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Versatility and Complexity: Common and Uncommon Facets of LysR-Type Transcriptional Regulators

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Abstract

LysR-type transcriptional regulators (LTTRs) form one of the largest families of bacterial regulators. They are widely distributed and contribute to all aspects of metabolism and physiology. Most are homotetramers, with each subunit composed of an N-terminal DNA-binding domain followed by a long helix connecting to an effector-binding domain. LTTRs typically bind DNA in the presence or absence of a small-molecule ligand (effector). In response to cellular signals, conformational changes alter DNA interactions, contact with RNA polymerase, and sometimes contact with other proteins. Many are dual-function repressor–activators, although different modes of regulation may occur at multiple promoters. This review presents an update on the molecular basis of regulation, the complexity of regulatory schemes, and applications in biotechnology and medicine. The abundance of LTTRs reflects their versatility and importance. While a single regulatory model cannot describe all family members, a comparison of similarities and differences provides a framework for future study.

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1. INTRODUCTION

LysR-type transcriptional regulators (LTTRs) were first described more than three decades ago, when few transcription factors had been characterized (49). Since then, it has become clear that a significant portion of bacterial genomic DNA encodes transcription factors (12, 16, 75). Of these transcriptional regulators, often classified into 15–20 families, LTTRs form one of the largest families (75, 96, 99). They are widely distributed in bacteria and are also in some (but not many) archaea and eukaryotes. This review focuses on bacterial regulators.

LTTR prevalence within individual bacteria does not correlate with any specific lifestyle or phylogeny, and LTTRs commonly represent 10–20% of the entire regulatory repertoire (99). In bacterial strains of *Acinetobacter*, *Agrobacterium*, *Burkholderia*, *Escherichia*, and *Pseudomonas*, as many as 40–120 LTTR genes are present per genome. This abundance correlates with involvement of LTTRs in diverse functions associated with essentially all aspects of bacterial life.

LTTR features have been reviewed elsewhere (24, 68, 80, 114) and are revisited here. Given the size of the family and diversity of its members, no single model can describe all LysR-type regulators (89). The goal of this review is to highlight progress in understanding the molecular basis of functionality in bacteria, the role of LTTRs in several complex regulatory schemes, and the development of LTTR-based applications in biotechnology and medicine. In addition, we highlight aspects of transcriptional control that remain unclear, including LTTR interactions with RNA polymerase (RNAP).

2. GENERAL LTTR CHARACTERISTICS: A FUNDAMENTAL FRAMEWORK

The following overview provides background for subsequent sections. However, for most traits, there are exceptions to the rule. Therefore, care should be taken in drawing inferences about any individual LTTR based on studies of homologs.

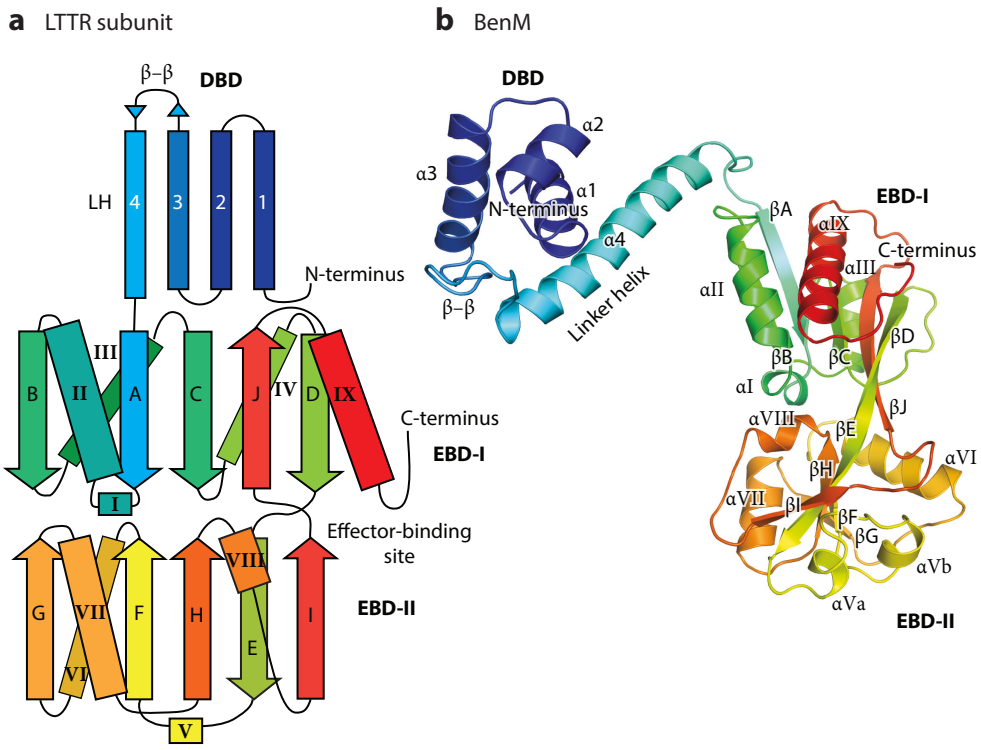


Figure 1

(*a*) Representative LysR-type transcriptional regulator (LTTR) subunit depicting common secondary structures. Each subunit (~300 residues) is composed of a DNA-binding domain (DBD), which is a three-helix bundle (~60 residues), followed by a β-hairpin (β–β) that extends into a linker helix (LH; ~20 residues). The LH is followed by two five-stranded β-sheets with helical crossovers making up subdomains of the effector-binding domain (EBD), EBD-I and EBD-II. Here, we introduce notation to establish a consistent naming scheme. Strands within the sheets are designated by letters A–J (sans serif font), and the crossover helices are designated by Roman numerals I–IX (serif font). Appending β and α symbols to distinguish unusual features should help with structural comparisons. The DBD–LH helices are numbered 1–4 (sans serif font). This scheme follows that introduced for CysB-EBD (126) and modifies notation that was first used for CbnR (91). Crossovers are shown in front of or behind strands with spatial orientations following a right-handed rule. The effector-binding site lies between the two β-sheets. (*b*) BenM illustrates nomenclature to address individual variations (109). In this structure, there is no helix associated with feature IV; it is a coiled-coil region. To cover N-terminal additions (not shown), primes can be introduced, going backward from helix α1. For example, in the full-length structure of AphB (122), which has a β-strand at the N terminus before α1, these additional regions would be denoted β1′ and β2′. Note that αV is split in BenM and is thus labeled αVa and αVb.

2.1. Domain Organization

LTTRs are typically homotetramers; a subunit is shown in **Figure 1**. The most highly conserved portion, the DNA-binding domain (DBD), is at the N terminus and contains a winged-helix–turn–helix (wHTH) motif (2, 68). This domain is connected by a linker helix (LH) to a domain that usually binds a small molecule (68). Since this molecule may be a coactivator, an inducer, and/or a corepressor, we use the term effector. The effector-binding domain (EBD) is sometimes called a regulatory domain, an inducer-binding domain, a ligand-binding domain, or a companion

domain. Regardless of terminology, this domain resembles a periplasmic binding protein in which a cleft between two subdomains binds an effector.

Different EBDs are structurally similar, despite individual recognition of diverse effectors and substantial variations in sequence. LTTRs can bind organic acids, metal ions, flavonoids, sugar phosphates, nucleotides, and many other metabolites (83). Moreover, the response can be highly specific. For example, two paralogs in *Acinetobacter baylyi* together regulate aspartate catabolism, yet one, DarR, responds to D-Asp and the other, AalR, to L-Asp (8, 61). Identifying specific effectors is challenging, and in most cases, signals to which LTTRs respond remain unknown.

2.2. Genetic Context

LTTR genes are often near regulated target genes. In the canonical model (114), an LTTR gene is divergent to a target gene or operon, as shown for BenM and CatM of *A. baylyi* (**Figure 2**). These LTTRs as well as a third, SalR, regulate clustered genes and operons for aromatic compound degradation. It is common for multiple LTTRs to participate in interrelated pathways. In this example, SalR is encoded by a gene near one regulated target, *salA*, and is convergently positioned relative to *salD* and *salE*, all needed for salicylate consumption (62). While most LTTR genes are monocistronic, *salA* and *salR* are cotranscribed. In *Rhodococcus equi*, an LTTR gene, *virR*, is part of an operon involved in virulence (15). The *virR* promoter allows five genes to be cotranscribed at low levels, whereas a second promoter within the *virR* coding sequence enables regulated expression of the four downstream genes. The precise role of VirR in positive regulation of the downstream genes is not clear (15). In other examples, LTTR genes are convergent to a target locus, such as for DarR and MdcR (8, 121).

Context can be used to predict LTTR function (8, 24, 61, 121). However, the entire complement of genes regulated by any specific LTTR cannot be revealed solely by genetic context. The evaluation of LTTR function is improved by methods of systems biology and gene cluster analysis integrated with phylogenetic footprinting, which evaluates conservation among diverse microbes (61, 105–107). One study investigated seven *Escherichia coli* LTTRs of unknown function (106). The workflow combined information from transcriptomics (110), determination of DNA-binding sites with an in vivo chromatin immunoprecipitation method (41), and mutants lacking specific LTTRs. Models based on metabolic and genetic contexts were further tested. This approach established the function of four new LTTRs and identified the targets of three others (106). The results highlight that individual LTTRs regulate multiple loci, have complex direct and indirect effects, and affect diverse aspects of metabolism.

2.3. Consensus LTTR Sequence (T-N_{11}-A)

The DNA-binding sites for LTTRs have been investigated using genome-wide protein–DNA-binding techniques (106, 107, 129). Not only do LTTRs often regulate multiple genes and operons, but also they sometimes serve as global regulators (39, 72, 82). In one study, BsrA from *Pseudomonas aeruginosa* PAO1161 was inferred to regulate 35 genes directly, and many others indirectly (88). Regulated functions included vital metabolic pathways, transport, and the formation of surface appendages. Binding sites were evaluated for possible matches to a known LTTR consensus sequence (T-N_{11}-A), sometimes called the LTTR box (114).

This consensus lies within a small region of dyad symmetry such that the exact sequence varies for different LTTRs. Conserved interactions between the DBD of an LTTR and its recognition box were first structurally characterized for BenM (2) and, more recently, CbnR (67). The BenM recognition box (ATAC-N_7-GTAT) corresponds to an operator site (OS_{R1}) in **Figure 2*c***. The dyad symmetry of this LTTR box, typically around 15 nucleotides in length, reflects interactions

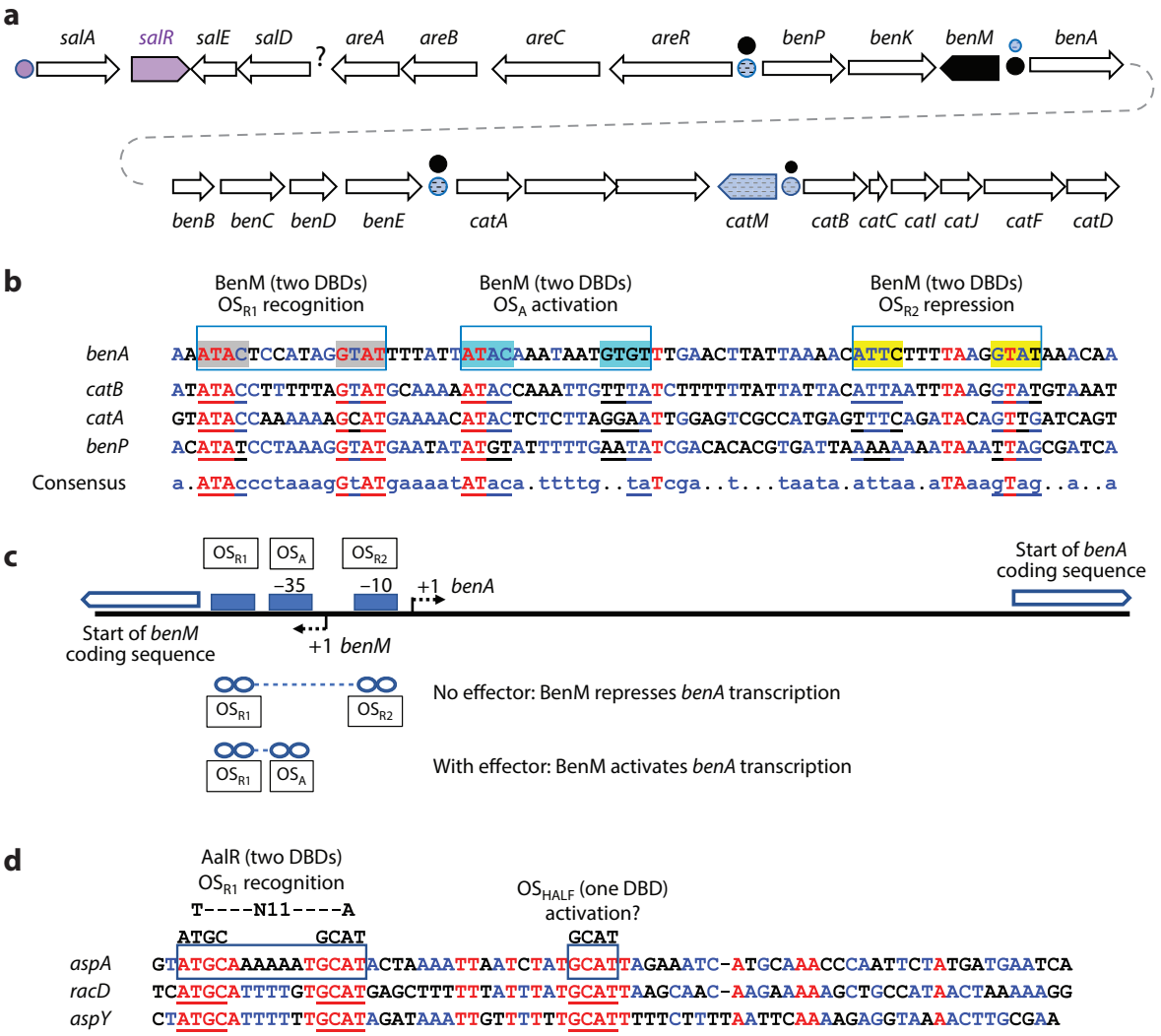


Figure 2

Examples of regulation by LysR-type transcriptional regulators (LTTRs) in *Acinetobacter baylyi*. (*a*) Clustered and functionally related chromosomal genes for aromatic compound degradation are regulated by three LTTRs, SalR, BenM, and CatM. BenM and CatM have overlapping functions at four loci (*black and gray circles*), with the extent of regulation indicated by the size of the circle. Transcriptional activation of *benA* is mediated primarily by BenM, with a minor role for CatM. The reverse is true for *catB*. BenM and CatM play equal roles in regulating *benP* and *catA* (24). SalR activates transcription of the *salAR* operon and may or may not regulate *salD* and *salE* (62). (*b*) Promoter regions are aligned relative to known transcriptional start sites. Each of three highlighted motifs for *benA* differs by no more than one nucleotide compared with the consensus LTTR box (ATAC-N_7-GTAT) for binding two DNA-binding domains (DBDs) of a CatM or BenM tetramer. (*c*) Relative positions of the start of divergent coding sequences for BenM and BenA. Three operator sites (OSs; *blue boxes*) are shown relative to the transcriptional start sites for *benA* and *benM* (+1). Open ovals indicate the binding positions for four DBDs of an LTTR tetramer in the absence or presence of effectors. (*d*) Promoter regions of genes involved in aspartate metabolism are aligned relative to known transcriptional start sites (8). A conserved LTTR box (ATGC-N_7-GCAT) could bind two DBDs of an AalR or DarR tetramer. A so-called half-site sequence (GCAT) is also conserved at a position that abuts the −35 site of the promoter, where one DBD of a tetramer may bind and interact with RNA polymerase to activate transcription.

with two wHTH motifs. This site is sometimes called the RBS, an abbreviation not used here to avoid confusion with a ribosome-binding site. Since LTTRs usually function as tetramers, one operator site accounts for binding of half the subunits.

A unified model of regulation is difficult to formulate, because of variations in the number, sequence, and position of operator sites. A repressor will bind DNA in a fashion that interferes with RNAP binding, whereas an activator is likely to contact and recruit RNAP (12). Thus, the relative positions of promoter and operator sites are critical. LTTRs that activate transcription when bound to effectors usually bind DNA and repress transcription in the absence of effectors. In these cases, deletion of the LTTR gene often causes low-level (approximately twofold) derepressed transcription of the target. However, high-level transcription most often involves repositioning of the activated LTTR and, presumably, contact with RNAP. Thus, LTTR activators are usually dual-mode regulators. In other examples, such as NdhR, LTTRs work principally as repressors (58). Furthermore, the relative operator–promoter positions can vary at multiple targets for the same LTTR, allowing different modes of regulation within the cell. Sequence analysis using a single T-N_{11}-A binding motif can be problematic, as this site neither indicates the binding position of the entire oligomer nor reflects possible RNAP contact.

Computational approaches to find LTTR-binding sites are more accurate in conjunction with properties such as oligomeric conformations, multiple binding sites, promoter positions, cooperativity, and constraints on DNA flexibility (94). A multifaceted computational approach was tested using a set of six LTTR-regulated genes. A refined regulatory model emerged that includes common features of tandem inverted repeats. Each gene in this set had an LTTR box centered at position −63 to −66 relative to the transcriptional start site (94). A second inverted repeat was identified (centered at −41 to −45), and, in some cases, a third inverted repeat was identified (centered at either −34 or −10). Sequence similarity was highest for the LTTR box (OS_{R1}), and reduced levels of conservation at other positions were attributed to lower-affinity binding interactions that were nonetheless important. In the case of OdcR, a single-nucleotide change in the LTTR box appears to modulate transcription for different promoters in its regulon (20).

Complexity is evident in an alignment of four promoter regions where BenM and/or CatM activate transcription (**Figure 2*b***). These paralogs have overlapping roles in the regulation of aromatic compounds, and they recognize the same LTTR box (OS_{R1}, ATAC-N_7-GTAT) (24). At the *benA* promoter, in the absence of effector(s), BenM binds the DNA with a long footprint, consistent with two DBDs of a tetramer binding OS_{R1} and the other two DBDs binding OS_{R2} (14). Since OS_{R2} overlaps the −10 region of the promoter, *benA* transcription is repressed when no effector is present. An effector-mediated response causes the tetramer to shift, consistent with binding of OS_{R1} and OS_A. This repositioning of the DBDs (**Figure 2*c***), called the sliding-dimer model (76), should promote interaction with RNAP to activate transcription (125). The signal-mediated compaction of the EBD affects the entire protein–DNA complex, and it changes the bending angle of the DNA (76, 124). The result, which may seem counterintuitive, is that effector binding relaxes the DNA bend angle.

Similar binding events (**Figure 3*a*,*b***) illustrate that OS_{R2} does not always overlap the −10 region of the target promoter (94). In this example, OS_{R2} blocks the −35 region of the promoter, and in response to the effector, repositioning occurs from OS_{R2} (**Figure 3*a***) to OS_A (**Figure 3*b***). This shift helps recruit RNAP (**Figure 3*c***).

For *benA*, both OS_{R2} and OS_A differ from OS_{R1} by one nucleotide (**Figure 2*b***). However, in the alignment of comparable BenM-regulated regions (such as *catA* and *benP*), sequence conservation is much lower for OS_{R2} and OS_A than for OS_{R1}. A pattern of multiple operator sites is not always evident. For example, in **Figure 2*d***, the promoter region of AalR-regulated *aspA* is aligned with comparable regions of other genes involved in aspartate metabolism in *A. baylyi* (8). In this

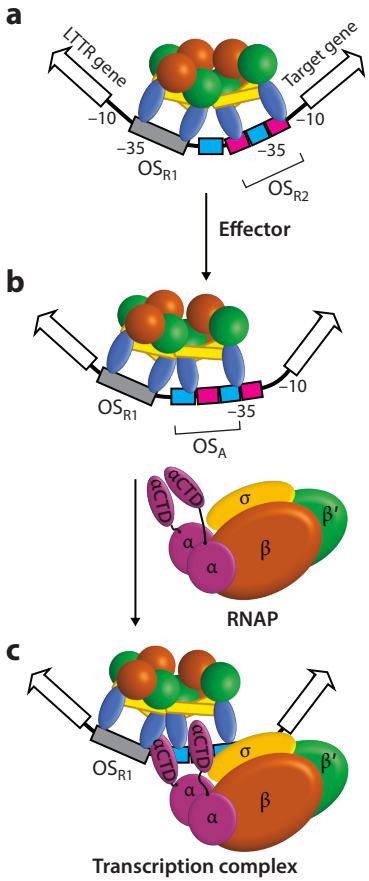


Figure 3

Binding of a tetrameric LysR-type transcriptional regulator (LTTR) to an operator–promoter region. (*a*) In the absence of effectors, both the LTTR gene and a divergent target gene are repressed. Two DNA-binding domains (DBDs; *ovals*) bind the LTTR recognition box (OS_{R1}; *gray rectangle*) and a repression site (OS_{R2}; *pink rectangles*). Each half-site of the OS binds one DBD. (*b*) The effector binds to the effector-binding domains (EBDs) (the two subdomains, EBD-I and EBD-II, are depicted as green and brown spheres). Effector-mediated conformational changes reposition two DBDs to an adjacent site (OS_A; *blue rectangles*). (*c*) Repositioning of the LTTR helps recruit RNA polymerase (RNAP) and activates transcription of the target gene. The different positions of OS_{R2} for this generic LTTR and for BenM (**Figure 2**) highlight some observed variations among different LTTRs (94).

example, the LTTR box (OS_{R1}) is ATGC-N_7-GCAT. However, no neighboring sequence obviously resembles the full OS_{R1} site. Interestingly, half of that sequence (GCAT) is conserved not only within these *A. baylyi* promoter regions but also in bacteria of other genera (8). This half-site could bind the DBD of one AalR subunit and might affect transcriptional activation. Many variations in operator–promoter sequence patterns, such as this one, have unknown significance.

2.4. Autoregulation and Other Control of LTTR Levels

LTTR expression tends to be low and subject to negative autoregulation. Such control makes signaling responsive to small changes in effector concentration and accelerates response times in transcriptional networks (108). The presence of a transcription factor–binding site upstream

of its own coding sequence can be used to discover the sequences of such *cis*-acting sites by bioinformatic analysis (5). For many LTTRs, a divergent gene configuration provides a simple mechanism whereby the same binding site(s) used for transcriptional regulation of the target can repress expression of the LTTR (**Figure 2*c***). A complicated type of negative autoregulation occurs for the nitrogen assimilation control protein (NAC) from *Klebsiella pneumoniae*. This unusual LTTR does not require an effector molecule, and regulation of its target genes depends on NAC expression levels (9). A two-component NtrBC regulatory system activates *nac* transcription from a σ54-dependent promoter when nitrogen is limiting and NtrC is phosphorylated. NAC represses transcription from its own promoter by binding a site that restricts the bending or flexibility of DNA, preventing necessary contact between phosphorylated NtrC and RNAP. With this exception, NAC-regulated targets have typical σ70-dependent promoters (9).

Autoregulation can also occur when LTTR genes are not divergent to a target. For example, binding of VirR in *R. equi* blocks its own promoter, repressing transcription of a five-gene operon to maintain low-level expression. Regulated transcription of the downstream genes is mediated at a second promoter (15). For SalR (**Figure 2*a***), the *salA* promoter also controls *salR* transcription (62). This arrangement results in positive autoregulation (52), which can also occur for other LTTRs, such as a regulator of linoleic acid production in *Lactobacillus plantarum* (77) and LrhA in *E. coli*, which controls transcription of genes for motility and flagellar synthesis (74). ThnR mediates positive autoregulation by transcriptional activation of genes, including its own, needed to degrade tetralin in *Sphingopyxis granuli* (38). Some LTTRs are not autoregulated; these include PA2206, which affects oxidative stress responses in *P. aeruginosa* (104), and HexA, a major regulator in *Photorhabdus luminescens* (70). Another nonautoregulated LTTR is ArgP in *E. coli*, which controls arginine export, DNA replication, and amino acid metabolism (92). The ArgP-mediated effect on disparate functions, as well as its lack of autoregulation, may relate to a role as a nucleoid-associated protein (NAP) (93), as discussed below.

While less is known about the cellular stability of LTTRs, proteolysis also contributes to regulation. Mutational approaches suggest that ClpAP-mediated proteolysis affects the stability of DarR either directly or indirectly (8). For HexA, in *P. luminescens*, differential proteolysis in two cell types appears to control regulation by this LTTR (70). In another example from *Pseudomonas protegens* H78, an LTTR, PltR, activates biosynthetic genes for synthesis of pyoluteorin (Plt), an antibiotic. The regulation of Plt production involves a regulatory cascade with multiple signals and transcription factors. The Lon protease reduces Plt synthesis by acting at several points in the cascade, including the direct degradation of PltR (131). In a different *P. protegens* strain, Plt synthesis was shown to be regulated by rare codons in *pltR* (133).

3. MOLECULAR BASIS OF REGULATION

Structural characterization is hindered by the inherent difficulty of obtaining pure, concentrated LTTR proteins that do not rapidly aggregate (34, 109). Initially, atomic structures were limited to the EBD regions. The first EBD structures of CysB (126) and OxyR (21) were soon followed by others. In 2003, a full-length LTTR crystal structure was characterized, that of CbnR from *Cupriavidus necator* (reclassified from *Ralstonia eutropha*) (91). In another advancement, molecular interactions were mapped between BenM-DBDs and their cognate DNA (2). The culmination of crystallographic study is the full-length CbnR complex with promoter DNA (44). Currently, structures are available for 16 different full-length LTTRs, 38 different EBDs, and 6 DBDs, not including structures of the same LTTR from different species, different variants, or the same regulator bound to different effectors. Structural features of LTTRs have been reviewed elsewhere (68), and we expand here on a few points of interest.

3.1. Assembly of Oligomers

Figure 1 depicts the DBD, LH, and EBD regions of a typical subunit. Although some LTTRs appear to have N-terminal extensions, these additions may reflect incorrect identification of the translational start. Problems can arise if expression constructs include extra residues at the N terminus, a region that interacts directly with DNA. Thus, N-terminal tags for LTTR purification should be avoided (14). Misannotation of homologs can result because of low sequence similarity among LH and EBD regions of homologs, even for LTTRs with the same function. In databases, many putative LTTRs are incorrectly designated CysB. While functional residues in orthologs are usually highly conserved, such as those for effector binding, they tend to be distributed throughout the sequence and difficult to detect.

We propose a general notation scheme to facilitate LTTR comparisons based on secondary structures (**Figure 1**). Strands βDE and βIJ extend between the EBD subdomains to create a flexible hinge. In many structures, strands D–E and/or I–J are continuous. LTTRs can have significant insertions or deletions in so-called crossover regions between the strands of the highly conserved β-sheets. Most crossover regions are helices, but a few include β-hairpin loops (as in the structure of an uncharacterized LTTR; Protein Data Bank identifier 3MZ1). Other crossovers are meandering coiled loops, as in PqsR (MvfR) (54, 132). Structural diversity in crossover regions is most common in EBD-II.

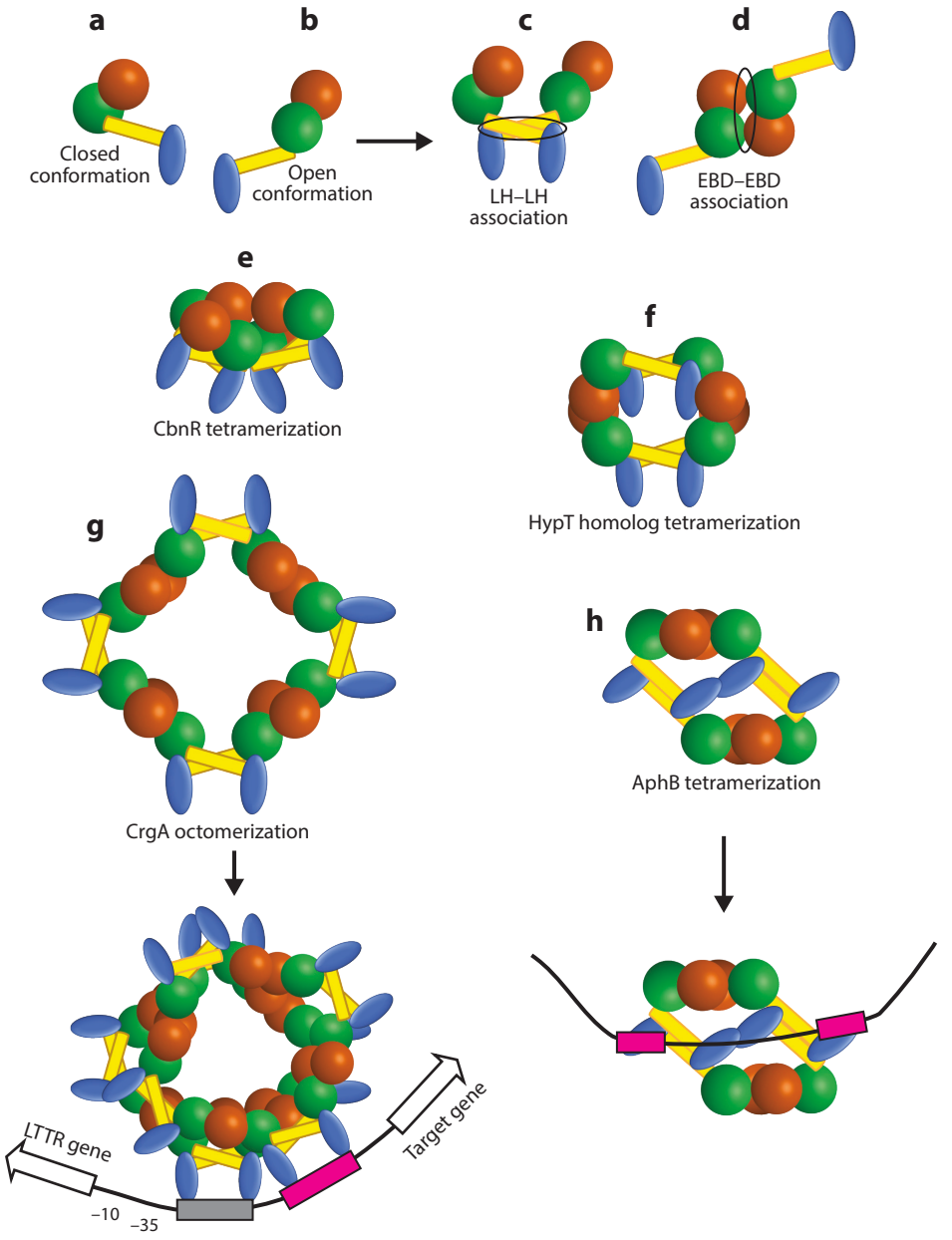
CbnR structures revealed that subunits of the same LTTR can assume different conformations within an oligomer (91) (**Figure 4*a*,*b***). In CbnR, the quaternary structure is a dimer of dimers with DNA-binding sites aligned along a concave surface (as in **Figure 3**). We refer to similar full-length structures (e.g., ArgP, TsaR, BenM, AphB, OxyR, DntR, HypT, and DarR) as CbnR-like. Of three oligomerization interfaces identified in 2010 (109), only two are characteristic of nearly all subsequently analyzed structures. At these interfaces, the types of interactions tend to be similar, but diversity in sequence may help prevent the misassociation of different LTTR subunits.

One interface, conserved in all known structures, consists of two DBD–LH domains that form homodimers through interactions between the LH faces (α4–α4′ in **Figure 1**), with the DBD region contributing to dimeric stability (**Figure 4*c***). This structure positions the DBDs to interact with dyadic DNA sequences in LTTR recognition boxes. The second conserved interface guiding quaternary assembly consists of two EBDs interacting head to tail in the region of βB–αII and αVII, previously termed α6–β2 and α11 (**Figure 4*d***) (109). This interface positions the two *trans*-EBD strands, βDE and βIJ, facing outward, with the effector-binding clefts oriented toward the interior of the dimeric unit. There is (at least) one exception to the presence of this interface, which is absent in crystal structures of the PqsR (MvfR) EBD from *P. aeruginosa* (54, 132). Instead, there is an interface consisting of antiparallel β-strands, βB–βB′, and a large coiled-coil region. In this example, the effector-binding clefts are oriented toward the exterior of the dimeric unit.

The third previously identified tetramerization interface, between αVI and αVI′ (**Figure 1**), earlier called α10–α10 (109), is not always observed. This interface may play a role in conformational changes associated with DNA binding and transcriptional activation in response to effector binding (65). However, since it is missing in many CbnR-like proteins, such as OxyR (59) and DarR (130), a role in quaternary structural assembly is more likely specific to individual LTTRs rather than a general feature.

Another structural feature concerns the interdomain angle between DBD–LH and EBD units (109). Rotation between α4 and strand βA creates a range of possible subunit structures from the same sequence. In full-length CbnR structures (**Figure 4*e***), two subunits have open conformations (**Figure 4*a***) and two closed (**Figure 4*b***). Changing the relative angles of the units

allows a range of quaternary structures, including a tetrameric HypT having a single subunit conformation; a CrgA octamer with a single subunit conformation that orients the DBD–LH dimers externally (**Figure 4*g***); a tetrameric AphB structure with both DBD–LH dimers oriented inside the tetramer using two subunit conformations (**Figure 4*h***); and a remarkable conformation with one DBD–LH dimer inside the tetramer and one dimer outside, as in a HypT homolog from



(*Caption appears on following page*)

Figure 4 (*Figure appears on preceding page*)

Oligomerization schemes for LysR-type transcriptional regulators (LTTRs). LTTRs have significant flexibility between the DNA-binding domain–linker helix (DBD–LH) domain and the effector-binding domains (EBDs). In several atomic structures of full-length LTTRs, (*a*) a closed conformation and (*b*) an open conformation are sometimes observed. These distinctions in subunit conformations are not depicted in the other panels of the figure. In all known structures of DBD–LHs and full-length LTTRs, (*c*) an LH interacts with another LH, while (*d*) most structures use the same EBD–EBD interface. Dimeric LTTRs in solution are most likely LH dimers. Combinations of the structures shown in panels *c* and *d* can result in diverse oligomeric states. (*e*) Assembly of the CbnR tetramer (44) is prototypical of many LTTRs. However, changes in the DBD–LH relationship to the EBD create a wide range of alternative quaternary structures. (*f*) In a HypT homolog from *Vibrio vulnificus*, VV2_1132, the DBDs of two subunits are oriented inside the tetramer, while two DBDs are exposed (55). (*g*) CrgA forms an octamer. Biochemical studies support the binding of two octamers to DNA (111). (*h*) A hypothetical LTTR could bind to DNA in a nonprototypical way, for example, lacking the classic T-N_{11}-A spacing. The tetrameric structure of AphB assembles with the internal DBD domains sterically inaccessible to DNA (122). The remaining flanking DBDs, not associated directly through contacting LH domains, could bind DNA. Note that such binding has not been demonstrated for AphB, but it offers insight into other binding models. Other oligomerization schemes have been observed for LTTRs that are not represented here.

Vibrio vulnificus (**Figure 4*f***). As these structures illustrate, CbnR should no longer be considered the sole LTTR prototype.

In solution, many full-length LTTRs appear to be tetramers, whereas others, including MetR (85), CatR (97), AmpR (11), ClcR (22), CbbR from *Xanthobacter flavus* (127), and OccR (1), appear to be dimers. In the case of OccR, a tetramer associates with DNA (1). No structures of dimeric LTTR proteins have been observed, and it is difficult to explain the quaternary assembly of a functional dimer using the assembly interfaces described above, which would be precluded by steric hindrance. Additional studies are needed to determine definitively whether LTTRs function as dimers and, if so, to characterize such atomic-level structures.

3.2. Molecular Features of Effector Binding

Structures of BenM-EBD and CatM-EBD bound to their effectors established the presence of an effector-binding pocket between EBD-I and EBD-II (33). Dipoles from four helices of one EBD subunit are organized such that opposing charges in the effector contribute to protein–ligand binding. Some effectors, such as *cis*,*cis*-muconate and benzoate in BenM (33), *p*-toluenesulfate in TsaR (90), salicylate in DntR (29), citrate in CcpE (18), shikimate in QuiR (102), and sulfite in YeiE (51), are strongly polar and form significant hydrogen bond networks with amino acid side chains that determine ligand specificity. In other cases, lipophilic components of the effectors (or designed therapeutics) can reach far into hydrophobic cavities within the EBDs, as in PqsR structures (54, 66, 137). Effector binding can draw EBD-I and EBD-II together, inducing a large conformational change in the quaternary structure. A so-called rotary switch model is based on the large angular rotation between helices in the EBD structure of OccR (65). These conformational changes can occur without effectors, such as for LeuO hyperactive variants (39).

Not all effectors bind in the typical pocket. In BenM, a secondary site binds benzoate (33), and DntR has a similarly positioned binding site (29). In BenM, binding of *cis*,*cis*-muconate in the principal site and benzoate in the secondary site enables a synergistic response to both (14, 33). Allosteric regulation mediated by two effectors at different sites has also been suggested for CysB-EBD structures complexed with natural ligands (87). In another interesting example, NdhR is a repressor that coordinates nitrogen and carbon metabolism in autotrophic cyanobacteria via the mutually exclusive binding of two different effectors (58). One effector binds at the interface of two EBD subunits as a corepressor to enhance DNA binding. The other is an inducer that binds in

the typical effector-binding pocket to mediate conformational changes that release NdhR from its operator sites. In a different study of AdmX, two different indoles bind competitively, with only one causing large conformational changes (43). Several LTTRs, such as RipR, bind antagonists that inhibit the response to their functional effectors (64).

Not all LTTRs bind effectors, as noted above for NAC (9). In another example, OxyR controls transcription in a redox-dependent manner by responding to hydrogen peroxide (H_2O_2) (6, 138). A model based on the oxidation of cysteine residues has been reevaluated and altered (21, 59, 98, 112). Interestingly, OxyR catalyzes the reduction of H_2O_2, to mitigate further cellular damage and cause a conformational change needed for OxyR-activated transcription. A disulfide-driven allosteric structural change occurs at the EBD interface (98). Like H_2O_2, hypochlorous acid (HOCl) can generate hydroxyl radicals. To resist cellular damage, HypT, regulates multiple genes that help *Salmonella enterica* survive in macrophages. HypT is activated by HOCl-dependent oxidation of a methionine (60). In AphB from *Vibrio cholerae*, a virulence factor that senses low pH and low oxygen, cysteine residues may act as thiol-based switches (17, 69, 78).

Sometimes, effectors are found in crystal packing sites distant from the expected binding pocket, as in TsaR and CysB-EBD bound with *O*-acetylserine (87). Caution should be used in assessing such binding. During experimental procedures, small molecules, such as benzoate (33) and 2-morpholinoethanesulfonic acid monohydrate (130), may bind tightly and cause artifacts. Exhaustive dialysis is recommended to remove tightly bound ligands. While bound ions can affect data interpretation (23), they can also guide structural analysis (118).

3.3. Interactions with RNA Polymerase

We have reviewed research on LTTR–RNAP interactions elsewhere (24). Briefly, mutational studies and footprinting methods have been used with some LTTRs to assess contacts with key regions of RNAP holoenzyme (12). Interactions were indicated between the C-terminal domain of the α-subunit of RNAP (α-CTD) and LTTRs such as MetR, CysB, CatR, GcvA, and OxyR (40, 79, 86, 120). However, interactions between an individual LTTR and regions of α-CTD differed at multiple promoters (40, 79). Promoter-specific differences were also observed for GcvA, which interacted with the σ70 subunit at some promoters and α-CTD at others (120). In addition, amino acids in the turn region of the wHTH of the DBD of several LTTRs exerted positive control of transcription (79, 120). In studies of LTTR-mediated repression, CrgA in *Neisseria meningitidis* promoted RNAP binding via interactions with α-CTD that prevented promoter clearing and resulted in abortive transcripts (28).

LTTR interactions with RNAP remain largely uncharacterized. Based on structures of BenM-DBD bound to DNA, a model was built of an initiation complex with RNAP at the *benA* promoter (125). It appears that BenM-DBDs do not directly contact σ70. In this model, there is an "UP-element," a promoter feature that increases transcription via interactions with the RNAP α-CTD. However, the relative position of the LTTR to promoter elements differs from that of a classical class I promoter, in which a transcription factor contacts the RNAP α-CTD (12). Similar models, such as in **Figure 3*c***, address promoter elements of other LTTR-regulated genes (e.g., 94).

4. COMPLEX REGULATION AND INTRICATE CIRCUITRY

LTTRs participate in intricate regulatory circuits, but specific regulatory schemes differ significantly between bacteria, even closely related species. Such differences may reflect evolutionary pressures during niche adaptation. Selection during adaptation tends to favor global rewiring of regulatory networks (45). This section describes several examples of LTTRs involved in complex, multilevel regulation.

4.1. LTTRs and Regulation by Small RNA

Posttranscriptional regulation often involves sRNA molecules that alter mRNA translation and/or stability. Interestingly, *oxyS*, which is one of the best-studied sRNAs involved in pleiotropic regulatory circuits, was discovered because of its proximity to a divergently transcribed LTTR gene, *oxyR* (3). Sometimes, the Hfq chaperone promotes sRNA–mRNA pairing (63). LTTRs can affect this posttranscriptional regulation by controlling transcription of sRNAs. For example, VtlR plays critical roles in host–microbe interactions in diverse *Alphaproteobacteria*. VtlR (or its homolog, LsrB) regulates the transcription of multiple sRNAs, influencing posttranscriptional control of hundreds of genes (13, 32, 42). Another example of LTTR-mediated control of sRNA transcription is provided by HexA of *P. luminescens* (70).

LTTR-encoding mRNA can also be affected by sRNA. In *Dickeya dadantii*, a plant pathogen, an Hfq-dependent sRNA represses the translation of an LTTR, PecT (136). In turn, PecT represses transcription of another sRNA, *rsmB*, that is part of a global posttranscriptional regulatory network. In this network, *rsmB* availability is further titrated through RsmA-mediated sequestration. In this system, which controls many virulence factors, additional regulatory interplay involves cyclic di-GMP diguanylate cyclases (136).

ThnR, mentioned above, controls genes for tetralin consumption. However, in the presence of preferred growth substrates, carbon catabolite repression is mediated by an sRNA that binds *thnR* transcripts in an Hfq-dependent manner (38). Lowered translation of this LTTR thereby decreases expression of the tetralin catabolic enzymes (38).

4.2. LTTRs as Global Regulators

While the definition of a global regulator is imprecise, many LTTRs have pleiotropic effects, control diverse functions, and regulate many genes. LeuO from *E. coli* activates and/or represses more than 100 targets affecting functions such as pathogenicity, CRISPR-Cas immunity, nitrogen metabolism, and biofilm formation (39). There is an overlap in genes affected by LeuO and H-NS, a NAP (50). NAPs bend DNA, bind many DNA sites, and affect genome architecture. Moreover, NAPs also regulate gene expression. H-NS forms extended nucleoprotein complexes with AT-rich DNA, thereby repressing transcription. There are many intriguing H-NS-LeuO interactions, some antagonistic (113). For regulators that bind numerous genomic sites, questions arise about the relative importance of specific nucleotide operator sequences compared with indirect readout, a recognition process based on shape. Such issues suggest that there may not always be a distinction between transcription factors and NAPs (31). For example, ArgP, an LTTR also known as IciA, is a global regulator that can bind DNA in a non-sequence-specific fashion, and it has been designated a NAP (93).

When engineered for high-level expression, LeuO, and other transcriptional regulators, can cause nucleoid compaction (135). However, LeuO expression is normally very low, despite the observation of multiple *leuO* promoters (113). A typical palindromic site was identified for LeuO at some promoters (39). Hyperactive LeuO variants were characterized that presumably mimic changes normally occurring in response to currently unknown effectors or signals.

ScmR of *Burkholderia thailandensis* is also a global regulator (72, 82). ScmR controls the synthesis of secondary metabolites, quorum sensing, pH homeostasis, and virulence. This LTTR, which is highly conserved in the *Burkholderia* genus, plays a major role in quorum sensing. Not only does it affect the production of signaling molecules such as *N*-acyl-L-homoserine lactones (AHLs) and LuxR-type regulators, but also expression of *scmR* is itself regulated by quorum sensing. ScmR regulates some genes independently of quorum sensing, and it affects the production of several other transcriptional regulators (72, 82).

4.3. LTTRs, Other Regulators, and Regulatory Cascades

PhcA, from a plant pathogen, *Ralstonia solanacearum*, is a master regulator affecting approximately 30% of the genes in its genome either directly or indirectly (100). PhcA-regulated genes include approximately 166 transcriptional regulators. PhcA has been studied because of its importance in controlling many virulence factors, yet the regulatory network extends beyond pathogenicity. When the bacteria are in the plant, PhcA-mediated gene expression differs from what is observed for bacteria cultured in nutritionally replete medium, and, in some cases, up- or downregulated effects are reversed under these conditions (100).

LTTRs are frequently implicated in quorum-sensing networks, yet the types of interactions can vary. In *V. cholerae*, an RNA sponge, called QrrX, binds multiple sRNAs that modulate quorum sensing. An RNA sponge is a type of noncoding sRNA that can base-pair with other regulatory sRNAs, thereby neutralizing their activities. In this example, an LTTR, QrrT, controls transcription of the QrrX sponge (53). In a different example in *P. aeruginosa*, PqsR (MvfR) is an LTTR that controls alkyl-quinolone-dependent quorum sensing (54). PqsR drives autoinduction of alkyl-quinolone signals by activating the transcription of genes encoding biosynthetic enzymes for these compounds. In contrast, the effect of an LTTR (ABUW_1132) in *Acinetobacter baumannii* on quorum sensing is less direct (123). This LTTR is a global regulator that affects multiple functions, including capsule expression and virulence. ABUW_1132 appears to control how the quorum-sensing signal, an AHL, is secreted from the cell (123). Additional studies are needed to clarify the regulatory mechanism(s) of this LTTR.

The ways that LTTRs participate in regulatory cascades, and their positions within these cascades, are diverse. For example, CrgA in *R. solanacearum* directly represses transcription of the master regulator (FlhDC) of flagellar biosynthesis, thereby negatively affecting cell motility and virulence at an early regulatory step (36). PltR, described above as an example of an LTTR regulated by proteolysis, is in the middle of a regulatory cascade (131). In this example, a two-component regulatory system (GacA/GacS) and several other regulators control PltR availability and activity. In some cases, there may be no obvious hierarchical scheme. In *Enterococcus faecalis*, iron homeostasis was studied using treatments of iron excess and limitation in the presence and absence of a non-LTTR regulator (Fur). Iron availability stimulated a large network of transcriptional regulators, including LTTRs (71). It is not always clear how varied signals are integrated, although some interactions involve protein–protein contacts between LTTRs and other regulators, such as ThnR–ThnY (38), CbbR–RegA (26), and AtzR–NtrC (101).

5. APPLICATIONS IN BIOTECHNOLOGY AND MEDICINE

Because of the abundance and importance of LTTRs, efforts to engineer bacterial metabolism often involve some aspect of LTTR-mediated regulation. While various types of bacterial transcription factors can be used similarly, the choice of LTTRs for biotechnology has recently proven effective (37, 99). In this section, we describe a few key roles for LTTRs in new methods for metabolic engineering and synthetic biology. Finally, we address the potential use of LTTRs in medical applications.

5.1. Metabolic Engineering and Synthetic Biology

Controlled gene expression is a central feature of metabolic engineering and synthetic biology (73). As the need to develop sustainable economic practice increases, so does demand for using bacteria as cellular factories. Traditional engineering involves "static" genetic modifications that do not alter expression levels according to variability in intracellular conditions. However, new approaches incorporate automatic and dynamic responses to intracellular variation (25).

One example that uses an LTTR in a bifunctional dynamic system illustrates how this type of regulation can distribute bacterial resources between growth and production (134). CatR, from *Pseudomonas putida*, is a *cis,cis*-muconate-responsive homolog of CatM and BenM. CatR was expressed in *E. coli* and used to regulate its native promoter to increase transcription of target genes. In a clever combination, this muconate-driven upregulation was paired with a different construct in which muconate-driven transcription generates antisense RNA to downregulate its target, genes funneling carbon to the tricarboxylic acid cycle. CatR-controlled genes expressed enzymes to synthesize muconate as an effector molecule. Additionally, muconate was the desired end product since there is commercial demand for this platform chemical. The antisense system decreases carbon entry into the tricarboxylic acid cycle in a dose-dependent fashion as muconate concentration increases. Thus, an autonomous distribution of carbon flux between growth and biosynthesis results from variation in muconate concentration. This dynamic system increased the yield of muconate compared with an optimized static system (134).

Metabolic engineering in *P. putida* can generate muconate via multiple strategies (10). A CatM-based biosensor was developed to monitor the production of muconate. In this application, CatM detects intracellular muconate and responds by increasing transcription of a fluorescent protein gene. This output allows fluorescence-activated cell sorting to select strains with the best performance. Many biotechnology applications for LTTR-based biosensors exist (4, 27, 117, 119), and the sensitivity, specificity, and dynamic range of the biosensor can be optimized (57, 95, 115). Mutational approaches have typically focused on the regulated promoter, the DNA-binding properties of the LTTR, and the ligand specificity of the EBD. A new strategy optimizes LTTR biosensors by focusing on the LH region (103). High-throughput methods, computational approaches, and systems biology techniques are being used to identify new transcriptional regulators and clarify LTTR–effector interactions (47, 48, 56).

5.2. Drug Targets and Therapeutics

Biosensors also have many environmental, diagnostic, and medical applications (46). One intriguing approach is the development of LTTR-based applications in chromosome-free cells called SimCells (simple cells). In such devices, the chromosome is specifically degraded, yet, for a while, expression of a synthetic genetic circuit continues. SimCells do not replicate and may be used to avoid problems that could be caused by whole bacteria. SalR from *A. baylyi* (**Figure 2**) was used in SimCells derived from *E. coli* to demonstrate that catechol can be synthesized and safely delivered to various cell lines as a potent anticancer drug (35). SalR had already been optimized in *E. coli* and SimCells for use as an aspirin-inducible biosensor (19). To generate catechol, SalR-positive autoregulation was exploited to activate transcription of *salA* and a fluorescent reporter such that salicylate was hydroxylated to generate catechol (35).

LTTRs have additionally drawn attention as drug targets to treat infections. Traditional therapeutics, such as antibiotics, affect essential bacterial growth pathways. However, with the rapid development of antibiotic resistance, new strategies are focusing on bacterial virulence factors (30). The aim is to disrupt pathways or compounds that cause disease by damaging the host or helping the pathogen evade the immune system. As indicated above, several LTTRs play key roles in the expression of virulence factors, often as part of complex regulatory circuits. One approach is to determine inhibitors that bind LTTRs, or other transcriptional regulators, to prevent the expression of virulence factors. This approach was used to search for nonlethal inhibitors of transcriptional virulence regulators in a bacterium causing a widespread and incurable disease in citrus (7). In another example, drugs that are already approved for human use were repurposed to determine possible inhibition of an LTTR from *V. cholerae*, AphB, which regulates genes for cholera toxin and a coregulated pilus (81). In this case, an antiviral drug, ribavirin, suppressed *V. cholerae*

pathogenesis in animal models. This same drug also inhibited an LTTR (Hrg) from *S. enterica* serotype Typhi (81).

A different LTTR in *P. aeruginosa* is similarly being used as the target of antivirulence drugs (66, 84, 128). This regulator, described above as PqsR for its role in alkyl-quinolone quorum sensing, is also known as MvfR, based on its regulation of multiple virulence factors. Using computational and screening methods, promising inhibitors were identified and are now in preclinical trials. Many new studies are focusing on the exciting promise of addressing infections causing critical illness with drugs that inhibit MvfR (116).

6. CONCLUSIONS AND FUTURE PERSPECTIVES

LTTRs are among the most abundant and widespread types of bacterial transcriptional regulators. Their prevalence and participation in complex regulatory networks highlight their importance, as they participate in essentially every type of physiological and metabolic function. Great progress has been made in characterizing LTTR structures. However, in most cases, signaling molecules remain unclear. Future studies are needed to clarify the mechanistic basis of transcriptional control and interactions with RNA polymerase. Detailed differences between the operator–promoter regions of specific LTTRs are often downplayed in efforts to generate a uniformly applicable model of regulation. Similarly, questions about oligomerization, assembly, and quaternary complexes do not appear to be answered by a single model. As molecular details emerge, it becomes increasingly important to characterize and highlight differences as well as similarities among these ubiquitous bacterial proteins. The promise of exciting applications for LTTRs in many areas of biotechnology, biomanufacturing, environmental remediation, and medicine are being realized through novel strategies of metabolic engineering and synthetic biology.

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