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Natural selection on crosstalk between gene regulatory networks facilitates bacterial adaptation to novel environments

Tiffany B Taylor[1], Matthew J Shepherd[1], Robert W Jackson[2] and Mark W Silby[3]

At the level of the gene, mutation is the raw material for natural selection. However, at the level of the gene regulatory network (GRN), variation is revealed to selection via promiscuous regulator activity ('crosstalk'), which creates opportunities for genetic innovation that can facilitate adaptation. Many genetic and environmental features can contribute to increasing potential for crosstalk by facilitating non-cognate interactions between regulatory elements. If a novel interaction provides a fitness benefit, rewired GRNs with strengthened affinity for newly forged connections can be selected. Here, we identify factors that facilitate opportunities for crosstalk and rewiring between GRNs, consider whether features of some GRNs make them more 'rewireable' than others and if these features might constrain evolution towards convergent outcomes. We explore patterns from laboratory and natural microbial populations that show changes within GRNs during adaptation. Finally, we discuss the prospects and open questions in the field.

Addresses
[1] Milner Centre for Evolution, Department of Biology & Biochemistry, University of Bath, Claverton Down, Bath, BA2 7AY, UK

[2] School of Biosciences and Birmingham Institute of Forest Research (BIFoR), University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK
[3] Department of Biology, University of Massachusetts Dartmouth, 285 Old Westport Road, North Dartmouth, MA 02747, USA

Corresponding author: Taylor, Tiffany B (t.b.taylor@bath.ac.uk)

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Features that facilitate adaptation of gene regulatory networks

Gene regulatory networks (GRNs) evolve through changes to regulatory connections [1]. 'Wiring' (i.e. the specific connections between regulatory elements) within a cell's regulatory network is as susceptible to adaptation as genome organisation [2,3]. However, factors that create and determine the evolutionary potential for GRN **rewiring** (for this and other terms used in the review please refer to the Glossary in Box 1) are poorly understood. Extensive sequence and structural similarity within protein families exist in prokaryotes between components of different pathways due to past cycles of gene duplication, divergence, and horizontal gene transfer [4]. This process of GRN expansion can also result in many regulators controlling overlapping **regulons** [5–8]. Together this confers 'built-in' promiscuity and modularity of GRNs that facilitates rapid evolution (Figure 1d(3)).

Promiscuous binding between regulators and non-cognate binding sites leading to transcriptional activation of non-target genes is termed **crosstalk**. In general, crosstalk between GRNs is low, because misregulation of genes is likely to reduce fitness in an environment where an organism is well adapted, and consequently there are mechanisms in place that aid their insulation [9–11]. However, crosstalk can make new interactions available to selection that (if beneficial) can facilitate the evolution of GRNs [12]. Therefore, there exists a balance between maintaining specificity between TFs and their **cognate binding sites** to ensure network efficiency, and relaxing specificity to enable adaptation of networks. Where this balance lies remains an open question.

The role of TF binding affinity and range in facilitating crosstalk

One required change for evolutionary rewiring of a GRN is for a TF to gain new regulatory function that selection can act upon. As with **neofunctionalisation** in enzymes and other proteins [13], this is commonly thought to occur through the gain of low-level promiscuous side-activity (i.e. crosstalk) [14–16]. If new connections are beneficial, selection can act on mutations in the TF binding domain or on mutations that change the promiscuously regulated promoter sequence, to strengthen the binding potential between the TF and consensus binding sequence — increasing affinity between the pair [17•]. Promoter sequences can evolve through very few mutational changes [18], and shared structural homology between TFs means binding domains can be modified towards new targets readily [19] demonstrating the ease with

Box 1 Glossary:

Autoregulatory loops: where a transcription factor regulates its own expression.

Crosstalk: a change in transcription of non-target genes that results from promiscuous binding. Although there are other mechanisms that can confer crosstalk, they are not relevant to the perspective of this review.

Cognate binding-sites: an established, co-evolved interaction site between transcription factor and DNA-sequence that optimises gene expression in a given environment.

***De novo*:** translating to 'a new', in evolution it describes when a genetic variant or a mutation arises in a population.

DNA-binding domain: a folded protein domain that matches and binds to a region of DNA, allowing protein–DNA interactions.

Global transcriptional regulators: transcription factors that control many essential genes in response to environmental changes.

GRN architecture: the interaction profile of regulatory elements within a GRN.

Neofunctionalisation: when a paralog gene takes on a novel function after duplication.

Orthologous: homologous genes in different species as a product of shared common ancestry. These genes often retain original function.

Paralogous: homologous genes in the same genome as a product of gene duplication and specialisation. These genes often have different functions.

Promiscuous binding: when a transcription factor binds to a non-cognate site

Regulons: a group of genes that are regulated as a single unit.

Rewiring: the emergence of new regulatory interactions between different GRNs.

Two-component regulatory systems: a subset of GRN that enables a cell to sense and respond to changing environmental conditions. Typically, it consists of a sensor histidine-kinase (HK) that will sense an environmental cue, and upon activation phosphorylate an associated response regulator (RR) that will initiate transcriptional changes in response to stimulus.

which beneficial connections can be strengthened under selection [20,21].

TFs engage with DNA sequences via a **DNA-binding domain** [22]. However, these binding sites are not uniform in sequence, instead being variations of consensus sequences [23] with differing binding affinities for the TF involved [24,25]. In particular, A/T-rich consensus sequences show lower binding specificity and therefore promiscuous binding by multiple TFs is more likely [26••] (Figure 1d(4)). This could explain the high incidence of A/T rich promoter regions in horizontally acquired genes that may facilitate expression through crosstalk via non-native TFs [27]. In addition, TFs can also vary in their potential to bind to multiple sequences. Detailed analysis of 182 TF-DNA interactions in *Pseudomonas aeruginosa* showed that the number of binding sites varied substantially, with most TFs binding fewer than 100 targets; however, a considerable proportion (~36%) bound to more, with 8 exceeding 1000 binding sites in the genome [28]. In a similar study from Fan *et al.* looking at TF binding affinity in *Pseudomonas syringae*, out of 100 TFs with identified binding motifs most bound fewer than 200 binding sites with 5 showing more than 500 binding sites [29•]. The consequence of this variation, however, is unknown in terms of whether TF binding affinity and range might afford greater opportunity for GRN adaptation and optimisation in variable environments through the facilitation of crosstalk.

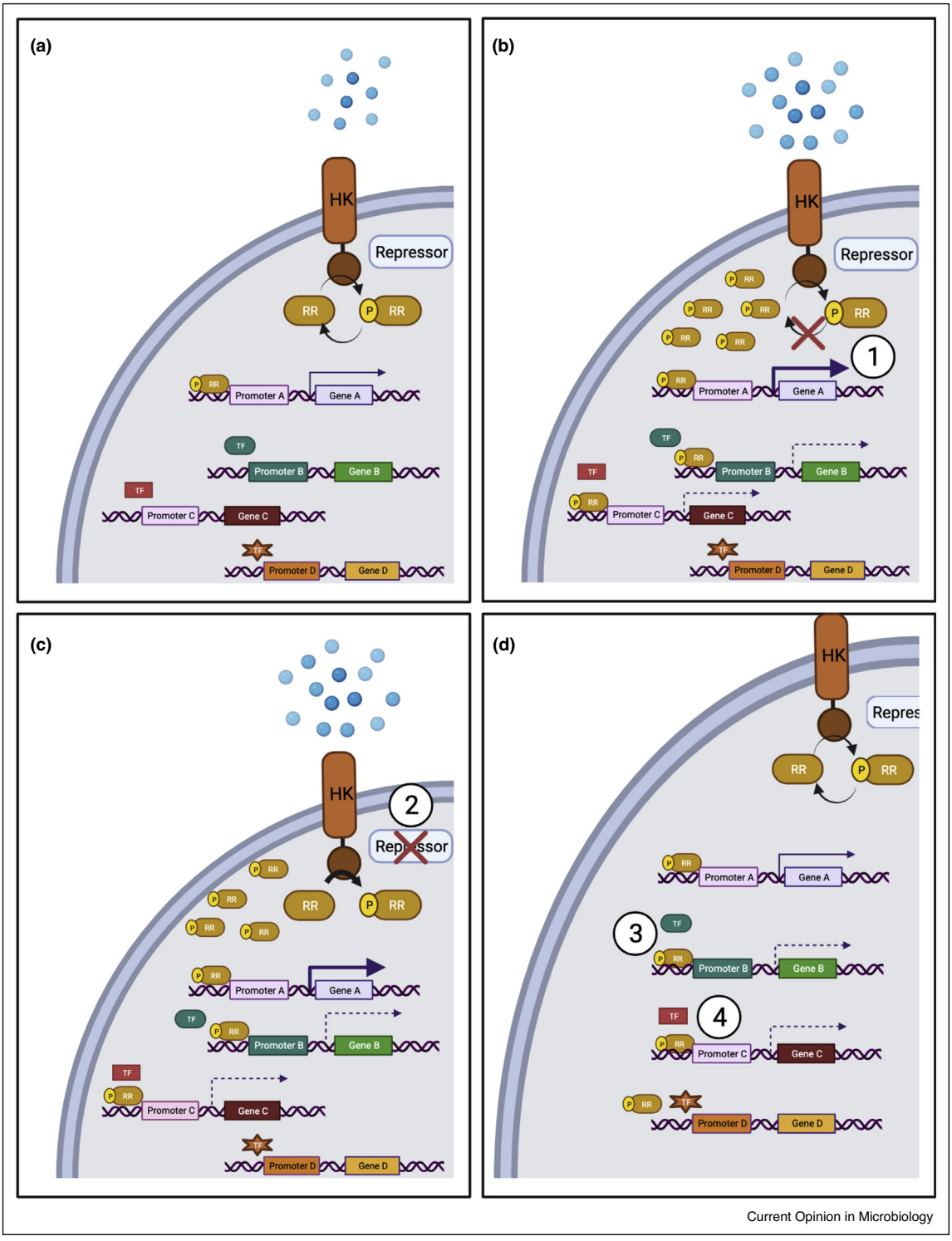
The role of environment in revealing pre-existing crosstalk to selection

The interaction of **GRN architecture** (i.e. abundance and nature of network connections) and transduced environmental signals determines the concentration of active TF for controlling the activity of a particular gene [30–32,33•]. Commonly found in prokaryotes, **two-component regulatory systems** (TCSs) are a subsystem of GRNs that specifically link environmental change to gene expression as they enable organisms to sense and respond to changing environmental conditions.

TCSs comprise a sensor histidine kinase (HK) and their cognate response regulator (RR) (Figure 1a). As with TFs, more complex environments select for systems with more precise control and thus more TCSs [1,34], and they are frequent sites of adaptive mutations during ecological niche shifts [35,36]. These simple GRNs offer a good model system to empirically explore the role of the environment in selecting for novel or rewired GRNs in terms of the mutational targets and architectures that readily facilitate adaptation.

Several elegant experimental studies highlight the role of environmental conditions in determining GRN adaptive routes, by utilising pre-existing indirect links (which may be considered crosstalk). For example, the evolutionary recovery of pili-mediated motility in a *Myxococcus xanthus ΔpilR* strain [37]. In wildtype, NmpR (a RR in the multicomponent system NmpRSTU) indirectly modulates *pilA* expression under low oxygen. In normal oxygen, HK NmpU is active and phosphorylates a hybrid RR-HK NmpS, but in low oxygen NmpS adopts its role as a HK to phosphorylate its RR (NmpR) – phosphorylated NmpR then interacts with *pilR* binding sites to express pili associated genes. In a Δ*pilR* mutant under selection for pili-mediated motility, suppressor mutations in HK NmpU were recovered in seven of eight motile mutants and in six of these were the only mutation reported. In addition, activation of NmpR in evolved strains and deletion of *nmpU* in the parental Δ*pilR* strain is sufficient to restore motility, which strongly suggests the ***de novo*** mutations as the causative agent of motility phenotype [36].

Figure 1



(a)–(d) Environmental and genetic features that create opportunity for crosstalk using a two-component system as an example GRN. (a) Under normal regulation, the sensor histidine kinase (HK) will receive an extra-cellular signal, leading to phosphorylation of the response regulator (RR), which in its activate state can bind to its cognate DNA promoter region to initiate transcription of target genes. In some cases, HK can also act as a phosphatase to de-activate RR. (b) (1) suppressor mutations to phosphatase activity of HK, or (c) (2) loss-of-function mutations to HK repressor genes lead to an elevated concentration of active TF in the cell that increases the chance of non-cognate interactions. In both these cases, sufficient environmental signal is necessary to initiate HK activity (represented by extracellular blue spheres); (d) (3) Sequence/ structural homology between TFs confers 'built-in' promiscuity and modularity of GRNs that can facilitate promiscuous binding; (d) (4) Similar

(Figure 1 Legend Continued) promoter sequences, or those with low binding affinities for example, those with high A/T content, show higher propensity to be bound promiscuously by multiple TFs. The strength of transcription is indicated by the thickness of line (with dotted lines representing weak transcription due to non-cognate binding). Created with BioRender.com.

Similarly, in a non-flagellate Δ*fleQ* strain of *Pseudomonas fluorescens*, promiscuity and rewiring of nitrogen RR NtrC was able to rescue flagellar gene expression after a hyper-activating mutation of its cognate HK NtrB or loss of the NtrB-repressor GlnK [20], which led to increased *ntrBC* expression due to autoregulation. Transcriptome analysis shows nitrogen compound transport genes are downregulated in the Δ*fleQ* mutant compared to wildtype, suggesting pre-existing indirect links between the flagellar and nitrogen regulatory networks. Without any mutations to the RR NtrC, Δ*fleQ* mutants were able to recover some activity of the flagellar pathway via mutations that increased intracellular levels of active NtrC. Once revealed to selection, second step mutations increased the affinity of NtrC towards FleQ and away from NtrC binding sites, which had the dual benefit of improving flagellar-mediated motility and compensating for an overactive ntr system. These studies suggest that pre-existing links between GRNs, regulated by environmental signals, provided an opportunity for rewiring. It may also suggest that some ecological settings are more likely to result in selection of rewired GRNs than others through activation of promiscuous pathways that, to some extent, are already present.

It is possible that some TFs may be 'primed'/more accessible for rewiring within a GRN, due to a combination of abundant activating signals and local network architecture that enables a high concentration of a TF in its active state. TCSs are simultaneously promiscuous and stringent [38]. Crosstalk is prevented by HK positive feedback loops, but mutations that remove repression or provide a source of constant activating signal are likely to permit promiscuity through runaway feedback leading to high regulator expression and activity [2,39–42] (Figure 1c(2)). These types of mutation are frequently seen in TCS rewiring events [20,37,43••,44]. Should promiscuous activity be advantageous, by increasing the flexibility and adaptability of GRNs, preexisting activating signals and architectures would likely constrain which regulators are available for evolutionary innovation in response to an environmental change, as these rewiring routes will be revealed to selection first without requiring time to acquire mutations that alter TF binding specificity. An important step towards unlocking promiscuity potential of a RR is increasing its concentration of active form in the cell; this can be achieved through mutation (Figure 1b(1) and c(2)) or through an increase in activating signal (Figure 1c(2)).

Flexible GRNs facilitate adaptation

Recent studies have highlighted rewiring of regulatory networks as an essential adaptive feature for some examples of host colonisation and establishment. For example, Gopalan-Nair *et al.* found that adaptation of *Ralstonia solanacearum* to a resistant tomato cultivar led to convergent GRN evolution between independently evolved lines [45•]. Although each line acquired different mutations, they resulted in similar rewiring patterns of the virulence regulatory network and associated gene expression profiles. Similarly, Cottalorda *et al.* followed the evolution of 108 clinical *P. aeruginosa* isolates (starting from a single clone type) within the urinary tract between 48 and 488 days and found that adaptive mutations were preferentially located in genes encoding transcriptional regulators, TCSs, and carbon metabolism [46]. Host adaptation is a barrier not only faced by pathogens, but also symbionts. Pankey *et al.* followed the adaptations in *Vibrio fischerii* that enhanced colonisation and establishment in a squid host (*Euprymna scolopes*). They found multiple independent lines had point mutations in HK BinK that promoted traits known to support colonisation and immune evasion, but also altered quorum sensing that resulted in increased luminescence at lower cell densities [47••]. The fact that multiple traits were altered through mutations in one HK suggests that BinK might participate across more than one regulatory pathway that assist a symbiotic lifestyle.

These studies followed the adaptation of bacteria to a host, but what do we know about how a new pathogen emerges from an environmental ancestor? Bryant *et al.* followed the evolution of a recently emerging pathogenic species of nontuberculous mycobacterium, *Mycobacterium abscessus*, from a free-living origin [48•]. Rather than acquiring mutations in regulatory genes *de novo*, they found that horizonal gene transfer of **global transcriptional regulators** was an important mechanism for generating large scale phenotypic variation that created opportunities for adaptive change. Looking at the process in reverse, Yebra *et al.* [49•] discovered that genome remodelling in the bacterium *Staphylococcus aureus* subsp. *anaerobius* (which evolved from an *S. aureus* ancestor) accompanied its transition from a versatile multi-host bacterium to a fastidious niche-restricted pathogen of ruminants. In particular, acquisition of insertion sequence elements interfered with expression of downstream genes; reduced expression was predicted via altered promoter or DNA binding sequences of an inducer, and increased expression by elimination of repressor binding sites. These mechanisms effectively uncoupled target genes from their regulatory elements which enabled substantial rewiring of the genome through disruption of promoter regions and operon structure [49•].

GRNs also play an important role in antimicrobial resistance [50,51] and are a potential target for new antimicrobials. Patel *et al.* [43••] showed that in *Escherichia coli*, mutations in the feedback regulator protein MgrB preceded and facilitated the evolution of drug resistance to the antibiotic trimethoprim. Normally, MgrB attenuates PhoPQ through negative-feedback, however inactivation mutations in MgrB resulted in PhoPQ-mediated trimethoprim tolerance. Of particular interest is the role of the selective environment in the outcome of GRN adaptation under further exposure to trimethoprim. Under strong selection, MgrB mutants acquire resistance via additional mutations in dihydrofolate reductase; however, under weak selection, compensatory mutations in response to an overactive PhoPQ system inactivate its target RpoS [43••]. The role of the selective environment in determining the evolutionary trajectory of GRN rewiring events remains understudied and is of likely crucial importance to understanding bacterial adaptation to ecological shifts more generally.

Conclusions

GRNs are dynamical such that connections are forged and lost frequently across an evolutionary timescale. Recent research shows that GRN restructuring is an essential part of ecological niche switching and highlights the importance of rewiring in newly emerging pathogens and host adaptation. However, we know very little about the evolutionary drivers behind these rewiring events and the role of the environment in shaping GRN arrangement.

Crosstalk is necessary to reveal new TF-DNA binding interactions to selection, an essential process in GRN rewiring. A TF's binding affinity and position within a GRN are emerging as important factors for creating opportunity for crosstalk, but how these factors interplay to drive GRN evolution are far from clear. In particular, there is a lack of data that openly links the ecological setting with opportunity for GRN rewiring via 'tinkering' [52] of pre-existing non-cognate interactions. We have discussed the enormous range of binding potential between different TFs within a bacterium; as an example, *P. aeruginosa* shows the number of binding sites per TF range between 1 and 2407 [28]. We have also mentioned that features of GRN architecture, such as **autoregulatory loops**, can make some GRNs easier targets for selection [40], especially when pre-existing links exist. But this leads to a number of unanswered questions that suggests exciting opportunities for research in this field: Why does such variation in the average number of TF binding sites exist, is this variation a legacy or a product of selection and what does it mean in combination with architecture and ecological conditions in terms of rewiring potential between GRNs? Combining insights from laboratory studies with natural datasets is an opportunity to uncover both the mechanisms and consequences of genetic and ecological factors driving opportunity for crosstalk that may ultimately facilitate GRN rewiring and ecological niche-shifts.

Conflict of interest statement

Nothing declared.

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Using HT-SELEX to identify binding motifs for 100 TFs, this study mapped functional interactions between virulence associated TFs and their targets. They found most TFs targeted fewer than 200 genes, but five bound more than 500 sites in the genome.

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Using a synthetic biology approach, this study moves transcriptional units (the coding sequence, promoter and terminator) to new locations within a GRN in *E. coli*, and measures the outcome on phenotype and gene expression. Moving the transcriptional units had dramatic impacts on phenotype, despite interactions within the GRN remaining the same.

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This study highlights the role of selection pressure in determining the adaptive trajectory of mutations within a GRN. In *E. coli*, tolerance to trimethoprim evolves via mutations in *mgrB* which attenuates PhoPQ signalling. Under further strong selection, mutations in folA result in trimthoprim resistance. Under weak selection, compensatory mutations correct for reduced PhoPQ signalling and *E. coli* remains tolerant (but not resistant) to antibiotic exposure.

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Using experimental evolution, the authors look at the adaptation of plant pathogen, *Ralstonia solanacearum*, to infect a resistant tomato cultivar. Over 300 generations they noticed that independently evolved clones had little genetic diversity between them, but across clones they showed convergent changes in gene expression.

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This study uses experimental evolution to observe the adaptation of ecologically distinct strains of *Vibrio fischerii*, within the light organs of *Euprymna scolopes*. Adaptive mutations were all within the sensor kinase *binK* that promoted colonisation and immune invasion as well as altered quorum sensing. This suggests prexisting links exist between traits essential for symbiotic life history and provides a mechanism for rapid adaptation to a host.

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Exploring the transition of environmental mycobacterial species to a pathogen, this study conducts genomic analyses on the recent emerging pathogen *M. abscessus*. Initially, horizontal acquisition (of mainly genes with transcriptional effects) drove phenotypic changes with large effects. Adaptation to the lung environment resulted in parallel evolution; genes under strongest selection were those involved in global regulation.

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This study explores extensive remodelling of the *S. aureus* genome that faciliates transition away from an opportunistic pathogen to a host-restricted lifestyle in *S. aureus* subsp. anaerobius. This life history transition was accompanied by multiple large insertion sequences that altered control of flanking genes critical for host establishment.

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