA-DNA and Rob-DNA complexes, this group constructed a model of the conserved DNA-binding domain. Docking methodology followed by *in vitro* screening of commercially available molecules was then used to identify compounds with inhibitory activity against these regulators [210]. N-hydroxybenzimidazole and derivative compounds were shown to prevent the binding to DNA by SoxS, MarA, and Rob. Moreover, one analog was shown to decrease the bacterial load of *E. coli* in an animal model of infection [210].

More recent studies have also demonstrated that N-hydroxybenzamidazole derivatives were active against other regulators of virulence. Inhibitors of the SoxS-DNA interaction were modified to develop compounds targeting LcrF [211], a regulator of the AraC family protein which controls virulence in *Y. pestis* and *Y. pseudoturbeculosis* (see above) [204, 205, 211, 212]. These compounds were also shown to reduce the virulence of *Yersinia* in a whole cell assay. Later, Grier and colleagues used the same strategy to identify new N-hydroxybenzimidazole derivatives active against ExsA [213], a regulator for which the DNA-binding domain displays 85% of identity with *Yersinia* LcrF. ExsA is necessary for full virulence in *Pseudomonas aeruginosa,* an opportunistic Gram negative pathogen [214-216]. The study also demonstrated that these compounds significantly reduced cytotoxicity of *P. aeruginosa* to infected [213]. Furthermore, encouraging results have shown that some of these compounds displayed good metabolic stability with *in vitro* human liver microsomes [213].

8. CONCLUSION

MarA, SoxS and Rob are three global TRs that through their hierarchical response to external stimuli such as antibiotics, oxidative stress, pH and host signals such as bile and CAMPs and through the complex network of interactions with other transcriptional regulators, can steer *E. coli* through changing environments, to produce a finely tuned but robust response mechanism [217]. The importance of their roles can perhaps be understood because of their conserved nature in Gram negative *Enterobacteriacae* [171] and by the finding that in other Gram negative

and a few Gram positive bacteria, where direct homologs are not found, genes that code for similar proteins exist. For example, in *Pseudomonas spp.*, a SoxR protein is involved in metabolism of endogenous molecules that act as an intercellular signaling molecule, though a SoxS protein is absent. In another example, Shin and colleagues [59] report that the SoxR homolog in *S. coelicolor* has five identified regulon members, and requires the presence of secreted antibiotic acthinorhodin to cause regulon member transcription. Other Gram positive bacteria have proteins with fairly high identity to MarA, SoxS or Rob. A FASTA search with MarA reveals a *Bacillus spp.* protein with 41% identity but little work has been done with these proteins. In this period of increasing antibiotic resistance, these three TRs and their relatives also show potential for innovative therapeutics to combat resistant infections. Like MarA, SoxS and Rob in *E. coli*, a subset of the AraC family of proteins regulate the expression of virulence genes in many clinically important bacterial species. Given that these regulators are not required for bacterial survival outside of a host, inhibitors of these proteins are less likely to apply the selection pressure for resistance development.

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