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## The Evolution of MarR Family Transcription Factors as Counter-Silencers in Regulatory Networks

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### Abstract

Gene duplication facilitates the evolution of biological complexity, as one copy of a gene retains its original function while a duplicate copy can acquire mutations that would otherwise diminish fitness. Duplication has played a particularly important role in the evolution of regulatory networks by permitting novel regulatory interactions and responses to stimuli. The diverse MarR family of transcription factors (MFTFs) illustrates this concept, ranging from highly specific repressors of single operons to pleiotropic global regulators controlling hundreds of genes. MFTFs are often genetically and functionally linked to antimicrobial efflux systems. However, the SlyA MFTF lineage in the *Enterobacteriaceae* plays little or no role in regulating efflux but rather functions as transcriptional counter-silencers, which alleviate xenogeneic silencing of horizontally-acquired genes and facilitate bacterial evolution by horizontal gene transfer. This review will explore recent advances in our understanding of MFTF traits that have contributed to their functional evolution.

### Keywords

Gene regulation; gene duplication; Transcription factor; counter-silencing; Transcription; evolution

### Introduction

In his book *Evolution by Gene Duplication* [1], Susumu Ohno argued that gene duplication is a driving force in evolution, suggesting that “natural selection (has) merely modified, while redundancy created.” He posited that gene duplications and the redundancy they produce provide the evolutionary space necessary for functional diversification and

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innovation. Duplication allows the exploration of otherwise “forbidden” mutations, generating novel functions unique from those of the ancestral gene, ultimately resulting in greater organismal complexity. In the absence of gene duplication, mutations are limited to those that do not disrupt essential gene function, constraining potential evolutionary trajectories. Although more recent evolutionary studies suggest that horizontal gene transfer has a greater impact on bacterial evolution than classical intragenomic duplication [2–4], Ohno’s ideas are relevant here as well, as the expansion of a gene family by extensive lateral transfer also provides valuable evolutionary space. These processes are exemplified by the evolution of gene regulatory networks. Transcription is typically regulated by transcription factors (TFs), which bind near gene promoters to modulate their transcription by RNA polymerase (RNAP). Although one genome may encode hundreds of unique TFs, these belong to as few as 10 unique TF families [5,6]. The TFs contained within each family are the products of gene duplication, and the maintenance of these duplicates implies a fitness advantage. This can occur through neofunctionalization, wherein a duplicate acquires a novel function not present in the original gene, or subfunctionalization, wherein the function of an original gene is divided between two or more copies via mutational divergence [7]. TF gene duplication allows both *in cis* variation, resulting from changes in the promoter driving expression of a TF, which affects the binding and activity of upstream TFs and RNAP, or *in trans* variation, resulting from changes in the coding sequence of the TF, which alters interactions with cognate targets or other interaction partners. The net result of these duplication events and the resulting variation are increasingly complex regulatory networks that are able to respond to a variety of environmental and physiological stimuli.

The MarR (*Multiple antibiotic resistance Regulator*) family of TFs (MFTFs) exemplifies these processes. MFTFs are ancient, predating the divergence of Archaea and Bacteria [8] and presently comprising one of the most common TF families in bacteria. Although the average bacterial genome encodes 7 unique MFTFs [9], the number can vary widely: *Bacillus subtilis* and *Streptomyces coelicolor* encode at least 20 each (depending on the strain), whereas *Salmonella enterica* serovar Typhimurium encodes 7, and the related enteric species *Yersinia pseudotuberculosis* encodes only 3. Even endosymbiotic species, which have undergone substantial genome loss, encode multiple MFTFs, including *Sodalis glossinidius*, which encodes 5 [10,11]. Despite their significant presence in bacteria, MFTFs exhibit limited sequence conservation between lineages, typically with less than 30% identity. This variability may reflect the inherent versatility of the MFTF backbone that allows them to interact with a variety of targets and respond to a variety of physiological and environmental signals. The ubiquity of MFTFs, particularly in the reduced genomes of endosymbiotic species, suggests that they serve an underappreciated role as central regulators of bacterial gene expression.

MFTFs were first recognized when *E. coli* mutants exhibiting heightened resistance to multiple antibiotics were observed to encode mutations in MarR, the prototypical MFTF [12]. Since that discovery, MFTFs have been found to play a role in a number of important biological processes, including antibiotic resistance, virulence [13], oxidative stress [14], central metabolism [9,15] and the catabolism of a variety of aromatic compounds [9]. Although MFTFs were originally regarded as classical repressors of transcription, often of very small regulons, more recent studies have demonstrated that some members of the

family can also function as global regulators, both positively and negatively modulating gene expression [11,16,17]. This scenario is perhaps best exemplified by the SlyA MFTF lineage in *Enterobacteriaceae*, which has evolved to function as transcriptional counter-silencers [10,16,18,19]. SlyA proteins alleviate xenogeneic silencing of horizontally-acquired genes by proteins such as H-NS [20,21], thereby playing a vital role in the regulatory integration of horizontally-acquired genes. This allows a bacterial cell to realize a potential fitness benefit from horizontally-acquired genes, which might be detrimental if expressed in an unregulated fashion. Notably, SlyA is strongly conserved, as it is present in most species in *Enterobacteriaceae*, including endosymbionts such as *S. glossinidius* and *Wigglesworthia glossinidia*, in which it is under strong selective constraints [22]. The SlyA lineage is not unique in controlling large numbers of genes. ScoC, which belongs to a distinct MFTF lineage, positively and negatively regulates more than 560 genes in *B. subtilis*, which are involved in sporulation, transport, motility, and metabolism [23], although the mechanistic basis for its pleiotropic function is unknown. The existence of MFTFs functioning as both small regulon repressors and global counter-silencers provides a clear example of functional innovation and diversification as a consequence of gene duplication.

### The MFTF DNA binding motif is highly variable.

Regardless of their specific function, MFTFs are generally defined by four common features: (1) a single globular domain, containing both (2) a winged-helix-turn-helix (wHTH) DNA-binding motif [24,25] and (3) a ligand-binding site that allows allosteric inhibition by environmental or physiological signals [26], and (4) genetic linkage to a multi-drug efflux pump. The chimeric wHTH (Fig. 1) domain consists of a classical prokaryotic helix-turn-helix motif, in which the recognition helix ( $\alpha 4$ ) engages extensively with the major groove of the DNA duplex, acting as the primary determinant of specificity. The wing domain, more commonly observed in eukaryotic proteins, interacts closely with the minor groove and appears to increase the affinity of the interaction via indirect readout, wherein the shape of the DNA rather than the DNA sequence itself is the primary determinant of the DNA-TF interaction [25,27–30]. This is notable, as xenogeneic silencers such as H-NS and Lsr2 [31] also rely upon indirect readout, detecting a narrowing of the minor groove that is associated with TpA steps in AT-rich DNA. The emergence of MFTFs as counter-silencers may be due in part to the ability of the wing region to recognize sequences similar in structure to those recognized by xenogeneic silencers. Upon MFTF binding, the DNA major groove widens by 2–4Å to accommodate insertion of the recognition helix, while the minor groove can similarly widen and overtwist as a result of interactions with the wing [25,32]. These structural changes distort the DNA duplex, bending the DNA by  $\sim 15^\circ$  and underwinding it by 1.2 to 1.4° [25,32]. This distortion may be important for counter-silencing function, which is accompanied by the formation of a bend within the silenced H-NS DNA complex [18]. A helix-helix (HH) motif, comprised of helices  $\alpha 3$  and  $\alpha 2$  and their connecting residues, also contributes to the MarR-DNA interaction via contacts with the phosphate backbone of the DNA duplex [25,28]. Genome-wide data characterizing MFTF-DNA interactions are limited. However, a number of studies have examined MFTF binding at specific loci and reveal that MFTFs typically recognize palindromic binding sites with an elevated AT-content [33–39]. This is partially due to a conserved arginine residue in

the wing of many MFTFs (Fig. 1), which is essential for SlyA function [40], This residue makes multiple contacts with adenine and thymine bases in the minor groove [27]. However, the  $\alpha 4$  recognition helix can vary significantly among MFTFs in both the number and type of interactions made with the major groove. Some MFTFs, such as OhrR of *B. subtilis* and SCO3205 of *S. coelicolor*, encode multiple residues (4 and 3, respectively) in  $\alpha 4$ , which form relatively sequence-specific hydrogen bonds with DNA. However, others are more reliant on the less specific or lower energy van der Waals interactions to form a MFTF-DNA complex. These include SlyA, which has only one hydrogen bond-forming residue but two van der Waals-interacting residues, and MepR of *S. aureus*, which does not form any hydrogen bonds but is instead reliant on 4 van der Waals contacts [27]. MarR, which exhibits high sequence specificity with only a single binding site in the *E. coli* chromosome [41] exhibits both types of interaction, forming 3 hydrogen bonds and 5 van der Waals contacts [28]. More unusual interactions are also possible, as the recognition helix of ST1170, an MFTF from *Sulfolobus tokodaii*, does not insert into the major groove at all, but rather makes non-specific contacts with the sugar-phosphate backbone of DNA [42]. The DNA passes over the wHTH domain of ST1170 to make contact only with the wing. Given these observations, it is conceivable that MFTF gene duplication has allowed  $\alpha 4$  to become reliant upon hydrogen bonds when more specific interactions of a classical repressor are required, or upon van der Waals contacts when performing a more pleiotropic global role, as in SlyA and other counter-silencers (Fig. 2).

### MFTFs are allosterically inhibited by multiple stimuli.

The mechanism of allosteric inhibition, which sensitizes MFTFs to physiological and environmental stimuli, is a point of conjecture for MarR. Early studies observed that multi-drug resistance could be induced by the addition of salicylate to *E. coli* cultures [43], which was later shown to result from the inhibition of MarR-mediated DNA binding and repression [44]. Because MFTFs are comprised of a singular globular domain, ligand binding can easily impact interaction with DNA. The binding of small molecules like salicylate causes the  $\alpha 4$  recognition helix to rotate out of register with the major groove of DNA, thereby inhibiting DNA binding [10,24,45,46]. Subsequent studies have identified endogenous aromatic acid metabolites that are structurally similar to salicylate and can also inhibit MarR [47,48]. More recently, it has been postulated that MarR is inhibited by cytoplasmic copper(II) liberated from membrane-bound proteins during envelope stress [38]. These copper(II) ions oxidize the conserved C80 cysteine residue of MarR, promoting the formation of disulfide bonds between MarR dimers to form a tetramer that is unable to interact with the DNA major groove. Inhibition by aromatic carboxylates was also suggested by these authors to result from cysteine oxidation, perhaps as a result of salicylate-induced membrane stress and copper(II) release. However, although the cysteine oxidation model of inhibition is attractive, as similar models have been observed for other MFTFs such as OhrR of *B. subtilis*, which utilizes cysteine oxidation to sense organic hydroperoxides [14,49], it is not universal. Mutation of the lone conserved cysteine residue in SlyA has no effect on its counter-silencing activity nor on its inhibition by aromatic carboxylates [10]. Mutagenesis of an analogous cysteine residue in the *Staphylococcus aureus* MFTF MhqR similarly had no impact on its function as a quinone-responsive regulator of antimicrobial resistance [50], and

non-cysteine-dependent aromatic carboxylate inhibitory mechanisms have been described for an array of MFTFs [9,51]. Although we cannot specify ancestral endogenous ligands with certainty, we note that both archaeal MFTFs and counter-silencing MFTFs such as SlyA and RovA have retained the ability to bind and be inhibited by aromatic carboxylates [10,42,45], suggesting that the ancestral ligand(s) is a similar molecule. It is possible that some MFTFs are subject to inhibition by both cysteine and aromatic ligand binding, due to an inherent promiscuity of the ligand-binding site [46]. This may be the case with MarR [24,38], and if true for other MFTFs, could contribute to their functional versatility.

## Genetic linkage to transporters suggests a physiological function for MFTFs.

The third common feature of MFTFs, genetic linkage to efflux pumps or transporters, may reflect the primordial function of this protein family. In *S. Typhimurium*, 4 out of 7 MFTF genes are genetically linked to multi-drug efflux pump or transporter-encoding genes (Fig. 3), including the pleiotropic counter-silencer SlyA, although this linkage has been lost in most other enteric species, including endosymbiotic species. This suggests that, although SlyA may be under selective constraints [22], the YdhIJK efflux pump is no longer important for SlyA function [10]. Similar linkages to pumps and transporters are observed in other species, including Archaea. In *Sulfolobus sulfataricus* P2, 4 out of 6 MFTFs are linked to MFS or ABC family transporters. Even when MFTFs are not directly linked to efflux pump coding genes, they are often functionally linked to efflux, as is MarR. Although the *marRAB* operon is not genetically linked to efflux pump genes, MarR represses the expression of the activator MarA, which up-regulates synthesis of the AcrAB-TolC efflux pump, a primary determinant of intrinsic antibiotic resistance in *E. coli* [52,53]. This suggests an ancestral role for MFTFs in maintaining physiological homeostasis by sensing and regulating the efflux of both toxic metabolites as well as xenobiotics, a hypothesis supported by studies identifying aromatic metabolites as endogenous ligands of MarR [47,48]. However, it is notable that these transporters are not all genetically related. AcrAB, regulated by MarR, is a member of the RND (Resistance-Nodulation-Division) superfamily, whereas many other regulated transporters are members of the Major Facilitator Superfamily (MFS), suggesting that some of these linkages are a product of convergent evolution.

## Variation *in cis* has contributed to pleiotropic function.

As mentioned above, *cis*-level variation, which alters the expression of individual MFTFs, can also contribute to regulatory evolution and functional adaptation. This was recently demonstrated in an analysis of SlyA alleles from *S. Typhimurium* and *E. coli*. Although allelic exchange demonstrated that both alleles are capable of functioning as counter-silencers in *S. Typhimurium* [10], SlyA does not play a significant role in the *E. coli* regulatory network [46–48], apparently because of low levels of expression. The *S. Typhimurium* *slyA* promoter has evolved to provide high expression levels under conditions found in the intra-phagosomal environment [10], which corresponds to the essential role of SlyA in resistance to macrophage killing [54]. This is supported by studies of *hlyE*, which encodes a cytolytic toxin in *S. enterica* and *E. coli*. Although non-pathogenic *E. coli* strains

encode a functional *hlyE* allele, it is silent except in the absence of *hns* [55] or during the over-expression of *slyA* [56,57]. This was shown to be due to SlyA-mediated counter-silencing of the *hlyE* promoter [58]. However, non-pathogenic *E. coli* strains are unable to express *slyA* at levels sufficient for counter-silencing. In contrast, even under non-inducing conditions, expression levels of SlyA in *S. Typhimurium* are typically higher than that of any other MFTF [59] (Fig. 4). Similarly, the *Y. pseudotuberculosis* SlyA ortholog, called RovA, is the most strongly expressed MFTF in that species [60]. Even in *B. subtilis*, where the functions of most MFTFs are presently uncharacterized, the pleiotropic regulators ScoC [23] and MhqR [61,62] are highly expressed [63].

## Conclusions

Gene duplication appears to have facilitated the asymmetrical adaptation of MFTF lineages (Fig. 2). Although recent studies have highlighted the prominence of horizontal gene transfer in bacterial evolution [2,4], these studies are typically limited to the last 100 million years. We do not dispute that horizontal gene transfer has played a role in MFTF expansion, as some lineages are suggested to have been acquired via horizontal gene transfer, such as HucR of *Deinococcus radiodurans* [64]. However, MFTFs, along with the AsnC family, represent the original wHTH TFs and are thought to have been present in the last universal common ancestor before the divergence of Archaea and Bacteria, more than 3 billion years ago [8,65,66]. Gene duplication must have occurred to allow the emergence of other wHTH TF families and their subsequent expansion. Indeed, evolutionary studies acknowledge that intragenomic gene duplication may have played a more prominent role in initial network establishment [4]. Horizontal gene transfer may have then allowed the subsequent refinement of MFTF function. Ohno's model [1] remains applicable, with extensive horizontal transfer providing new evolutionary space for functional adaptation. Although some MFTFs such as MarR exhibit tightly controlled expression circuits and highly specific DNA interactions with the  $\alpha$ 4 helix, others such as SlyA possess a promiscuous recognition helix and more robust expression levels to accommodate their global regulatory roles. The conserved feature of allosteric MFTF regulation by small aromatic molecules provides a potential mechanistic linkage between the regulation of drug resistance and virulence. The further analysis of this fascinating ancient family of regulators promises to provide important new insights into transcriptional regulation as a driving force in bacterial evolution.

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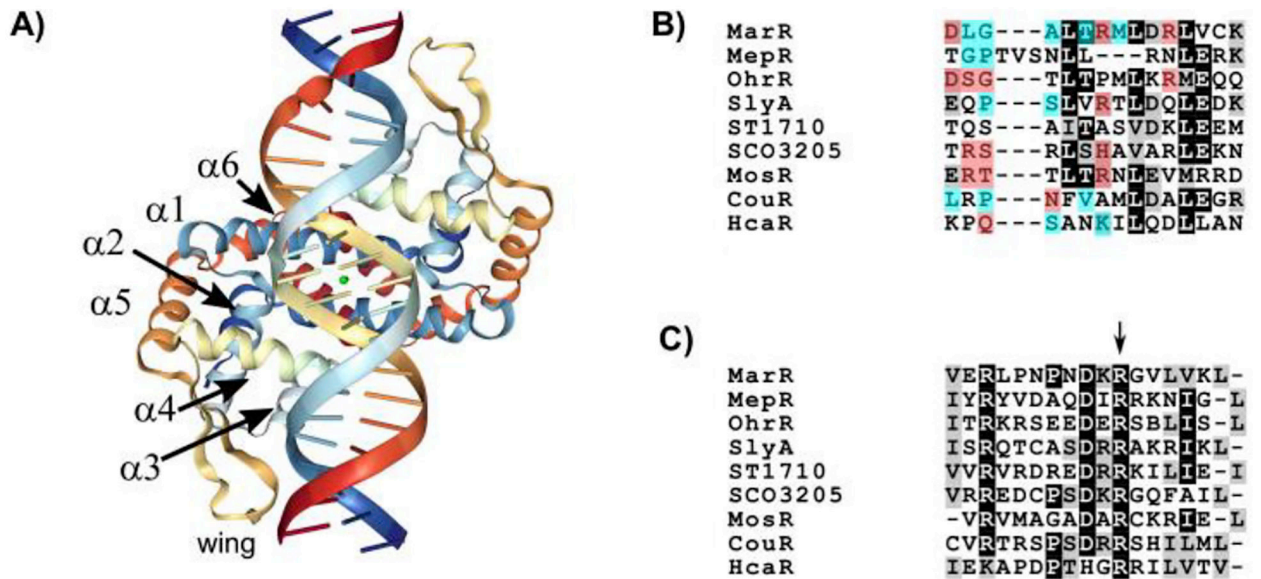
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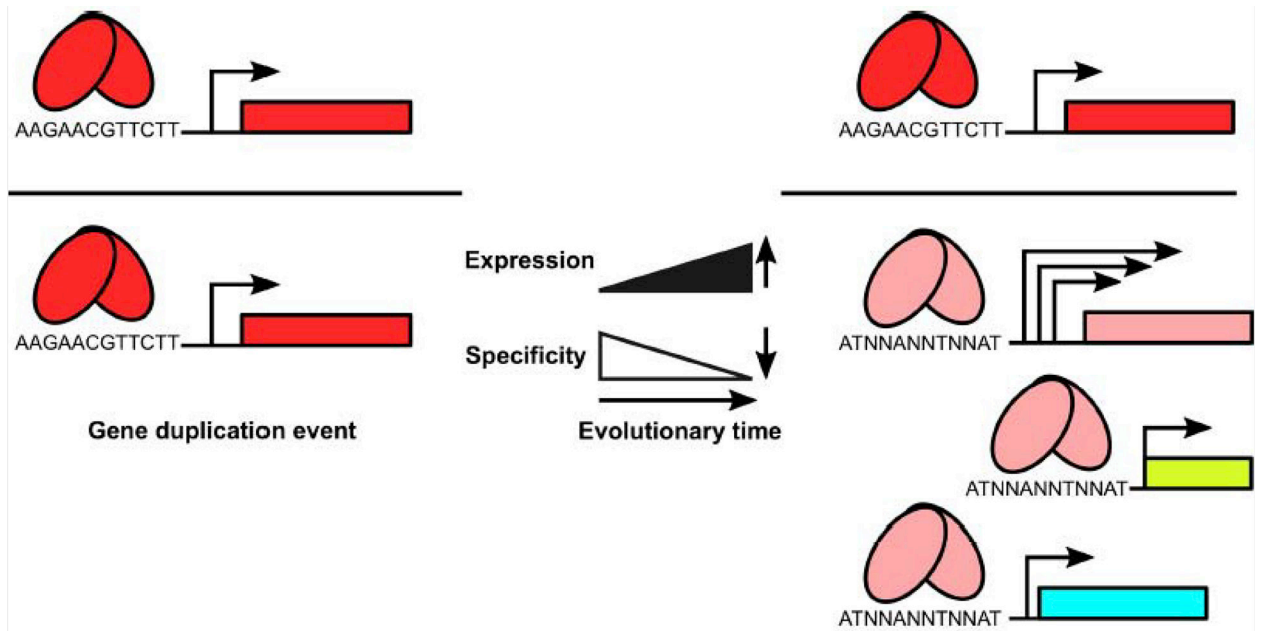
### Highlights

- MarR family transcription factors (MFTFs) are ancient and ubiquitous regulatory proteins, predating the divergence of Archaea and Bacteria.
- Gene duplication has accommodated the adaptation of MFTFs to multiple regulatory functions.
- Allosteric inhibition, typically by small aromatic molecules, confers MFTF responsiveness to environmental and physiological stimuli.
- Variation in DNA-binding domains and promoters contributes to MFTF specificity.



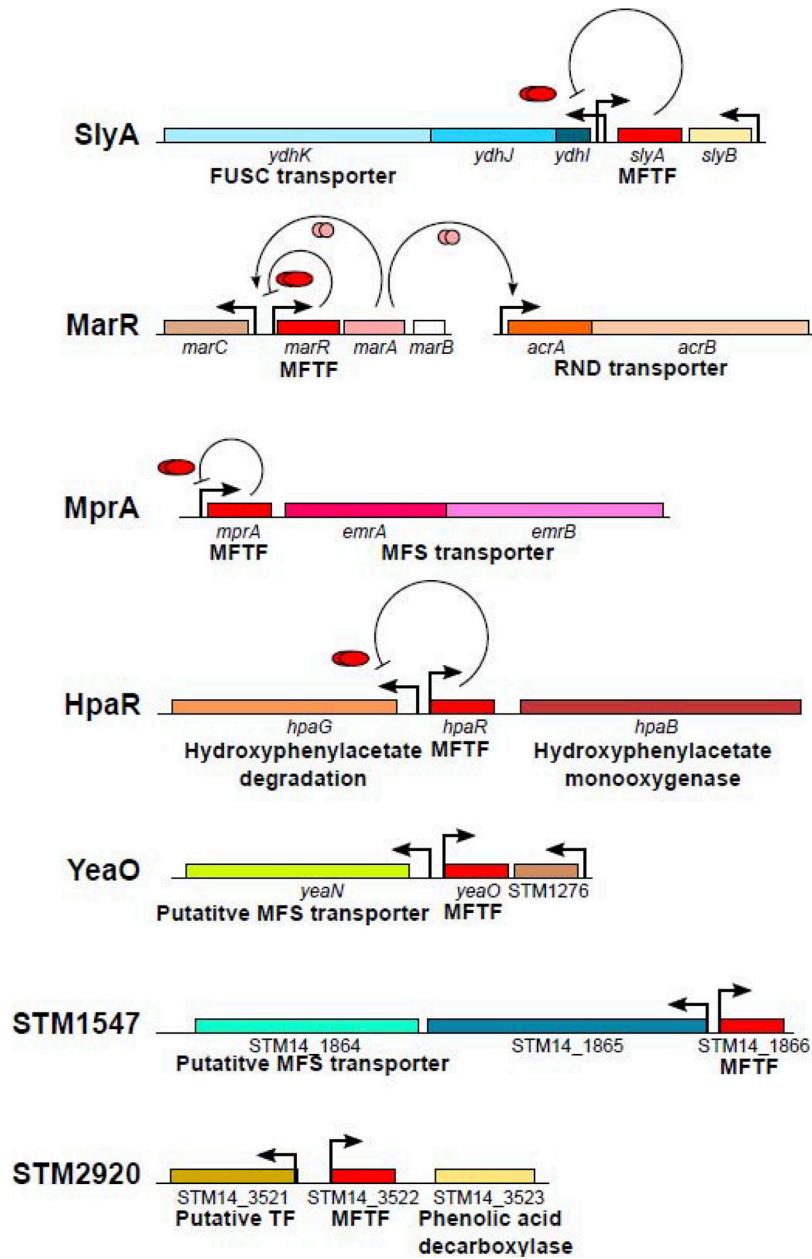
**Figure 1. MFTF DNA-protein interaction.**

The crystal structure of MarR C80S is shown in complex with its operator DNA (PDB ID: 5H3R) [28] (A). The image was generated using the NGL viewer [67]. The axis of symmetry is indicated by a green circle. Helices and the wing of one MarR monomer are indicated. The aligned recognition helices ( $\alpha 4$ ) of representative MFTFs are shown in (B). Residues forming hydrogen bonds with the DNA major groove are highlighted in red. Residues forming weaker and less specific van der Waals contacts are highlighted in blue, based on data originally presented by [27] and the following structural data: *E. coli* MarR [28]; *S. aureus* MepR [27]; *B. subtilis* OhrR [25]; *S. Typhimurium* SlyA [32]; *S. tokodaii* ST1710 [42]; *S. coelicolor* SCO3205 [68]; *Mycobacterium tuberculosis* MosR [69]; *Rhodopseudomonas palustris* CouR [70]; *Acinetobacter* sp. ADP1 HcaR [71]. The aligned wings of the same MFTFs are shown in (C). A conserved arginine residue responsible for interacting with the DNA minor groove is indicated with an arrow.



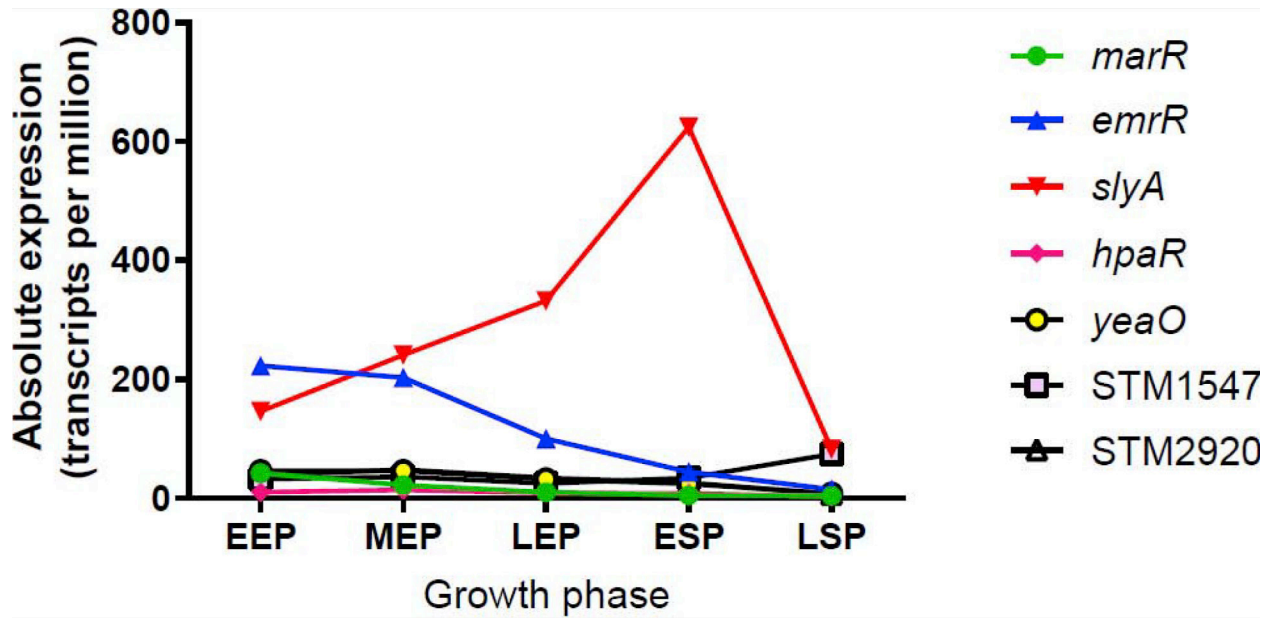
**Figure 2. Model of functional evolution by gene duplication.**

Pleiotropic regulators (pink ovals; pink box represents the coding sequence) such as SlyA may have arisen following duplication of a gene encoding a more specific classical repressor (red ovals; red box represents the coding sequence). This duplication allows one gene copy to accumulate mutations that reduce its binding specificity and thereby increase its potential target repertoire, as well as increasing its expression level, while the other copy retains its ancestral function. Eventually, the duplicate copy evolves to perform a unique role as a regulator of multiple genes.



**Figure 3. MFTFs linked to transporters.**

*S. Typhimurium* encodes 7 MFTFs, each of which is depicted with its relevant linked gene, with known or hypothetical functions indicated below. Promoters are indicated by arrows. Local regulatory functions are shown if characterized and indicated by curved lines ending in bars (repression) or arrows (activation; e.g., MarA). The *slyA* gene is divergently transcribed from the *ydhIJK* operon, which encodes an FUSC superfamily transporter [72]. The *marR* gene is not linked to a pump gene but indirectly regulates *acrAB*, encoding an RND superfamily transporter [73], via MarA [74]. MprA functions as an autorepressor and is co-transcribed with *emrAB*, which encodes an MFS family transporter [75,76]. *yeaO* and STM1547 are also linked to putative MFS family transporters. Diagram is not to scale.



**Figure 4. SlyA is the most highly expressed *S. Typhimurium* MFTF in late exponential and early stationary phases of growth.**

Raw expression levels (in transcripts per million) of each *S. Typhimurium* MFTF were measured during early exponential phase (EEP), mid-exponential phase (MEP), late exponential phase (LEP), early stationary phase (ESP) and late stationary phase (LSP). Data are from previously published transcriptomic data [59], which are available online ([http://bioinf.gen.tcd.ie/cgi-bin/salcom.pl?\\_HL](http://bioinf.gen.tcd.ie/cgi-bin/salcom.pl?_HL)).