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Review

Plastic Circuits: Regulatory Flexibility in Fine Tuning Pathogen Success

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Bacterial pathogens employ diverse fitness and virulence mechanisms to gain an advantage in competitive niches. These lifestyle-specific traits require integration into the regulatory network of the cell and are often controlled by pre-existing transcription factors. In this review, we highlight recent advances that have been made in characterizing this regulatory flexibility in prominent members of the Enterobacteriaceae. We focus on the direct global interactions between transcription factors and their target genes in pathogenic *Escherichia coli* and *Salmonella* revealed using chromatin immunoprecipitation coupled with next-generation sequencing. Furthermore, the implications and advantages of such regulatory adaptations in benefiting distinct pathogenic lifestyles are discussed.

Transcriptional Regulation in Bacterial Species

The central dogma of molecular biology describes the basic process underpinning gene expression, whereby DNA first acts as the template for transcription of mRNA by RNA polymerase (RNAP) followed by the translation of this genetic information by ribosomal machinery into functional proteins in the cell. However, this fundamental framework is an incomplete view of the complexities that regulate each step. Transcriptional regulation is the first checkpoint in gene expression and must be dynamically controlled in order for the cell to coordinate many processes in tandem [1]. There are several ways in which transcription is regulated. These include RNAP transcription initiation by sigma factors in response to particular growth states or stresses, transcription termination by Rho-dependent/independent mechanisms, riboswitches, DNA methylation, alterations in chromosome topology by DNA supercoiling and nucleoid-associated proteins (NAPs), regulation of RNA degradation, post-transcriptional control of gene expression by small noncoding RNAs, and arguably the most prominent method, the action of DNA-binding transcription factor (TF) proteins, which are the focus of this review [1–12].

Members of the Enterobacteriaceae encode large numbers of TFs to cope with the vast variety of regulatory roles required. For instance, in *Escherichia coli* K-12 over 300 TFs have been classified in the 4.6 Mbp chromosome [13]. TFs function to activate or repress transcription in response to specific triggers that are usually indicative of environmental or cellular signal shifts. This allows rapid changes in gene expression to benefit the lifestyle of the individual bacterium. TFs can act on a local or global scale depending on the number and range of genes under their control, known as the regulon [14]. There are several classes of TFs in bacteria but broadly they can be split into three groups (Figure 1). The first group are cytoplasmic regulators that typically bind small molecules in the cell and control gene expression by directly interacting with RNAP (activation) or by blocking RNAP activity at the promoter element (repression) (Figure 1A). The details of RNAP function and regulation have been extensively reviewed elsewhere but briefly the RNAP holoenzyme (consisting of the multisubunit RNAP apoenzyme in conformation with a sigma factor) is recruited to a recognisable gene promoter element consisting of the −10/−35 consensus sequence relative to the transcriptional start site. Transcription initiation is then regulated either by direct interaction of the TF with RNAP, indirect activation by manipulating DNA topology upstream of the promoter, by

Highlights

Bacteria encode hundreds of conserved transcription factors that are routinely repurposed to regulate horizontally acquired virulence genes in bacterial pathogens.

These transcription factors are often nonessential for growth but can be adapted to regulate genes essential for virulence.

Global approaches to map DNA-binding proteins reveal details of direct regulatory reactions that occur *in vivo* and, combined with gene expression studies, can decipher complete transcription factor regulons.
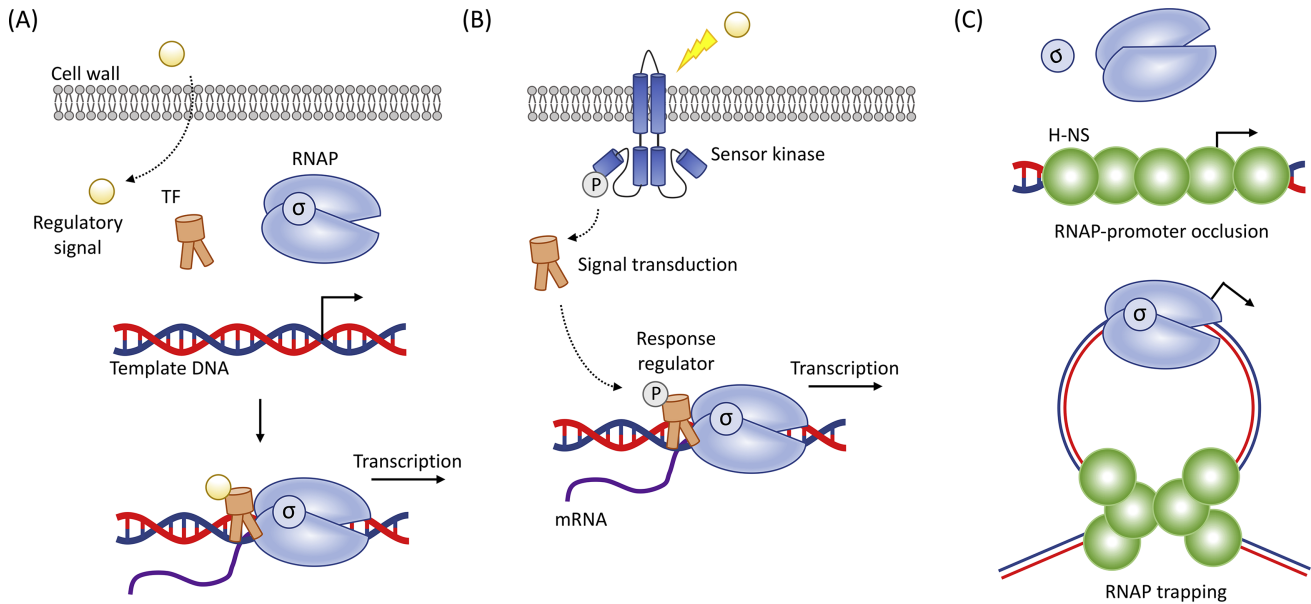
Regulatory rewiring occurs not only between species encoding orthologous genes but also within species, creating very personalised circuits of regulation that benefit individual pathogenic lifestyles.

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Figure 1. Generalised Mechanisms of Bacterial Transcription Factor (TF) Gene Regulation. (A) Signal sensing by TFs in the cytoplasm. A signal (typically a small molecule or metabolite) is recognised inside the bacterial cell by its cognate TF, which in turn interacts with RNA polymerase (RNAP) to either activate or repress transcription of target genes. (B) Signal transduction by two-component systems. An extracellular signal is sensed by a membrane-exposed sensor kinase that is phosphorylated in response. This phosphoryl group is then transferred to a cognate response regulator (TF), which alters expression of target genes. (C) Example of chromosome organisation by nucleoid-associated proteins (NAPs). The histone-like nucleoid-structuring protein (H-NS) can function to repress transcription by several mechanisms, including occlusion of RNAP from a promoter element or trapping of RNAP by DNA bridging around the target region.

blocking RNAP binding to the promoter, or by relieving this repression [3]. The second group includes two-component regulatory systems which consist of a membrane-bound sensor kinase that recognises environmental signals, subsequently phosphorylating a cognate response regulator TF driving its function [15,16] (Figure 1B). The third group are globally regulating NAPs which contribute to the dynamic structure of the chromosome and thus can influence transcription profoundly [9] (Figure 1C).

Responding to environmental signals and cues is critically important for bacteria as it allows rapid functional adaptation to a preferred niche. This results in maximising competitiveness by altering nutrient uptake and metabolism, responding to stress, or determining when to utilise macromolecular nanomachines such as flagella, colonisation factors, and secretion systems [17]. This is of particular importance for pathogens as specific virulence factors are often involved in host colonisation of a strict niche, and this occurs in tandem with tailoring core genome expression and metabolism in order to limit competition with the host microbiota [17]. As virulence factors are typically encoded on horizontally acquired genomic elements, they must be integrated into the regulatory network of the cell in order to be expressed at the most appropriate time during host contact [18]. Arguably, the best example of this process is regulation of type 3 secretion in the gastrointestinal pathogen enterohaemorrhagic *E. coli* (EHEC). The type 3 secretion system (T3SS) of EHEC is carried on a 35.6 kb pathogenicity island known as the locus of enterocyte effacement (LEE) and encodes a full injectisome structure used to attach intimately to host cells [19,20]. EHEC typically encounters susceptible hosts through contaminated food but must counteract several innate and microbiota-derived defences in order to establish its preferred niche at the epithelial surface of the large intestine [17]. This is mediated through a complex molecular interplay between numerous signal-

sensing regulatory systems and the LEE [21–23]. Several TFs converge on the extended promoter region of the LEE-encoded master regulator, Ler, directing environmental sensing that is intrinsic to *E. coli* and coordinating this with the regulation of virulence. This leads to a highly orchestrated scenario that allows EHEC to use alternative sources of energy and express the T3SS at the correct location for establishment of a successful niche and subsequent expansion of the population.

This complex system is just one of several employed by distinct pathogens, each requiring appropriate transcriptional fine tuning. In recent years the vast extent of direct TF interplay with virulence regulation has begun to emerge thanks to global TF binding-site mapping in parallel with transcriptome studies (see Figure I in Box 1). This review focuses on the most prominent advances in this field, deciphering how TFs directly contribute to virulence gene regulation by integrating transcriptional networks and signal sensing, thus tailoring gene regulation to benefit the pathogenic lifestyle.

Distinct Intra- and Inter-Species Mechanisms of Virulence Regulation in Pathogenic *E. coli* and *Salmonella*

E. coli encodes hundreds of TFs but their precise roles in regulation of virulence gene expression, and how this benefits the pathogenic lifestyles of mechanistically distinct pathotypes, are often elusive. Gene regulation in *E. coli* K-12 has often been used as a paradigm to study TF networks using ChIP-seq (Box 1). However, recent research has begun to describe the vast array of mechanisms by which TFs can fine-tune virulence in pathogens, revealing regulatory adaptations that apply to individuals rather than to a species as a whole.

A current example of this concept describes an *E. coli* core-genome encoded TF, the LysR-type transcriptional regulator YhaJ, which, despite being very highly conserved (>80% identity in 1486/1581 genomes analysed), was found to regulate entirely unique gene sets between two distinct pathotypes under identical conditions. While being dispensable for growth, YhaJ controlled expression of key virulence genes, namely the LEE-encoded T3SS of EHEC that is essential for intestinal colonization and type 1 fimbriae (T1F) of uropathogenic *E. coli* (UPEC) that are critical for attachment to the bladder epithelium [24,25]. This begged important questions surrounding the ability of nonessential TFs to be recycled and acquire new functions in the cell, which are highly beneficial to the individual's lifestyle. ChIP-seq analysis revealed 23 distinct binding sites in EHEC with a consensus motif matching the canonical LysR T-N11-A sequence and correlated strongly with the transcriptome [25,26]. YhaJ bound upstream of *nleA,* which is located on a cryptic-prophage and encodes a T3SS effector essential for virulence in EHEC, as well as two foreign autotransporter genes implicated in pathogenesis, indicating recruitment of YhaJ to regulate multiple horizontally acquired virulence loci. Other binding sites included known target *yqjF* (hydroquinone metabolism) and *gadX,* a key regulator of acid tolerance [26,27]. The latter site explained a second phenotype driven directly by YhaJ in EHEC (repression of acid tolerance gene expression), which is particularly important for EHEC as GadX is also capable of repressing the T3SS [28]. ChIP-seq analysis of UPEC YhaJ revealed a minimised chromosomal binding profile that included UPEC-specific genes and, importantly, a lack of YhaJ binding to the *gadX* promoter region despite the sequence being identical to EHEC. This finding was accompanied by no role for YhaJ in UPEC acid tolerance. This suggests important regulatory adaptations involving conserved genes that benefit individual pathogens within the species. Another seemingly paradoxical finding was that T1F expression was downshifted in the UPEC *yhaJ* mutant, yet there was a distinct lack of binding enrichment at the *fimA* gene (encoding the major subunit of the T1F apparatus). This was in contrast to EHEC, where significant YhaJ binding was found upstream of *fimA* in EHEC.

Box 1. ChIP-Seq as a Tool for Exploring Global TF Targets and Mechanisms

While it is well understood that TFs regulate essential processes in the cell in conjunction with virulence factors, it can often be a guessing game as to which proteins do this directly or indirectly and what are the associated mechanisms underpinning pathogen behaviour. A powerful tool for studying global regulation by TFs is chromatin immunoprecipitation coupled with next-generation sequencing or ChIP-seq (for a detailed overview see [57]). ChIP involves fixing a population of cells under a chosen condition, crosslinking DNA-bound proteins, and immunoprecipitating a TF using a specific antibody for that protein or an epitope-tagged allelic variant (such as FLAG or HA). As the TF is purified from live cells, any DNA that is associated with binding of the TF under that condition is also obtained. A protocol is then carried out to reverse the crosslinking of the protein to DNA, creating a size-selected library of TF-bound chromosomal fragments and finally analysis of these by next-generation sequencing (ChIP-seq). The reads are then mapped back to a reference genome for the organism in question and compared with a control sample, revealing regions of sequence-enrichment around TF-bound regions of the chromosome. This results in a genome-wide map of TF-binding sites *in vivo*, revealing crucial detail of the regulatory networks that the TF is involved in directly (Figure I). Variants of the technique, including ChIP-chip (ChIP coupled with microarray analysis, used prior to the emergence of next generation sequencing) and ChIP-exo (a modified protocol that employs an exonuclease step to degrade TF-bound DNA in the 5′ to 3′ direction after ChIP in order to increase the base-resolution of the binding site), have also been used for similar purposes [58,59].

In recent years, ChIP-seq has been used extensively in bacterial species to define TF regulatory networks. A large number of flagship studies have focused on exploring transcriptional regulation by known global regulators in *E. coli* K-12 as a model organism, such as the NAPs FNR, H-NS, Fis, IHF, HU, and Lrp, as well as several TFs and sigma factors [60–69]. These studies have revealed large-scale networks of globally regulated systems in *E. coli*, and more recent work is beginning to emerge, characterising how more discrete TFs are contributing to both core genome and virulence gene regulation by integrating transcriptional networks and signal sensing.
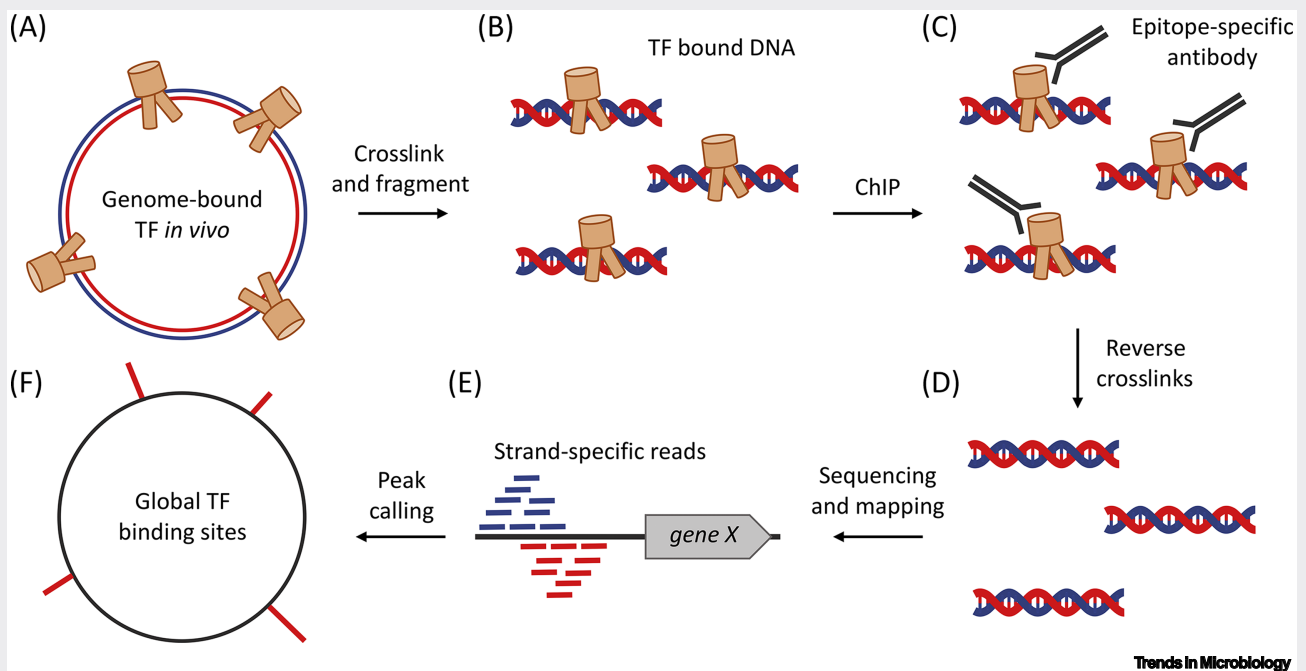

Figure I. Transcription Factor (TF) Binding Site Mapping by Chromatin Immunoprecipitation-Coupled Sequencing (ChIP-Seq). (A) Bacterial cells (typically encoding an epitope-tagged TF) are cultured in a desired condition, fixed, and the chromosomal DNA obtained. (B) The chromosomal DNA is fragmented. (C) Antibodies specific for the TF or the epitope tag are used to immunoprecipitate the TF-bound fragments. (D) The crosslinking step is reversed to remove the TF from the fragment pool. (E) The fragment pool is used to generate a library for next-generation sequencing, and the reads are mapped back to the reference genome. Reads corresponding to TF-binding sites form a canonical bimodal-peak clustering pattern on the forward and reverse strands of the genome site. (F) Bioinformatics are used to call statistically significant peaks and generate a global map of TF-binding sites.

However, T1Fs are nonfunctional in EHEC due do a 16 bp deletion in its invertible promoter element known as the *fim* switch, rendering the promoter permanently silent. A molecular analysis of this phenomenon revealed that YhaJ bound to the *fim* switch exclusively in the OFF orientation, leading to phase inversion of the *fim* switch promoter and T1F expression. This also explained the strong binding of YhaJ to the EHEC *fim* switch region, as all cells in this population would be in the OFF phase and bound by YhaJ [25]. Thus, single ancestral TFs are actively repurposed to benefit individual scenarios, resulting in pleiotropic regulation of virulence (Figure 2).
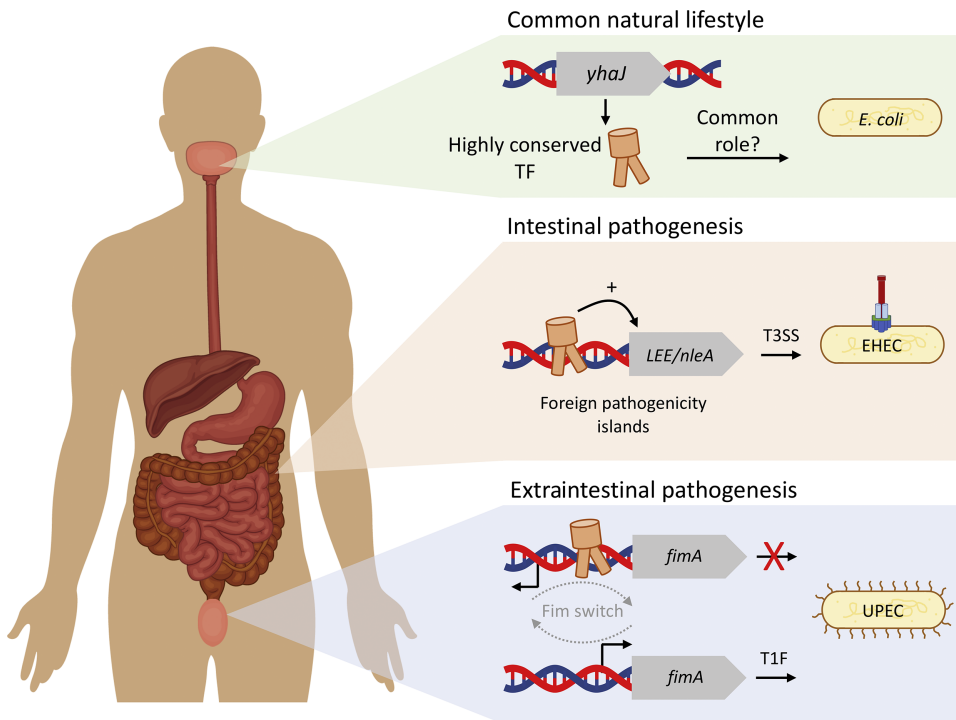
Figure 2. An Exemplary Model for Regulatory Adaptation and Transcription Factor (TF) Recycling in Pathogens. The TF YhaJ is highly conserved in all strains of *Escherichia coli* but does not have a clear common role in the normal lifestyle of the bacterium. However, its function has been tailored to suit the lifestyles of individual pathotypes of *E. coli* that colonise distinct niches in the host. In intestinal-pathogenic enterohaemorrhagic *E. coli* (EHEC), YhaJ has been adapted to directly regulate virulence genes encoded on pathogenicity islands, thus promoting expression of the type 3 secretion system (T3SS). In contrast, extraintestinal-pathogenic uropathogenic *E. coli* (UPEC) recruits YhaJ to drive expression of T1F by binding directly to the phase-variable promoter region, resulting in reorientation of this genetic element. The signal responsible for YhaJ regulation is unknown. This model demonstrates how nonessential TFs can be repurposed to regulate distinct virulence factors that are critical for the pathogens' lifestyle.

Regulatory rewiring is widespread between bacterial species that carry orthologous genes [18]. While sharing much genomic content and similarities in the typical Enterobacteriaceae lifestyle, pathogenic species of *Salmonella* have clear differences from *E. coli.* For instance, *Salmonella enterica* serovar Typhimurium utilises two distinct T3SSs (also encoded on horizontally acquired pathogenicity islands) – SPI-1 to first attach and invade intestinal host cells and SPI-2 for subsequent intracellular survival [29]. Regulation of pathogenicity island-encoded T3SS expression is crucial for the fitness and virulence potential of pathogenic species [30]. LeuO is a LysR-type TF that regulates diverse gene sets in members of the Enterobacteriaceae, including integrating environmental sensing of butyrate to form a complex feedback loop with Ler, thus controlling the T3SS of EHEC [31]. In *S.* Typhimurium, ChIP-chip analysis of LeuO revealed 178 binding sites that included the SPI-1-encoded repressor *hilE* [32]. Conversely, regulators encoded on pathogenicity islands can also control other chromosomal genes themselves. The global binding profile of the LEE master regulator Ler has been mapped to 59 chromosomal sites in EHEC, linking direct regulation of both foreign DNA elements encoding virulence factors and core genome targets by this horizontally acquired TF [33]. However, gene expression analysis revealed that over half of the genes affected were not bound by the TF, suggesting extensive indirect regulatory effects. It is important to note here that overexpression of Ler (or any TF) for experimental purposes may not accurately represent genes truly regulated under native conditions.

Furthermore, binding site identification by ChIP-chip analysis lacks the fine resolution of ChIP-seq or ChIP-exo. Therefore, context and caution must be taken when interpreting such data. In a similar study, global binding of SPI-1-encoded activator HilD revealed 17 binding sites, with the majority being located outside of SPI-1 [34]. Furthermore, HilD directly regulates six other TFs (HilA, HilC, InvF, RtsA, SprB, and RtsB) that collectively form a complex, interconnected network of distinct regulons for each TF with a core set of SPI-1 invasion genes [35]. It is worth reiterating (as discussed for YhaJ) that adaptive regulation, either by core encoded or horizontally acquired TFs, often results in pleiotropic regulation of factors that can drive distinct phenotypic lineages. A recent example of this elegantly describes how the StdE and StdF TFs of S. Typhimurium control a bistable fimbrial operon that leads to two phenotypic subpopulations of cells [36]. However, several global binding sites were identified for both TFs – 171 for StdE, 105 for StdF, and 60 overlapping. The consequence of this is that StdE/F regulate other traits such as motility, chemotaxis, biofilm formation, or conjugation, and that these functions are linked to the aforementioned fimbrial phase variation leading to the hypothesis that phenotypic variation can drive populations of both chronic and acute infection. While these phenotypes were validated experimentally, engineered constitutive expression of TFs, as described here, prohibits addressing the question: Under what conditions does such regulation naturally occur?

Studies on YhaJ revealed key mechanisms of virulence gene regulation by a conserved TF in distinct E. coli pathotypes. The response regulator OmpR plays a similar role. Despite the protein sequence being identical between E. coli K-12 and S. Typhimurium, functionally it was found to regulate vastly different networks of genes in each case [37]. Transcription of ompR was enhanced at acidic pH but only in S. Typhimurium, and this was driven by differences in the promoter region between the species rather than variation in OmpR itself. Global binding analysis revealed more than 200 binding sites (ChIP-chip) in S. Typhimurium (several of which were located with SPI-1/SPI-2-associated genes) whereas only 15 sites overlapped between the two species, demonstrating extensive regulatory rewiring. *Salmonella enterica* serovar Typhi is a human restricted pathogen responsible for systemic typhoid disease. S. Typhi favours invasion of the intestinal mucosa over colonization and is associated with the broad host-range gastrointestinal pathogen S. Typhimurium. The genomes of such host-restricted *Salmonella* isolates are subject to degradation, characterised by pseudogene accumulation often in genes required for gastroenteritis [38]. ChIP-seq analysis of OmpR in S. Typhi revealed 43 binding sites with the strongest peak located upstream of *tviA* on the horizontally acquired *viaB* capsular polysaccharide locus, a critical determinant of Typhi pathogenesis [39,40]. Furthermore, while OmpR-dependent regulation of SPI-1/2 was evident in S. Typhimurium, only one SPI-1 gene displayed altered expression in S. Typhi. It must be noted that culture conditions differed between the studies as well as resolution of the ChIP approaches, which may therefore possibly affect differences in binding site identification irrespective of the *Salmonella* strain tested. Regardless, these studies collectively challenge the assumption that regulators target the same regulon in alternative members of the species, demonstrating beneficial regulatory flexibility for individual pathogens.

Response to Stress as a Trigger for Regulatory Flexibility

Response to stress is a common cue for a TF to tailor gene expression. In UPEC, nitric oxide is a toxic water-soluble gas present in the urinary tract and thus must be detoxified ensuring survival from interference by nitrogen radicals. NsrR is a key TF involved in controlling the response to nitric oxide. Mehta et al. used ChIP-seq to map binding of NsrR to the UPEC chromosome and compared the binding profile with genes identified in response to nitric oxide stress by transcriptomic analysis [41]. They found 94 NsrR binding sites on the chromosome, with 49 of these located in promoter regions. Of 49 targets, 19 were differentially expressed in response

to nitric oxide (out of >1000 genes). Importantly, 29 NsrR-binding sites were not identified in *E. coli* K-12 by ChIP-chip analysis, and 10 of these sites were specific to UPEC strain CFT073 with no homologues in *E. coli* K-12 [42]. Another crucial point is that, while over half of the identified binding sites were intragenic or between convergent coding regions, these sites were not validated and thus the question of whether they are functional (i.e., in control of a small or antisense RNA for instance) remains unanswered. Interestingly, in EHEC, NsrR has also been previously shown to form part of a complex interplay with GadX (and the acid tolerance regulator GadE), modulating T3SS expression in response to nitric oxide [28].

Regulation of acid tolerance is a complicated affair, with several TFs interlinked in the regulation of four defined systems. In a comprehensive study, Seo *et al.* used ChIP-exo to reconstruct the genome-wide regulon of GadX and two other TFs of this system, GadE and GadW [43]. The study identified a total of 45 genes in 31 transcription units that were part of the tri-regulon. It was also demonstrated that 28 of these genes formed part of the RpoS (general stress sigma factor) regulon. What was perhaps most striking from the study was that transcriptional analysis of the three TF mutant strains revealed that 351 genes were collectively differentially expressed; however, only 25 genes were common to all TFs, suggesting that each regulator plays a distinct role in the response to acid and other cellular functions, such as proton flow, chaperones, and the general stress response. In addition to this, two other studies have also used ChIP-seq/ChIP-chip to map the regulatory networks of the TFs Nac, NtrC, OmpR, CsiR, and YdeO in *E. coli* [44,45]. The results revealed elegant interplay, both direct and indirect, between these TFs that leads to an interconnected regulation of acid tolerance, glutamate and nitrogen metabolism.

Additionally, it has been shown that the nitrogen starvation regulator NtrC is directly linked to the multiple nutrient starvation stringent response in *E. coli.* NtrC was found to bind directly to the *relA* promoter region, a key stringent response regulator, and activate its expression specifically under nitrogen starvation thereby coupling two major stress responses at the mechanistic level [46]. This study is also strengthened by extensive *in vitro* validation of newly identified ChIP-seq binding sites that were hypothesised to require other cofactors for promoter binding *in vivo.* It is important to highlight, however, that these latter studies have been carried out in *E. coli* K-12, and data specific to the aforementioned YhaJ has revealed that TF control of generalised stress responses in distinct pathogens, such as the GAD system, can be unique.

Interplay between Distinct TFs and the Genome Sentinel H-NS for Regulating Foreign DNA

As discussed above, beneficial traits such as virulence factors, fitness genes, or antibiotic-resistance mechanisms are often encoded on horizontally acquired DNA elements. The histone-like nucleoid structuring protein, H-NS, is a global NAP that binds genome regions with low GC content and can block transcription by occluding RNAP from promoters or creating DNA bridges that trap RNAP, thus acting as a global silencer of foreign DNA [9,47]. Lucchini and colleagues mapped global binding of H-NS and RNAP across the *S.* Typhimurium chromosome and found that H-NS associated with A-T rich regions rather than regions actively transcribed by RNAP, including heavy nucleation of SPI-1/SPI-2 [48]. Another study identified an indirect mechanism of relieving this repression through the extracytoplasmic stress response sigma factor, sigma E, which downregulates *hns* and upregulates the SPI-2 activator gene *ssrB* simultaneously [49]. This means that TFs regulating virulence gene expression must counteract H-NS binding to pathogenicity islands. For instance, nearly half of the global LeuO binding sites (discussed above) in *S.* Typhimurium overlapped with H-NS, indicating that LeuO may function by antagonising rather than displacing H-NS, although this was not directly tested experimentally

[32]. Uniquely, LeuO actually blocked SPI-1 expression via HilE, and was proposed as a 'backup' repressor of SPI-1 under H-NS impaired conditions [50]. In contrast, H-NS repression of the T3SS in EHEC is relieved by the Ler master regulator and thus drives T3SS expression in this pathogen [21,22].

The study of global TFs can extend far beyond simple activation or repression of target genes and this is particularly true of the NAP family of regulators [9]. Singh and Grainger previously used the EHEC pO157 plasmid-encoded enterohaemolysin operon *ehxCABD* as a model genetic system to study H-NS function [51]. The promoter region of this operon is extremely AT-rich (71%) and thus heavily bound by H-NS. In order to eliminate incorrect recognition of such DNA flanking the precise −10 element, RNAP takes advantage of this situation by only recognising the canonical binding element as the surrounding sequences are masked by H-NS. Further analysis revealed that the entire operon contained 95 putative intragenic promoters, 20 of which were active in both sense and antisense orientations and repressed by H-NS [52]. Using ChIP-seq to map both H-NS and sigma-70 binding (in wild-type and *hns* mutant backgrounds) across the genome, a striking observation was made: 668 promoters were identified in the *hns* strain that represent RNAP-bound promoters, many of which would otherwise be repressed by H-NS. Importantly, promoters identified in H-NS bound regions were far more likely to be intragenic in context. Thus, a major overlooked role of H-NS in *E. coli* and *Salmonella* is to silence the transcription of spurious noncoding RNA derived from intragenic promoters, which would otherwise be toxic to the cell by titrating limited resources of RNAP, causing a global downshift in gene expression and thus fitness [53]. This process is crucial for controlling pervasive transcription from intragenic promoters, which is in fact widespread among pathogens in the bacterial kingdom.

Enterotoxigenic *E. coli* (ETEC) causes severe diarrhoea in humans, and pathogenesis is driven largely by the action of two enterotoxins encoded on plasmids – heat-labile toxin and heat-stable toxin. Both toxins are secreted by ETEC and, via GM1 ganglioside endocytosis and binding to guanylate cyclase C receptor respectively, interfere with host cellular signalling, resulting in cAMP and cGMP overproduction in the affected cell. This ultimately leads to a loss of water and electrolytes from the gut epithelium [20]. The cAMP receptor protein (CRP) is an important global TF in *E. coli* that controls transcription in response to nutrient availability, primarily by increased cAMP-mediated DNA binding in the absence of glucose [3,54,55]. In a detailed study, Haycocks *et al.* used ChIP-seq to map the global binding profile of CRP in ETEC [56]. The analysis identified 111 CRP-binding sites in ETEC (7% of which were not found in *E. coli* K-12) that were surprisingly restricted to the chromosome. In contrast, H-NS (a global NAP that binds genome regions with low GC content, thus acting as a global silencer of foreign DNA) bound to both chromosomal and plasmid sites, which encode the aforementioned toxins. Combining bioinformatics with detailed genetics, the authors subsequently determined that two ETEC plasmids, p948 and p666, indeed encoded five functional CRP-binding sites (two of which targeted enterotoxin genes) but that H-NS occupied all five sites strongly *in vivo.* Another major function of H-NS is the occlusion of TF-binding sites on a global scale [9]. The study uncovered several distinct mechanisms of control over enterotoxin expression involving CRP and H-NS interplay, including direct repression of both toxins by H-NS, indirect repression by occlusion of CRP binding sites, direct activation and also indirect repression of toxin expression by CRP. The glue that binds these mechanisms together is an elegant model for integrating osmolarity and metabolism into gene regulation. During episodes of ETEC-driven diarrhoea, water, cations, and cAMP are released into the gut lumen while at the same time patients will consume electrolyte solutions of salt and glucose to counteract this. The

cAMP flux therefore creates a positive feedback loop on enterotoxin expression via CRP while the increased osmolarity of cation extrusion can relieve H-NS repression of toxin expression. On the other hand, electrolyte consumption provides an excess of glucose that in turn is concentrated enough to inhibit CRP activation of toxin expression, while the salt excess administered in oral hydration therapy is not sufficient to relive H-NS repression of both toxins [56]. This work delves into the impact that a clinical scenario can realistically have on the regulation of pathogen genetics. Collectively these studies have revealed the global interplay between H-NS and TFs for regulating foreign DNA, often encoding virulence factors.

Concluding Remarks and Future Perspectives

Tailoring the transcriptional response to environmental stimuli provides a primary strategy for bacterial lifestyle adaptation, and, coupled with the acquisition of genetic traits, such as virulence or antibiotic-resistance genes, is essential to maintain a stable functional regulatory network in the cell (Box 2). Global approaches to map TF networks have begun to reveal an overwhelming amount of complexity in bacterial regulatory networks, and key themes exist that glue certain aspects together, for instance integration of T3SS regulation with environmental signal sensing. However, the complexities are likely to expand as we begin to characterise the unknowns in the equation such as the functions of previously uncharacterised genes identified within bacterial regulons (see Outstanding Questions). Furthermore, this concept extends far beyond the Enterobacteriaceae, with very diverse pathogens adapting their regulation of virulence in unique and beneficial ways (Box 3). Finally, while interspecies divergence in regulatory networks is a well established phenomenon, intraspecies variation in transcriptional networks has been largely unexplored. This concept has begun to reveal important questions regarding regulatory mechanisms, and thus lifestyles, of individual isolates within a species that, for example, occupy unique niches rather than the species itself as a whole.

Box 2. Regulation beyond Virulence – Emerging Regulatory Control of Antibiotic-Resistance Mechanisms

Much like canonical virulence factors, antibiotic-resistance genes are often horizontally transferred and require specific regulatory control [70]. Cross-regulation of antibiotic resistance with virulence can provide highly tailored mechanisms of regulatory adaptation and is an emerging subtheme. The multiple antibiotic (*marRAB*) locus of *Escherichia coli* confers resistance to several antibiotic classes, including quinolones, tetracyclines, and β-lactams through the regulation of efflux pumps and porins. Sharma *et al.* recently mapped the global binding profile of the MarA and MarR TFs in ETEC using ChIP-seq, defining an extensive *mar* regulon [71]. A single MarR-binding site was identified within the *marRAB* promoter; however, 33 MarA-binding sites were located throughout the chromosome, including four targets within ETEC-specific prophage remnants. Antibiotic phenotyping of MarA-regulated genes revealed novel determinants of resistance to specific antibiotics. XseA encoding exonuclease VII was found to mediate DNA damage repair in response to ciprofloxacin, whereas the *mlaFEDCB* operon encoding a lipid trafficking ABC transporter was identified as a mediator of doxycycline resistance via reducing excessive cell surface hydrophobicity and thus permeability. Importantly, in both cases, the phenotypes were dependent on MarA regulation via its DNA-recognition sequence located next to the promoter –35 sequence, known as the marbox. Additionally, the study observed that MarA targets, including *xseA* and the *mla* operon, were highly conserved throughout the Enterobacteriaceae, suggesting widespread mechanisms of enhanced antibiotic resistance. Intriguingly, a follow-up study identified a new target for MarA activation, the *ycgZ-ymgABC* operon, which in turn represses biofilm formation via direct interaction with the Rcs phosphorelay system, subsequent expression the RprA small RNA, and finally inhibition of curli fibre production. Biofilms are normally considered beneficial for tolerance to antibiotics; however, establishment of this matrix can take 24 h. The authors proposed a fascinating model to explain their data, suggesting that during planktonic growth nascent biofilm expression would be a poor strategy to overcome an immediate threat, therefore the cells favour alternative MarA-mediated strategies (efflux, DNA repair, and membrane permeability) in order to permit short-term gain [72]. The *E. coli* two-component system ZraPSR was recently found to mediate the envelope stress response by providing enhanced resistance to five classes of antibiotic. A total of 39 ZraR binding sites were identified in the study relating to several cell processes, including stress, metabolism, motility, and biofilm production [73]. Additionally, ZraR was found to directly activate GadW, further suggesting integration of stress response mechanisms.

Outstanding Questions

Do conserved TFs that regulate virulence have other condition-dependent regulatory roles?

To what extent are intragenic TF-binding sites functional?

What is the extent of global and virulence gene regulation by recycled TFs beyond model strains?

Why do certain TFs play discrete roles in gene regulation within and between species?

Are these regulatory mechanisms essential *in vivo*?

Box 3. Regulatory Flexibility in Diverse Bacterial Pathogens

Regulatory adaptation extends beyond the model organisms *Escherichia coli* and *Salmonella*. For instance, ChIP-seq has been used extensively to characterise TF networks in the opportunistic pathogen *Pseudomonas aeruginosa*. This pathogen forms biofilm via alginate production in response to signals such as the second-messenger cyclic-diguanylate-GMP and is typically associated with incidences of cystic fibrosis or burn wounds [74]. Three independent studies have directly linked the regulation of cyclic-diguanylate-GMP accumulation to biofilm production via the TFs AmrZ, AlgR, and a novel dual-regulator, PA1226/PA1413 [75–77]. *P. aeruginosa* also encodes over 100 two-component systems. Bielecki *et al.* identified cross-regulation between the response regulators PhoB and TctD, leading to direct integration of independent signal sensing on common target genes [78]. The regulons of 20 key virulence-associated *P. aeruginosa* TFs were defined using an extensive systems biology approach and identified a staggering 1200 gene promoter regions bound by the TFs. The network was linked to regulation of 83.7% of the entire genome (4775 differentially expressed genes). The data were compiled into a community resource known as the '*P. aeruginosa* genomic regulatory network' or PAGnet [79]. Additionally, several studies have focused on mapping global TF networks that integrate with a distinct T3SS in the plant pathogen *Pseudomonas syringae* [80–83].

Members of the Vibrionaceae are a family of gamma proteobacteria that occupy diverse ecological niches, including marine environments and the human intestine, where several virulence factors, such as mucinase, motility, pili, and toxins, are employed [84]. Manneh-Roussel *et al.* defined the CRP regulon in *Vibrio cholerae* and identified direct regulation of the acfA/acfD accessory colonization factors, haemolysin and the RTX-toxin exporter. Importantly, the authors found that these factors were activated in response to host colonisation but were repressed in a planktonic environment [85]. *V. cholerae* uses quorum sensing to regulate gene expression in response to population density. Haycocks *et al.* mapped global binding of the TF AphA, expressed at low cell density, and found that it activated the virulence cascade during colonisation as well as repressing natural competence. The latter is controlled by the TF HapR under high cell density and as such the study identified a mechanism by which *V. cholerae* directly regulates the marine-to-host lifestyle switch [86].

Examples of additional studies exploring regulatory adaptation using ChIP-seq in distinct pathogens have emerged, and key examples include control of fimbriae and type 6 secretion by MrpJ in *Proteus mirabilis*, integration of metabolism and the T3SS by BvgA in *Bordetella pertussis*, and modulation of a master regulator of virulence in *Listeria monocytogenes* CodY [87–89].

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