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Title
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Permalink
https://escholarship.org/uc/item/3fv645nj

Journal
Environmental Microbiology Reports, 12(3)

ISSN
1758-2229

Authors
Rajeev, Lara
Garber, Megan E
Mukhopadhyay, Aindrila

Publication Date
2020-06-01

DOI
10.1111/1758-2229.12838

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environmental microbiology reports

Environmental Microbiology Reports (2020) 12(3), 267–276 doi:10.1111/1758-2229.12838

Minireview

Tools to map target genes of bacterial two-component system response regulators

Lara Rajeev,[1] Megan E. Garber[1,2] and Aindrila Mukhopadhyay [1,2,3]*

[1]*Biological Systems and Engineering Division, Lawrence Berkeley National Laboratory, Berkeley, CA, 94720, USA.*
[2]*Department of Comparative Biochemistry, University of California, Berkeley, CA, 94720, USA.*
[3]*Environmental Genomics and Systems Biology Division, Lawrence Berkeley National Laboratory, Berkeley, CA, 94720, USA.*

Summary

Studies on bacterial physiology are incomplete without knowledge of the signalling and regulatory systems that a bacterium uses to sense and respond to its environment. Two-component systems (TCSs) are among the most prevalent bacterial signalling systems, and they control essential and secondary physiological processes; however, even in model organisms, we lack a complete understanding of the signals sensed, the phosphotransfer partners and the functions regulated by these systems. In this review, we discuss several tools to map the genes targeted by transcriptionally acting TCSs. Many of these tools have been used for studying individual TCSs across diverse species, but systematic approaches to delineate entire signalling networks have been very few. Since genome sequences and high-throughput technologies are now readily available, the methods presented here can be applied to characterize the entire DNA-binding TCS signalling network in any bacterial species and are especially useful for non-model environmental bacteria.

Introduction

Vast numbers of bacterial genomes are being sequenced, providing valuable information on an organism's physiology and evolution. However, knowledge of bacterial regulatory networks has not kept pace with genome sequence availability. We have incomplete pictures for the signalling networks for even well-studied bacteria such as *Escherichia coli* and *Bacillus subtilis*. The knowledge gap is even wider for non-model organisms, particularly environmental microbes. Bacteria employ signalling systems to perceive and respond to changes in their environment such as the presence of nutrients or stressors. Thus, information on the signalling and regulatory networks is required for the understanding of an organism's physiology. The number of signalling systems that a given genome encodes independent of its genome size has been assigned a metric called the 'bacterial IQ' (Galperin, 2005). The bacterial IQ score can vary dramatically even between closely related species as it reflects the adaptation of each species to its ecological niche (Galperin, 2005; Galperin *et al*., 2010). In general, pathogens whose environments tend to be more stable than those of their free-living counterparts tend to have fewer signalling systems.

Two-component systems (TCSs) are the most prevalent signalling systems in bacteria. TCS function in essential physiological processes, stress responses, secondary metabolism and virulence. A typical TCS consists of a membrane-bound sensor histidine kinase (HK) and a cytoplasmic response regulator (RR) (Stock *et al*., 2000). The HK perceives environmental or intracellular signals and is auto-phosphorylated. The HK then transfers the phosphoryl group to the receiver domain of its partner RR, which then effects a corresponding change through its output domain. The most common output domains for RRs are DNA-binding domains through which the RR mediates transcriptional changes. Other output domains may be enzymatic, such as those synthesizing or hydrolyzing the second messenger cyclic-di-GMP, or the RR may lack an output domain altogether, acting then as phosphate sinks or mediating phosphorelays or effecting changes through protein–protein interactions (Galperin, 2010). TCSs are also attractive candidates for developing antibacterial drugs since they are absent in humans, and targeting TCSs can inhibit virulence without killing the

*For correspondence. E-mail amukhopadhyay@lbl.gov; Tel. (510) 495-2628; Fax (510) 486-4252.

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bacteria, thus potentially not allowing resistance to develop (Gotoh *et al*., 2010).

As an increasing amount of genomic data is available for uncharacterized species, it is critical to have the tools to interrogate and understand an organism's signalling network quickly. To understand the TCS network in a bacterial species, we would need to know what signals the sensor kinase perceives, what partner RRs the HKs recognize and what the functional/effector output for each RR is. Since RRs with DNA-binding domains make up the majority of the RRs, knowing the transcriptional targets for an RR can provide broad understanding of the regulatory picture. Identifying gene targets could give information on the function of a hitherto uncharacterized TCS and the signals that are most likely sensed by the TCS. This review focuses on the methods and tools available to decipher the regulon and binding sites of TCSs. We cover methods that fall into categories such as expression profiling methods, binding site mapping methods and targeted profiling methods (Fig. 1), and span approaches that maintain the native cellular context to those that do not but have been used successfully to provide useful insights into the regulatory and networks of microbial systems.

Expression profiling methods

Target mapping by transcriptomics

Transcriptomics is very routinely employed to elucidate the regulons and functions of TCSs. Typically, gene expression in a deletion or knock-out mutant in either the RR gene (Liu *et al*., 2015; Low *et al*., 2016; Antoraz *et al*., 2017) or both HK–RR genes (Richmond *et al*., 2016; Chen *et al*., 2017; Moon *et al*., 2017) is compared with that of the wild-type (WT) strain (Fig. 1). This method is effective if the TCS of interest is active under the conditions tested; either the TCS is expressed under normal growth conditions or the conditions that activate the TCS are known, in which case the WT and the mutant strains are examined under normal and activating conditions. This deletion approach cannot be used for essential TCS genes.

Alternately, constructs overexpressing the RR gene may also be used to compare gene expression changes with that of WT. For example, Kaihami *et al*. examined target genes for an atypical RR by overexpressing it (Kaihami *et al*., 2017). An overexpression construct may be a better choice when the activating conditions for the TCS are unknown. The rationale behind this is that an overexpressed RR will not need its activating signal to exercise gene expression changes. However, studies that examined both deletion and overexpression mutants have found that deletion mutants give more reliable results, whereas overexpression mutants can have effects that are not physiologically relevant. For example, a study that compared constitutively-on and constitutively-off mutants in the LiaRS TCS system in *B. subtilis* found that the targets identified by the analysis of the constitutively-on mutant (where the repressor for the TCS was deleted) had weak binding sites for LiaR and were not physiologically relevant (Wolf *et al*., 2010). In some cases, overexpression of an RR has little effect on gene expression because its native cognate HK gene serves to inhibit the RR or act as a phosphatase to deactivate the RR in the absence of the activating signal (Ogura *et al*., 2001). Instead, overexpressing the RR in the background of the HK-RR deletion mutant can modulate the expression of the target genes in the absence of the inducing conditions (Kobayashi *et al*., 2001; Ogura *et al*., 2001). This approach helped to determine the target genes for 24 TCSs in *B. subtilis*, gain valuable insights into the functions for previously uncharacterized TCSs and discover interconnected networks (Kobayashi *et al*., 2001).

Transcriptomics analysis can inform whether the RR of interest functions as an activator, repressor or both. However, RRs often affect the expression of other transcription factors (TFs); such transcriptional cascades can lead to observation of indirect effects of the RR deletion/overexpression.

Target mapping by proteomics

Similar to the transcriptomics approach, targets for RRs may be identified by measuring protein abundances between WT and mutant strains. Proteomics studies are often accompanied by transcriptomics, metabolomics or ChIP-seq analyses (Wang *et al*., 2016, 2018; Sepulveda and Lupas, 2017; Sun *et al*., 2017; Reed *et al*., 2018).

Binding site mapping techniques

In vivo methods

ChIP-seq. Chromatin Immunoprecipitation (ChIP) combined with sequencing has been used to determine the in vivo binding sites for a large number of TCS RRs. (Wang *et al*., 2016; Pelliciari *et al*., 2017; Fishman *et al*., 2018; Fu *et al*., 2019). In contrast to transcriptomics analysis, which indirectly hypothesizes targets of transcriptionally active RRs, ChIP-seq directly detects the DNA-binding targets through protein–DNA interactions (Fig. 1). Bioinformatic analysis of the ChIP-seq output can then be used to identify the binding site motif that determines the specific target of protein–DNA interaction. Electrophoretic mobility shift assays and/or DNase I footprinting assays are often carried out to validate the binding site motif, and qRT-PCR assays may be used to verify changes in gene expression (Pelliciari *et al*., 2017; Fu *et al*., 2019).

A few points to be considered while designing a ChIP-seq experiment are as follows and have also been

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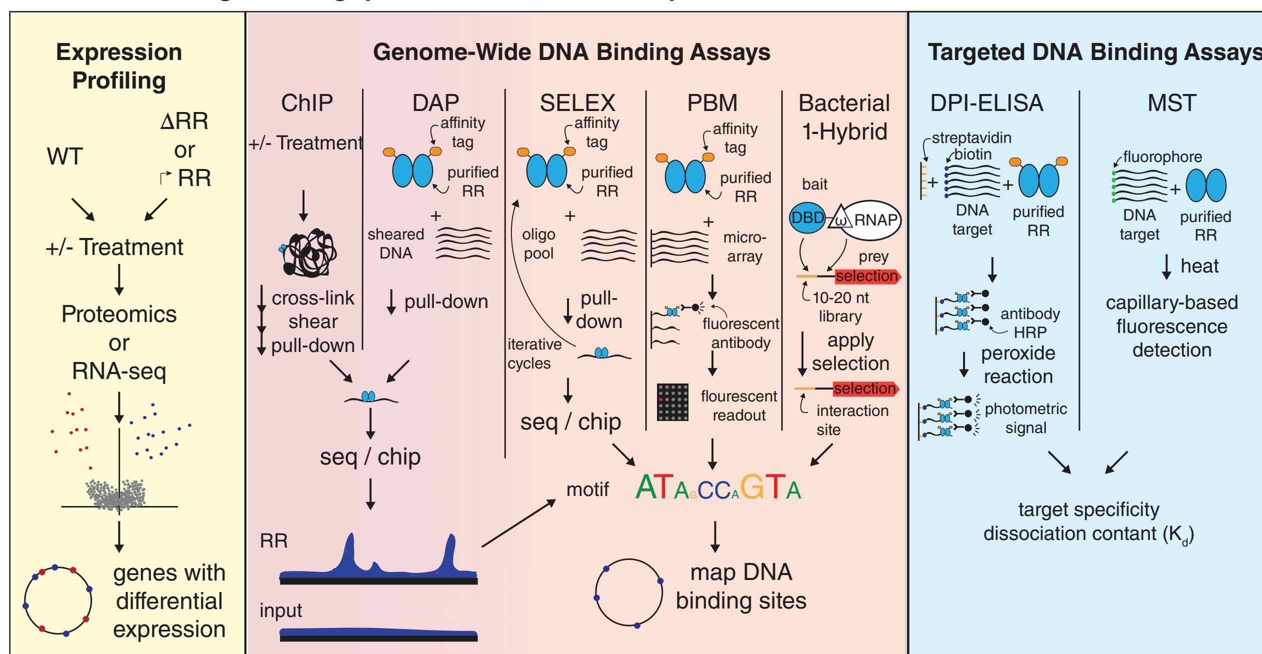

Fig. 1. High-throughput methods for the evaluation of transcriptionally acting TCSs. There are many methods to query genome-wide DNA-binding targets directly. In ChIP-seq or ChIP-chip, the cells are grown under the appropriate conditions, the RR is cross-linked to the DNA and then affinity-purified or immune-precipitated. RR-bound DNA is identified by hybridization to a chip or sequencing. In DAP, purified and tagged RR is mixed with sheared genomic DNA, the RR-bound DNA is affinity-purified and the binding sites are identified by sequencing or hybridization to a chip. In SELEX, the tagged and purified RR may be mixed with either genomic DNA fragments or a synthetic oligonucleotide pool, and the RR-bound DNA is subjected to iterative cycles of selection, before sequencing or hybridization to a chip. In a PBM, the tagged and purified RR is bound to an oligonucleotide microarray, and RR-bound spots are identified by fluorescent anti-tag probes. High-throughput methods for targeted DNA-binding assays include the DPI-ELISA and MST. In DPI-ELISA, biotin-labelled DNA are bound to streptavidin-coated plates, the tagged and purified RR is allowed to bind and the bound RR is detected by anti-tag antibody conjugated to a peroxidase enzyme that produces a photometric signal. In MST, the DNA target is fluorescently labelled and mixed with the purified RR, and the movement of the molecules during heating are measured in capillaries.

described in earlier reviews (Myers *et al*., 2015). First, the quality of the ChIP-seq data depends on the quality of the antibody. Antibodies can be raised to the RR in question, or commercial antibodies that recognize affinity tags can be used against tagged proteins. For essential TCS genes that do not allow modifications or modulations, raising antibodies to the RR itself may be the optimal method (Pelliciari *et al*., 2017). Antibodies to the RR should not cross-react to other proteins in the bacterium. Commercial antibodies against affinity tags bypass the cross-reactivity problem, but they require a genetically tractable organism that permits either the gene encoding the RR to be chromosomally tagged or the RR/TCS to be expressed on a plasmid. Second, for a successful ChIP experiment, the TCS needs to be expressed and active under the growth conditions used. Alternately, the RR may be overexpressed, which can override the requirement for activating conditions (see Zhou *et al*., 2015, for an example of ChIP-seq with overexpressed RR in a background strain where the HK is deleted). An overexpressed RR may also bind to weaker affinity binding sites that are not physiologically relevant, as discussed above. Nevertheless, overexpressed RRs have been used effectively to elucidate physiologically relevant RR targets. The regulons for 80% of *Mycobacterium tuberculosis* TFs – 154 TFs, including some RRs – were determined by ChIP-seq with overexpressed proteins (Galagan *et al*., 2013b; Minch *et al*., 2015). For some TFs, including the RR DosR, the authors found that the ChIP-seq data with overexpressed RR agreed well with the ChIP-seq data from WT strain with native RR levels (Galagan *et al*., 2013b). Since the TF genes were expressed under an inducible promoter, they also measured the mRNA levels of the TFs under different inducer concentrations and found that they were comparable to the mRNA levels seen under physiological inducing conditions; thus, the authors argued that for most of their TFs, the observed binding sites were physiologically relevant. With the highest inducer concentrations, weaker affinity sites were observed in addition to strong binding sites; however, parallel transcriptomics analysis showed that many of the weaker sites also showed regulatory effects (Galagan *et al*., 2013b). This large-scale ChIP-seq revealed several interconnected regulatory networks and binding site motifs for more than 50 TFs (Minch *et al*., 2015).

ChIP-seq offers the advantage of detecting direct binding events, unlike transcriptomics analysis, where indirect transcriptional effects are also observed. However, motif detection in ChIP-seq may be complicated if the RR interacts

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with other TFs, thus pulling down DNA fragments with multiple TF motifs (Wolberger, 1999; Bailey and Machanick, 2012).

Combining ChIP-seq with expression profiling. Many recent studies carry out both RNA-seq and ChIP-seq analyses and compare the results (Minch *et al*., 2015; Fishman *et al*., 2018; Fu *et al*., 2019). These combination studies help to reveal if the RR is activating or repressing the genes at the detected binding sites. ChIP-seq typically results in a large number of hits which may be narrowed down to define a more reliable regulon by also examining which of the hits showed regulatory effects by RNA-seq or which of the hits have a binding site motif (Bielecki *et al*., 2015). Quite often, not all ChIP-seq targets will have regulatory effects by RNA-seq. Sometimes, ChIP-seq targets may show differential expression when examined closely by reporter gene fusions rather than by RNA-seq (Fishman *et al*., 2018). For essential RRs, RNA-seq with deletion mutants are not possible, but ChIP-seq data may provide clues as to the function of the TCS and the conditions under which it is active, and RNA-seq analysis may be then performed under the suggested conditions to validate the functional predictions obtained by ChIP-seq (Pelliciari *et al*., 2017).

Bacterial one-hybrid screen. The bacterial one-hybrid screen is an in vivo heterologous genetic screen that consists of two vectors (Meng *et al*., 2005; Meng and Wolfe, 2006) (Fig. 1). One is the 'bait' vector where the protein of interest (usually only the DNA-binding domain) is expressed as a chimera fused to the ω subunit of RNA polymerase. The other is the 'prey' vector, which consists of a library of randomized DNA sequences 18–28 bp in length cloned upstream of the *his3-ura3* yeast genes. These two vectors are transformed into an *E. coli* expression strain that is deleted for the *his3* and *pyrF* genes and the ω subunit of RNA polymerase. The *his3-ura3* genes are transcribed from the prey vector only if the chimeric RR recognizes the upstream DNA sequence. The *ura3* gene allows for counterselection against self-activating sequences that allow expression in the absence of RR binding. The *his3* allows selection for RR-bound sequences. A binding site motif can be determined from the sequences obtained and then the bacterial genome can be scanned for the motif to identify target promoters (Meng *et al*., 2005; Meng and Wolfe, 2006). The bacterial one-hybrid screen has been successfully used to determine the regulon for a few RRs (Tomljenovic-Berube *et al*., 2010; An *et al*., 2014; Svensson *et al*., 2015; Hebdon *et al*., 2018). This screen can also be used to determine the TFs that bind to a particular promoter of interest, as was done for *M. tuberculosis* (Guo *et al*., 2009). The main advantage of the bacterial one-hybrid screen over the other in vitro methods described below is that the proteins do not have to be enriched or purified.

In vitro methods

In vitro assays that map binding sites, such as DAP-seq, SELEX/genomic SELEX and protein-binding microarrays (PBM) described below, can circumvent the limitations of in vivo methods. For insights into context-dependent protein–DNA interactions, ChIP-seq experiments must be performed under conditions in which the TCS is active, requiring prior knowledge of the inducing signals/conditions. In in vitro assays, no prior knowledge of activating conditions is required, making these tools very attractive for studying uncharacterized TCSs. To simulate activation, a heterologously expressed full-length RR may be activated in vitro by phosphorylation – by its cognate HK (if available) or small-molecule phosphate donors such as acetyl phosphate (Da Re *et al*., 1999). For many RRs, phosphorylation enhances DNA binding (Schaaf and Bott, 2007; Barbieri *et al*., 2013). An alternative to activation by phosphorylation is to generate phosphorylation mimics that are constitutively active. For example, treatment with beryllium fluoride creates a phosphorylation mimic (Yan *et al*., 1999). In some RRs, substitution of the Asp residue at the phosphorylation site with a Glu generates a constitutively active RR (Klose *et al*., 1993; Lan and Igo, 1998). However, this approach is not universally applicable because there are examples of RRs where the Asp-Glu substitution results in an inactive RR (Pazour *et al*., 1992; Webber and Kadner, 1997). There are also instances where the Asp-Glu mutant retains a fraction of the activity of the WT, and such mutants can be useful in generating insights on the relationship between phosphorylation and regulation of different targets (Horstmann *et al*., 2017). The in vitro assays may be performed with and without activation to determine the effect of phosphorylation on DNA binding. Alternatively, experiments employing truncated RRs with only DNA-binding domains may avoid the need for activation altogether; however, such experiments are further removed from the native context, and conclusions should be drawn with caution.

In vitro assays can be implemented in high-throughput and are amenable to automation. They also allow determination of binding site affinities, which are difficult to measure with ChIP-seq because of binding limitations introduced by interfering proteins in vivo. While ChIP-seq could pull down DNA fragments that are bound by secondary proteins that interact with the RR under study, in vitro experiments only identify direct binding events. Most importantly, in vitro binding experiments neither require the organism of interest to be genetically tractable nor any antibodies to be raised against the RR of interest. Eliminating these requirements makes in vitro methods the most implementable for non-model and environmental microbes, a large number of which have no genetics or even cultivation conditions in place. Environmental microbes also have complex signalling inputs that would be challenging to implement as would be required in in vivo assays.

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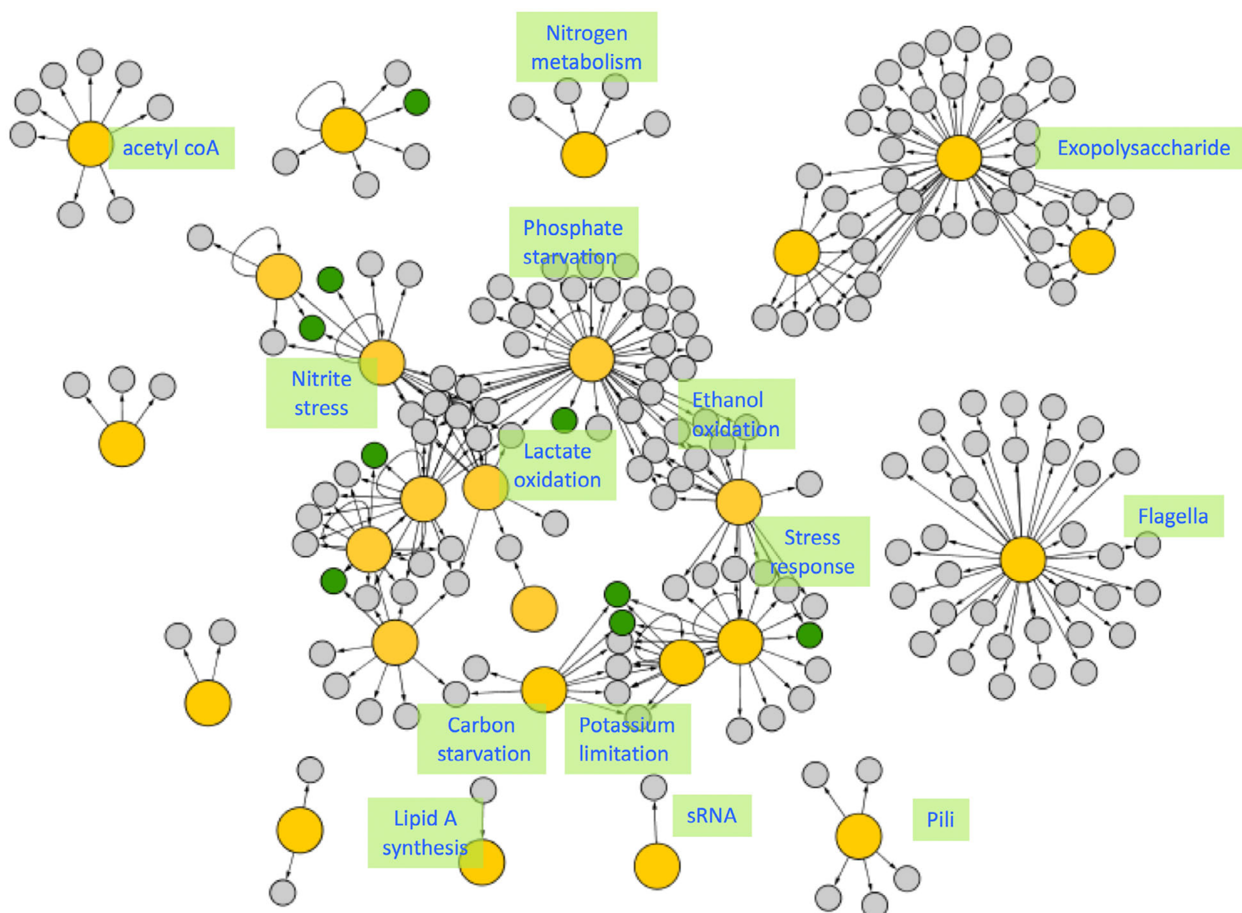

Fig. 2. Illustration of the DAP-chip results of multiple transcriptionally acting TCSs in *Desulfovibrio vulgaris*. Yellow circles represent the RR gene, green circles represent the cognate HK gene and grey circles represent all other target genes. Arrows represent regulatory interactions. Of the 29 RRs with a DNA-binding domain, DAP-chip analysis revealed target genes for 24 of them. Based on functions of the target genes, the functions for some of the TCSs could be predicted as shown in the green boxes. This analysis identified the TCSs involved in lactate and ethanol oxidation, nitrogen metabolism, general stress response, responses to carbon, phosphate and potassium starvation, nitrite stress, lipid A and acetyl-coA metabolism, flagella and pili synthesis, exopolysaccharide synthesis and regulation of small RNAs.

The main limitation of the in vitro methods is that they do not provide a cellular context. This lack of a cellular environment may limit the binding sites determined if a particular RR requires other in vivo interactions or specific cellular conditions for binding. in vitro assays require heterologously expressed proteins, which can pose challenges that have been addressed in a few new approaches. Ionic strength can affect both the affinity and specificity of DNA–protein interactions (Leirmo *et al*., 1987; Richey *et al*., 1987). While designing in vitro experiments to determine binding sites, the ionic strength of the reaction may need to be optimized to allow for selection of only specific binding sites.

DNA-Affinity-Purified (DAP). In this approach, affinity-tagged and purified RR is mixed with sheared genomic DNA from the native organism. RR-bound DNA is then affinity-purified, and the sequences are determined either by labelling and hybridization to a tiling microarray [DNA-affinity-purified (DAP)-chip] or by preparing libraries for next-generation sequencing (DAP-seq; Fig. 1). The DAP-chip assay was originally described for determining binding sites for a yeast TF (Liu *et al*., 2005; Gossett and Lieb, 2008). DAP lends itself particularly well to bacterial TCS, especially in non-model environmental strains which often contain a large of number of these signalling proteins (Galperin *et al*., 2010) but where there is little or no knowledge of the activating conditions. DAP-chip was adapted for bacterial RRs and used to systematically analyse and determine the target genes for the majority of transcriptionally acting RRs for a key sulfate-reducing microbe, *Desulfovibrio vulgaris* Hildenborough. Specifically, 24 of the 29 previously uncharacterized DNA-binding RRs in *D. vulgaris* were examined in a single study (Rajeev *et al*., 2011, 2014). Identifying the target genes also led to functional predictions and binding site motif determinations for many RRs, most of which represent novel motifs. Most importantly, the data also revealed several interconnected TCS networks that would have been otherwise missed in individual studies of TCSs (Rajeev *et al*., 2011; Fig. 2).

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Combining DAP with next-generation sequencing (DAP-seq) enables high-throughput analysis of large numbers of TFs. The steps involved in DAP and library preparation are fully automatable, potentially allowing very rapid elucidation of RR networks. The binding site motifs and targets for 529 TFs in Arabidopsis (O'Malley *et al*., 2016), 14 TFs in maize (Galli *et al*., 2018) and MAP-kinase pathways in *Neurospora crassa* (Fischer *et al*., 2018) were determined with DAP-seq assays. In some of these studies, the challenges associated with heterologous expression and purification of the TF protein have been addressed by employing in vitro translations, which further assists in the automation of this approach. Among bacterial TCSs, DAP-seq has been applied to map the target genes for metal-responsive TCSs in *Pseudomonas stutzeri*, enabling the discovery of co-regulated networks (Garber *et al*., 2018), and to determine the targets for the RR NarP of *Actinobacillus pleuropneumoniae* (Zhang *et al*., 2019). Application of DAP-seq to more than one RR often reveals aspects of signal integration. In the environmental denitrifying microbe *P. stutzeri*, the overlap in the regulons of copper- and zinc-responsive RRs was revealed through DAP-seq and may have remain hidden when examining only single RRs of interest (Garber *et al*., 2018).

Systematic evolution of ligands by exponential enrichment (SELEX) and Genomic SELEX. With SELEX, preferred binding sequences can be rapidly selected from a pool of random sequences. Multiple rounds of selection exponentially increase the selection of the best binding sequences (Fig. 1; Tuerk and Gold, 1990). SELEX and genomic SELEX differ only in their choice of DNA – SELEX uses a pool of synthetic randomized oligonucleotides, whereas genomic SELEX uses genomic DNA fragments (200–300 bp) providing the advantage of genomic context (Shimada *et al*., 2005). A purified and affinity-tagged RR is allowed to bind the DNA, the protein-bound DNA is then separated by affinity purification and is amplified and the selected sequences are subsequently used for another round of SELEX. The SELEX cycle may be repeated two to eight times to enrich the DNA; with each successive cycle, only the sites with the highest binding affinity are retained. The DNA-binding site sequence is identified either by cloning and sequencing the fragments or by hybridization to a tiling array (Shimada *et al*., 2005). A few examples where SELEX was used to identify the consensus binding site for RRs: PhoP from *M. tuberculosis* (He and Wang, 2014), RegR from *Bradyrhizobium japonicum* (Emmerich *et al*., 2000), HemR from *Leptospira* (Morero *et al*., 2014) and AlgB from *Pseudomonas aeruginosa* (Leech *et al*., 2008).

In recent years, the genomic SELEX screening has been extensively used to determine the gene targets and binding sites for ~200 TFs in *E. coli*, including several TCS RRs (Ishihama *et al*., 2016; Shimada *et al*., 2018), and all the promoters for five sigma factors (Shimada *et al*., 2014, 2017). Examples of TCSs analysed by genome SELEX are EnvZ-OmpR (Shimada *et al*., 2015), KdpDE and TorSR (Shimada *et al*., 2018), BasSR (Ogasawara *et al*., 2012), PyrSR (Miyake *et al*., 2019) and RstBA (Ogasawara *et al*., 2007). Genomic SELEX has thus dramatically expanded the known regulatory network in *E. coli*.

Traditional SELEX may be replaced by an improved SELEX-seq that was recently described (Riley *et al*., 2014). SELEX-seq, as the name suggests, combines SELEX with next-generation sequencing, enabling large numbers of DNA sequences to be characterized by sequencing at each round of selection. SELEX-seq can thus determine binding sites with a full range of binding site affinities for a given TF, in just one to two rounds of selection. It has been applied to different eukaryotic TFs but is yet to be implemented in TCS studies.

Protein-binding microarrays. In this high-throughput method, affinity-tagged purified RR is allowed to bind to a double-stranded DNA microarray. Bound protein is detected by a fluorophore-conjugated anti-tag antibody, and the amount of protein at each DNA spot is then measured (Berger and Bulyk, 2009; Fig. 1). PBMs may be made with either synthetic DNA or with genomic DNA-derived fragments. The most commonly used array is a universal PBM that is made of 44,000 double-stranded oligo spots, each being 10 bp long. The main limitation of this method is that it uses short DNA strands, so it does not allow for the recognition of longer motifs. Examples of RRs whose binding sites were determined by PBMs include four RRs from *Burkholderia thailandensis* (Nowak-Lovato *et al*., 2012), LuxR from *Vibrio harveyi* (Pompeani *et al*., 2008) and ArcA from *Shewanella oneidensis* (Wang *et al*., 2008).

Targeted profiling methods

The above methods focus on identifying genome-wide binding sites for an RR of interest. Conversely, it can also be useful to query a promoter of interest to determine what TFs regulate its expression. Shimada *et al*., 2013 developed an in vitro *promoter-specific TF screening system* to identify the TFs that bind to a specific promoter. This screening test performs gel-shift assays with the promoter of interest and as many purified TFs as available. However, gel-shift assays are limited in scale.

An alternate assay that can examine protein–DNA binding in a high-throughput manner is the DPI-ELISA (or *DNA–protein interaction ELISA*; Fig. 1) (Brand *et al*., 2010). It utilizes a 96-well plate format where the plate is coated with streptavidin, and biotin-labelled DNA (which can be short synthetic substrates representing the binding site motif or promoter fragments) is bound to the plate. The purified tagged protein is added and allowed to bind (along with any effectors needed), and after washing unbound protein, the DNA-bound protein is detected by enzyme-conjugated antibodies that recognize the affinity tag. This assay was originally developed to study plant TFs (Brand *et al*., 2010) but has also been applied to TCS RRs (Garber *et al*., 2018). The DPI-ELISA provides quantitative information and can also be automated easily (Brand *et al*., 2013). The assay may be made faster and with less variation across laboratories by the

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use of fluorophore-conjugated proteins rather than antibodies (Fischer *et al*., 2016).

Another method that measures protein–nucleic acid interactions, with potential for high-throughput use, is *microscale thermophoresis* (MST) (Mueller *et al*., 2017). MST allows quantitative analysis of any biomolecular interaction (Fig. 1). It optically measures the movement of fluorophore-tagged molecules in temperature gradients. Such thermophoretic movements depend on the molecule size, charge and hydration shell; these factors change when a ligand is bound, so distinct thermophoresis movements are seen in the DNA bound vs. unbound state. The concentration of the labelled target molecule is kept constant, and serial dilutions of the ligand to be tested are mixed in and then the samples are loaded in capillaries (volumes of ~5 μl) and analysed by the instrument. MST has been used to study HK–RR interactions (Hörnschemeyer *et al*., 2016), binding of ligands to RRs and HKs (Correa *et al*., 2013; Rotem *et al*., 2016) and DNA-binding affinities of RRs (Davlieva *et al*., 2015; Kühne *et al*., 2016; Wang *et al*., 2016). DNA-binding affinities may be measured by MST even without isolation of the protein from the cell lysate if the protein is expressed as a fluorescent-tagged protein (Khavrutskii *et al*., 2013). MST has also been employed for high-throughput screening for ligand binding (Linke *et al*., 2016).

Concluding remarks

The tools we have discussed when applied systematically to an organism can dramatically expand the known regulatory networks – as seen with the ChIP-seq experiments in *M*. *tuberculosis* (Minch *et al*., 2015), the genomic SELEX experiments in *E*. *coli* (Ishihama *et al*., 2016), the DAP-chip experiments in *D*. *vulgaris* (Rajeev *et al*., 2011; Fig. 2) and the transcriptomics (microarray) analysis of RR overexpression strains in *B*. *subtilis* (Kobayashi *et al*., 2001). These tools are often used to study individual TCS of interest, but we propose that their application to multiple TCS and regulator proteins can lead to large-scale determination of signalling and regulator networks in a large number of bacteria. Large-scale studies often reveal surprising observations. The studies mentioned above all revealed interconnected regulatory networks and assigned functions to previously uncharacterized TCSs. In *D*. *vulgaris* Hildenborough, core carbon metabolism was found to be regulated by four TCSs (Rajeev *et al*., 2011, 2019). The *E*. *coli* studies showed that each TF binds more promoters than was previously known and that each promoter is regulated by more TFs than was previously known (Ishihama *et al*., 2016). In both *E*. *coli* and *M*. *tuberculosis*, some TF binding sites are located within operons and even within open reading frames. Only 25% of the DNA binding sites determined for *M*. *tuberculosis* were intergenic and proximal to a promoter (Galagan *et al*., 2013a). Weak binding sites were prevalent in the *M*. *tuberculosis* networks and contributed to transcriptional regulation (Galagan *et al*., 2013a). Such deep insights are made possible with the systematic use of the tools we have discussed here.

Acknowledgements

This work was part of the ENIGMA, Ecosystems and Networks Integrated with Genes and Molecular Assemblies (http://enigma.lbl.gov), a Scientific Focus Area Program at Lawrence Berkeley National Laboratory and is supported by the U.S. Department of Energy, Office of Science, Office of Biological & Environmental Research under contract number DE-AC02-05CH11231 between Lawrence Berkeley National Laboratory and the U.S. Department of Energy.

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