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To ~P or Not to ~P? Non-canonical activation by two-component response regulators

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Summary

Bacteria sense and respond to their environment through the use of two-component regulatory systems. The ability to adapt to a wide range of environmental stresses is directly related to the number of two-component systems an organism possesses. Recent advances in this area have identified numerous variations on the archetype systems that employ a sensor kinase and a response regulator. It is now evident that many orphan regulators that lack cognate kinases do not rely on phosphorylation for activation and new roles for unphosphorylated response regulators have been identified. The significance of recent findings and suggestions for further research are discussed.

Introduction

Two-component regulatory systems are fundamental signal transduction systems in bacteria and they are also found in archaea and eukarya. The first component is a sensor kinase, often a membrane protein. The sensor kinase (HK) is autophosphorylated by intracellular ATP on a conserved histidine residue. Recent studies suggest that although many HKs are membrane proteins, they may be responding to cytoplasmic signals (Wang *et al*., 2012; Foo *et al*., 2015). Upon activation, a phosphoryl group is transferred from the HK to the N-terminal receiver domain of the second protein, a response regulator (RR). RRs are most often DNA binding proteins that activate or repress transcription upon phosphorylation at a conserved aspartate residue, although some output or effector domains have enzymatic activity or other functions. For example, RR FrzS has a C-terminal coiled-coil domain that is hypothesized to interact with a cytoskeletal motor (Mignot *et al*., 2005). In some systems, the HK can also dephosphorylate the phosphorylated RR, but this is less well-established (Kenney, 2010).

Substantial structural and biochemical characterization of RRs exists to enable a description of a series of molecular events involved in activation upon phosphorylation (see Hoch and Silhavy, 1995 for reviews). X-ray crystal structures of several receiver domains from

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homologous RRs have been solved, and they all reveal an $(\alpha/\beta)_5$ topology (summarized in Bourret, 2010). The five parallel β-strands form a hydrophobic core, surrounded by two α-helices on one side and three on the other. The receiver domains also share a group of conserved residues at the active site that are important for phosphorylation and signal propagation (Lee *et al.*, 2001). These are (see Fig. 1): an aspartate residue that is the site of phosphorylation, Asp55 in OmpR, two additional acidic residues, Asp11 and Asp12 that coordinate a divalent metal ion (usually magnesium), these three aspartates form a catalytic or acidic triad, a lysine residue that interacts with the phosphoryl group, Lys105, an aromatic residue that functions as a rotamer, Tyr102, and a side-chain hydroxyl residue, Thr83, that also interacts with the phosphoryl group. Structural and functional analysis of activated response regulators has revealed the role of these conserved residues upon activation (Zhu *et al.*, 1996; Mattison *et al.*, 2002).

In the presence of the HK or a small molecule phosphodonor such as acetyl phosphate or phosphoramidate, the threonine side-chain moves to form a charge-dipole interaction with the newly arrived phosphoryl moiety, a movement that is transmitted to the β4–α4 loop and is also tracked by the closely packed tyrosine side-chain. This ‘aromatic switch’, where the tyrosine moves from an exposed to a buried position, may be a general mechanism for intramolecular signalling in many RR proteins (Lee *et al.*, 2001), although it does not appear to be valid for NtrC (Villali *et al.*, 2014; Pontiggia *et al.*, 2015; Vanatta *et al.*, 2015). What remains less clear at present is how this conformational change is propagated to the C-terminal effector domain. Phosphorylation of the conserved aspartate residue often drives dimerization and modulation of DNA binding affinity. Many RRs can bind DNA without phosphorylation, including OmpR (Head *et al.*, 1998), PhoP (Liu and Hulett, 1997), BvgA (Zu *et al.*, 1996; Boucher *et al.*, 1997), SsrB (Desai *et al.*, 2016) etc., although phosphorylation increases the binding affinity and sometimes alters the specificity (Kenney, 2002). This minireview focuses on non-canonical activation by RRs including RRs that are not phosphorylated, lack the phosphorylated aspartate residue or possess other modifications and briefly summarizes the strategies employed. It is not intended to serve as an exhaustive review. For the purpose of this review, we refer to orphan RRs as those lacking cognate HKs, whereas pseudo-receivers or atypical RRs and non-canonical RRs are used interchangeably and indicate that either active site residues are lacking or activation is not via phosphorylation. In some cases, orphan RRs are also atypical.

RRs with distinct functions in unphosphorylated and phosphorylated states

It has generally been assumed that phosphorylated RRs represented the active state, whereas the unphosphorylated state was inactive or inert. Recent studies have challenged this view. Some RRs make strong interactions between their N- and C-terminal domains, for example, MtrA and PrrA (Nowak *et al.*, 2006; Friedland *et al.*, 2007). Perhaps the best characterized of these is NarL, where N- and C-terminal interactions were first evident in the full-length crystal structure (Baikalov et al., 1996). Phosphorylation was required to reorganize the interface and promote dimerization and DNA binding. However, SsrB, a NarL homologue, was recently shown to bind and bend DNA in the absence of phosphorylation (i.e., when its kinase was not present or in a D56A mutant) (Desai *et al.*, 2016), emphasizing that structural homology does not indicate functional homology (Fig. 2).

NarL homologue SsrB

The two-component regulatory system, SsrA/B, present on the Salmonella Pathogenicity Island-2 (SPI-2), is responsible for the expression of a type three secretory system and the virulence effectors that are crucial for the survival of *Salmonella enterica* in macrophages. Activation of SPI-2 genes requires phosphorylation of SsrB at Asp56 by the tripartite sensor kinase, SsrA (Feng *et al.*, 2004; Walthers *et al.*, 2007). We recently discovered a novel role of unphosphorylated SsrB in the formation of biofilms, a multicellular lifestyle responsible for the establishment and maintenance of *Salmonella* in the carrier state in asymptomatic patients (Desai *et al.*, 2016). In the absence of the HK SsrA, SsrB was sufficient to activate the transcription of *csgD*, which encodes for the master regulator of biofilms. In *Escherichia coli* and *Salmonella*, CsgD is required for the synthesis of cellulose and curli fimbriae that form the extracellular matrix of mature biofilms (Romling *et al.*, 1998; Zogaj *et al.*, 2001). Expression of *csgD* in a D56A SsrB mutant was similar to the wild type strain, providing the first evidence that phosphorylation of SsrB was not required for its positive role in regulating formation of biofilms. This was due to the ability of SsrB to activate *csgD* by relieving silencing by H-NS (Histone-like Nucleoid Structuring protein) (see Fig. 2). Single molecule Atomic Force Microscopy experiments showed that unphosphorylated SsrB was actually bound to the *csgD* regulatory region and introduced a bend of 82°. Further, such an SsrB-mediated conformational change in the promoter was responsible for displacing H-NS from discrete sub-regions on the DNA, enabling transcriptional activation. Unlike its subfamily member, NarL, SsrB does not require phosphorylation to bind DNA and mediate transcriptional activation via anti-silencing. However, for direct transcriptional activation of the SPI-2 genes, which requires an interaction with RNA polymerase, phosphorylation of SsrB is necessary (Walthers *et al.*, 2007). Thus in *Salmonella*, the horizontally acquired RR, SsrB, has evolved to regulate the ancestral gene *csgD*, in a manner that is completely different from its classical function of regulating pathogenicity island genes (see Figs. 2 and 3A).

NarL homologue RcsB

This same paradigm described above for unphosphorylated SsrB playing a role in activating *Salmonella* biofilms, may exist with RcsB (Fig. 3A). The RR RcsB, with the sensor kinase, RcsC, and the auxiliary regulatory protein, RcsA, form a complex phosphorelay signalling system involved in the synthesis of extracellular polysaccharide and some membrane and periplasmic proteins (see Majdalani and Gottesman, 2005 for a review). Unphosphorylated RcsB was reported to drive biofilm formation in *Salmonella* Typhimurium by activating expression of *csgD*, the master regulator (Latasa *et al.*, 2012). If RcsB functions similarly to SsrB, then it would bind to the *csgD* promoter and de-repress H-NS. However, in this study (Latasa *et al.*, 2012), no evidence of direct binding of unphosphorylated RcsB to the *csgD* regulatory region was provided, nor was there evidence provided for how unphosphorylated RcsB might mediate transcriptional activation of *csgD*. Instead, it was proposed by the authors that the presence of RscB~P leads to *csgD* repression through the production of RprA, a small regulatory RNA, leaving the open question as to what is the function of unphosphorylated RcsB?

OmpR subfamily archetype member OmpR

Recent evidence suggests that a variation on this paradigm is also relevant to OmpR-repression of genes required for neutralization during acid stress (Chakraborty *et al.*, 2015). In the macrophage vacuole, the *Salmonella* cytoplasm acidifies via an OmpR-dependent repression of the *cadC/BA* operon. Expression of the *cad* genes would normally serve to neutralize the cytoplasm in response to acidification. OmpR binding and repression does not require phosphorylation (i.e., a D55A mutant is sufficient to repress the *cadC/BA* operon), although it does require the cognate HK EnvZ or the cytoplasmic domain of EnvZ, EnvZc. The assumption is that interaction of EnvZ with OmpR is sufficient to drive OmpR dimerization and DNA binding in the absence of phosphorylation (Chakraborty *et al.*, 2016). A similar mechanism appears to function with the closely related OmpR homologue CpxR, where studies in *Legionella pneumophila* showed that a D53A CpxR mutant was no longer capable of transcriptional activation, but was still competent for repression (Feldhelm *et al.*, 2015).

NarL homologue DegU

DegU is a RR in *Bacillus subtilis* belonging to the NarL subfamily. When phosphorylated by its atypical cytoplasmic HK DegS at conserved Asp55, it plays a central role in deciding cell fate by favouring biofilm formation. In the unphosphorylated form, DegU confers an ability to acquire foreign DNA via genetic competence (Dahl *et al.*, 1992). Binding of unphosphorylated DegU enhanced auto-activation by stimulating binding of ComK to the upstream sites at *comK* (Hamoen *et al.*, 2000). This cooperative effect of DegU on ComK binding to the *comK* regulatory region did not require direct protein–protein interactions and was presumed to occur through conformational changes in DNA (mediated by DegU-driven DNA bending?) (Hamoen *et al.*, 2000). Thus, as with SsrB and OmpR, the unphosphorylated and phosphorylated forms of DegU control distinct pathways (Fig. 3A). However, precise experiments involving single molecule (Desai *et al.*, 2016) or biochemical approaches (Will *et al.*, 2014) are lacking to determine whether DegU, like SsrB, bends the DNA to increase the affinity of ComK binding.

LytR homologue AlgR

Another example of unphosphorylated RRs having distinct regulatory roles is found in the opportunistic pathogen *Pseudomanas aeruginosa*. It is known that phosphorylation of AlgR by the HK AlgZ is required to activate the *fim* genes for twitching motility (Whitchurch *et al.*, 2002) and the *hcn* genes for hydrogen cyanide production (Cody *et al.*, 2009). AlgR~P seems to be required to bind the *fim* and *hcn* promoters. On the other hand, transcriptional activation of the *alg* operon, which is required for the synthesis of the capsular polysaccharide, alginate, is directly mediated by two unphosphorylated response regulators, AlgB and AlgR (Ma *et al.*, 1998; Leech *et al.*, 2008). *Pseudomonas* strains carrying the D59N AlgB substitution and the D54N AlgR substitution retained the ability to form alginate-rich mucoid colonies. In this case, it is possible that unphosphorylated AlgR and AlgB are capable of binding to the *algD* promoter and with the help of co-activators such as IHF bring about conformational changes that result in transcriptional activation (see Okkotsu *et al.*, 2014 for a review). IHF would then provide the DNA bending capability (Figs. 2 and

3A). However, the above predictions need to be tested experimentally using D59A AlgB or D54A AlgR strains, as de-amidation of Asn to Asp in CheY has been known to generate a wild type RR (Wolanin *et al.*, 2003).

Orphan RRs mimic an active interface

In RRs that lack a conserved phosphorylated aspartate (sometimes referred to as atypical or pseudo-receiver domains), the most common substitution is glutamate (Maule *et al.*, 2015). It remains enigmatic how some receivers are activated without phosphorylation, but many of these, including ChxR, HP1043, FrzS and AmiR are dimers in solution and appear to adopt an active conformation (Figs. 3B and 4), using a dimeric interface that is mostly similar to phosphorylated RRs (e.g., α4–β5–α5). NblR from *Synechococcus elongatus* sp PCC 7942 is a slight exception to these atypical RRs in that it retains an aspartate in the acidic pocket, but not the remaining residues required for phosphorylation (Ruiz *et al.*, 2008). Although the active pocket and the YT pair of non-canonical RRs is often reorganized, much of the canonical post-phosphorylation mechanism appears to have been retained (Maule *et al.*, 2015).

OmpR homologue HP1043

Helicobacter pylori HP1043 is an orphan RR (i.e., no HK has been identified) that is essential for growth (Beier and Frank, 2000). It is also an atypical RR. In the unphosphorylated state, it is a symmetric dimer that binds to the target *tlpB* promoter DNA (Hong *et al.*, 2007). The α4–β5–α5 dimer interface resembles that of the OmpR/PhoB subfamily (Figs. 3B and 4), although several conserved residues deviate from the typical interface (Hong *et al.*, 2007). The stable interaction interface involves electrostatic and hydrophobic interactions and buries 800 $Å^2$/monomer. Thus, the HP1043 dimer mimics the activated state, even in the absence of phosphorylation. The HP1043 dimer is structurally similar to the activated state of PhoB and ArcA (Toro-Roman *et al.*, 2005; Arribas-Bosacoma *et al.*, 2007) and the Tyr94 rotomer adopts the ‘active’ inward position. The fact that a stable dimer forms in solution probably constrains the structure, making it resolvable. This is unlike OmpR, in which the flexible linker presumably leads to crystals that diffract poorly.

An F87L mutant of HP1043, which substitutes a residue in α4 conserved in other RRs such as OmpR, is a monomer (Hong *et al.*, 2007) (see Fig. 4). Surprisingly, the monomer was capable of DNA binding. Because actual binding data was not shown (only ‘+’ or ‘−’), it is not known whether the mutant possessed altered DNA binding affinity. One would expect that the monomer would exhibit lower binding affinity than the activated dimer. In any case, the HP1043 RR regulatory domain does not inhibit DNA binding activity of the C-terminal DNA binding domain and the two domains appear to function independently, for example, few chemical shift changes in the C-terminus were observed by NMR in the presence of DNA (Hong *et al.*, 2007). This is unlike the archetype RR OmpR, for example, where even non-specific DNA resulted in many chemical shift changes (Rhee *et al.*, 2008).

OmpR homologues GlnR and RamR

The GlnR protein of *Actinomycetes* is an OmpR subfamily member that coordinates expression of genes related to nitrogen metabolism. It also forms a homodimer through the α4–β5–α5 interface and has a conserved Asp residue in the active site (Figs. 3B and 4). It is considered an orphan RR since no cognate HK has been identified. Although Asp50 is conserved, the phosphorylation pocket is not conserved and is altered by charge interactions of Asp50 with Arg52 and Thr9 (Lin *et al.*, 2014). Crystal structures of *Amycolatopsis mediterranei* GlnR or *MTb* GlnR indicated that except Asp50, conserved phosphorylation pocket residues were either not conserved or were not in the typical position. For example, conserved Mg-binding residues (OmpR residues Asp11 and Asp12, see Fig. 1) are replaced with Thr and Ala respectively. Furthermore, the 'Y-T' signalling residues Thr, Lys and Tyr (Fig. 5) are substituted with Val, Leu and Ile respectively (Lin *et al.*, 2014). The GlnRD50A mutant is a monomer by gel filtration analysis, indicating that the conserved aspartate is required for dimerization. Residues Asp50, Arg52 and Thr9 are also conserved in GlnR proteins from *Streptomyces coelicolor* and *MTb*, but are not present in Gram-negative OmpR subfamily members, suggesting a common reaction mechanism. Thus, GlnR appears to be unphosphorylated, but the aspartate is critical for homodimerization and downstream DNA binding activity.

RamR from *S. coelicolor* appears to be similar in this regard (Hudson and Nodwell, 2004; O'Connor and Nodwell, 2005). Attempts at phosphorylating the aspartate residue *in vitro* using phosphoramidate were unsuccessful and phos-tag analysis failed to demonstrate a phosphorylated GlnR from either *A. mediterranei* or *S. coelicolor* (Lin *et al.*, 2014). The difficulty with a negative result is that the phos-tag barely detects PhoP~P (Lin *et al.*, 2014) or OmpR~P (Adediran *et al.*, 2014) *in vivo*, so a low level of GlnR~P might have simply gone undetected in these studies. However, the crystal structures confirm an inadequate phosphorylation pocket (Lin *et al.*, 2014). Mutants that were unable to dimerize could not grow on minimal media in the presence of nitrate, nor were the GlnR gene targets activated, suggesting that phosphorylation-independent homodimerization was sufficient to activate GlnR (Fig. 3B).

Others

In *Myxococcus xanthus*, twitching motility is powered by type IV pili. The direction of movement is determined by the frequency of reversals along the long axis of the cell. Cell reversal correlates with RR FrzS accumulation at the new leading cell pole (Mignot *et al.*, 2005). A combination of *in vivo*, crystallographic and NMR data suggest that the FrzS receiver domain retains structural and functional features of canonical receiver domains, but does not modulate switching or signalling via aspartate phosphorylation (Fraser *et al.*, 2007). The FrzS receiver domain fold is highly similar to typical RRs, but the acidic triad (OmpR residues Asp11, 12, 55; see Fig. 5), the aspartate acid and the threonine residue in the active site are missing and the conserved Tyr102 is in an unusual conformation (Fraser *et al.*, 2007). However, the α4–β5–α5 interface was maintained, and substitutions along this interface abolished *M. xanthus* S-motility (Fraser *et al.*, 2007) (Figs. 3B and 5).

In a similar manner to FrzS, ChxR, a RR required for development in *Chlamydia trachomatis*, also appears to mimic the activated state in the absence of phosphorylation (Barta *et al.*, 2014). ChxR lacks most of the conserved active site residues, containing a glutamic acid residue instead of aspartate (Koo *et al.*, 2006), yet forms homodimers and binds to direct DNA repeats (Hickey *et al.*, 2011a, 2011b).

FruA is a LysR family member from the social bacterium, *Myxococcus xanthus*, that controls cellular responses to C signalling during development. It may also fit this new paradigm of phosphorylation-independent dimerization (Fig. 3B). It appears to be an orphan, and is missing a couple of conserved aspartate residues, although dimerization has not been demonstrated (Mittal and Kroos, 2009).

The RR circadian clock protein KaiA from *S. elongatus* is proposed to function as a timing input-device. Its N-terminus is a pseudo-receiver domain in which the interface helix α4 is replaced with an unstructured, solvent-exposed loop that may serve an interfacing role (Williams *et al.*, 2002). KaiA lacks the conserved aspartates for phosphorylation and magnesium binding (Fig. 5), these residues are instead replaced with Asn60, Glu12 and Ser13 respectively. The C-terminus of KaiA binds to KaiC, enhancing the KaiC rate of autophosphorylation (Williams *et al.*, 2002). Phosphorylation of KaiA is not required for this effect (Fig. 3C).

Another active orphan RR is the Rem protein of *S. meliloti*. Rem is in a chemotaxis pathway and affects motility. It is expressed during exponential growth (Rotter *et al.*, 2006) and is required for production of flagella. It has a glutamic acid in the active site. Potential phosphorylated aspartates Asp43, Asp45 and Asp47 were substituted with asparagine, but only combinations of multiple substitutions had an effect on swarming. The authors concluded that Rem was similar to HP1043. Dimerization was not examined, nor was the role of Glu50 in substituting for the phosphorylated Asp or a role in dimerization ever examined.

A question that arises from these discussions is what turns activated atypical receivers off? One mechanism is to only express the activated RR precisely when it is needed. For example, the RR Rem from *S. meliloti* is only expressed during exponential growth (Rotter *et al.*, 2006). It has a glutamic acid (Glu50) in place of aspartate. Tight control appears to be achieved by synthesis, but whether proteolysis might play a role in the regulation of Rem has not been examined.

Regulation by sequestration

Some RRs are regulated by removal from their site of action by sequestration. AmiR is an orphan RR in *Pseudomonas aeruginosa* that lacks all of the conserved catalytic residues of RRs, including (OmpR residues are in parenthesis): Ser59 (Asp55), Asn 19 and Pro 20 (Asp 11, 12), Val86 (Thr83) and Gln108 (Lys105) (O'Hara *et al.*, 1999). AmiR and AmiC constitute an amide-regulated transcription anti-termination system. AmiR is a coiled-coil dimer that binds RNA. AmiC binds amides at the interface of its N- and C-terminal domains (Pearl *et al.*, 1994). In the absence of inducing amides, AmiR anti-termination activity is inhibited by interaction with AmiC (Fig. 3C). Addition of an inducing amide disrupts this

complex *in vitro*. Once AmiR is liberated from AmiC, it multimerizes and binds to RNAs containing sequences upstream of an inverted repeat in the amidase promoter (Norman *et al.*, 2000). Thus, AmiR activity is controlled by sequestration by AmiC, rather than phosphorylation (O'Hara *et al.*, 1999).

A possible variation on the sequestering theme might occur during biofilm formation in *Pseudomonas aeruginosa*. The enzymatic output domain of the RR WspR~P is diguanylate cyclase activity, which is enhanced by the formation of higher order oligomers, visualized as distinct sub-cellular clusters. Cluster formation leads to synthesis of the exopolysaccharide PeI (Huangyutitham *et al.*, 2013). WspR is canonical in that phosphorylation at Asp70 by the HK WspA is required to synthesize the second messenger, cyclic-di-GMP, and in turn, PeI. Binding of cyclic-di-GMP to the active WspR~P tetramer turns off its enzymatic activity. The question that arises is whether oligomerization of WspR~P tetramers affects the binding of cyclic-di-GMP, thus preventing auto-inhibition? This remains to be determined.

Heterodimer formation

Both typical and atypical RRs are capable of heterodimer formation. In this section, we highlight a few examples.

BldM/WhiI—Two RRs belonging to the FixJ/NarL subfamily in *Streptomyces venezuelae* form heterodimers to activate expression of group-II genes in the late stages of development (Al-Bassam *et al.*, 2014). BldM and WhiI are orphan RRs that are also atypical, and heterodimer formation is an alternate way of regulating their DNA-binding activities. A BldM homodimer binds to group-I genes, whereas heterodimers of BldM/WhiI bind and activate group-II genes. WhiI alone does not activate transcription of group-II genes. Structural characterization of the BldM/WhiI heterodimers is lacking, and this would provide crucial information toward an understanding of the differences in activation and specificity mechanisms of heterodimers versus homodimers (Fig. 3B).

RcsB/BglJ and RcsB/GadE?—An example of heterodimerization of RRs occurs in the Gram-negative prototype, *E. coli*. The RR RcsB is part of a complex phosphorelay signalling system involved in the synthesis of extracellular polysaccharide and some membrane and periplasmic proteins (see above). In addition, unphosphorylated RcsB heterodimerizes with BglJ to activate the cryptic *bgl* operon by relieving H-NS silencing (Venkatesh *et al.*, 2010) (Figs. 2 and 3A,B). RcsB/GadE heterodimers might also be activating the acid-responsive *gadA* promoter by anti-silencing (Castanie-Cornet *et al.*, 2010). In contrast, RcsB homodimers activate environmentally-sensitive genes such as *rpoS*, *osmB* and *osmC* (Majdalani and Gottesman, 2005). Thus, homo and heterodimers of RRs can have different specificities.

Regulation by other covalent modifications

Post-translational modification of a RR at its conserved aspartate either by its cognate HK or small molecule phosphodonors such as acetyl phosphate modulates the activity of most RRs. However, to date, two additional covalent modifications have been discovered that also contribute to regulation of RR output activity.

Acetylation—In *E. coli*, the intracellular activity of RcsB is modified post-translationally by phosphorylation at Asp56, as well as by acetylation at Lys180 (Hu *et al.*, 2013). Acetylation of Lys180 inhibited the ability of RcsB to bind to the *rprA* regulatory region, encoding a small regulatory RNA (Hu *et al.*, 2013). However, a mutation in *yfiQ*, encoding the only known acetyl transferase in *E. coli*, did not abolish RcsB acetylation, suggesting perhaps a direct interaction of RcsB with acetyl-CoA. Although the precise mechanism by which acetylated RcsB controls gene expression is not known, it is interesting to speculate that acetylation of RcsB might prevent phosphoryl transfer from the RcsD histidyl phosphotransfer protein (HPT). It has not been established whether acetylation of the other six Lys residues in RcsB also affects its gene regulation functions (Hu *et al.*, 2013).

Serine/threonine phosphorylation—Signal transduction pathways based on Serine/Threonine phosphorylation in Gram-positive bacteria show convergence with eukaryotic signalling pathways and also variable evolution of two-component regulatory systems across different phyla. The RR CovR in Group B *Streptococci* is a classic example where aspartate or threonine phosphorylation has opposing effects on its gene regulatory ability (Lin *et al.*, 2009). Phosphorylation of CovR at Asp53 by the sensor HK CovS was required for DNA binding and activation of toxin gene expression. Phosphorylation of CovR at Thr65 by activation of the lone eukaryotic Ser/Thr kinase (eSTK) Stk1 drastically reduced its ability to bind DNA and activate gene expression, that is, phosphorylation at Thr65 of CovS reduced phosphorylation at Asp53. Reciprocally, Asp53 phosphorylation of CovR also reduced Stk1-driven Thr65 phosphorylation (Lin *et al.*, 2009). Allosteric regulation of CovR activity by differential phosphorylation at Asp and Thr residues was also observed in Group A *Streptococcus* (Horstmann *et al.*, 2014).

In contrast, in *Bacillus subtilis*, the activity of RR WalR is influenced by two separate classes of membrane-bound kinases, the cognate HK WalK, and the eSTK, PrkC (Libby *et al.*, 2015). The WalR/K two-component system is essential and controls cell wall metabolism. Phosphorylation at Asp53 or Thr101 had similar effects on the downstream expression of WalR regulon genes. In the exponential growth phase, WalK phosphorylated WalR at the conserved Asp53, resulting in activation or repression of the WalR regulon genes involved in cell wall metabolism. However, when cells entered stationary phase, PrkC-dependent phosphorylation at Thr101 influenced the regulatory role of WalR (presumably as WalK activity was low). It is still not clear how the different forms of WalR~P compare in their ability to bind DNA or undergo conformational changes.

Small ligand binding

In the antibiotic-producing species *Streptomycetes venezuelae* (ISP5230), production of the broad-spectrum jadomycins is determined by a large gene cluster containing a regulatory gene specific for the pathway. JadR1 is an orphan RR in the OmpR subfamily and lacks the two N-terminal catalytic triad aspartate residues required for metal binding (replaced with Glu49 and Ser50). Instead, ligands of the late biosynthetic products, including jadomycin B (JdB) and aglycone (JdA), bind to the N-terminus of JadR1 (Fig. 3C). JdA binding enhanced JadR1 activity, whereas JdB binding inhibited its DNA binding activity (Wang *et al.*, 2009). In another antibiotic pathway of *S. coelicolor*, the NarL homologue RedZ is also an orphan

RR that is modified by undecylprodigisines. It lacks two aspartates for metal binding and the lysine of the conserved phosphorylation pocket (Guthrie *et al.*, 1998). Thus, end product-mediated control of orphan RRs (through ligand binding) may be a widespread mechanism of regulation in antibiotic-producing bacteria, replacing or in some cases augmenting phosphorylation.

Final remarks

In summary, with more available structures and new assays and approaches for studying two-component regulatory systems, it is evident that there are many variations on the canonical theme. We are just beginning to appreciate that unphosphorylated RRs can evoke responses in pathways that are distinct from phosphorylated RRs and we presented some ways in which this has been shown to occur. Other orphan RRs can function without phosphorylation by mimicking an activated state, often via dimerization using the α4–β5–α5 interface.

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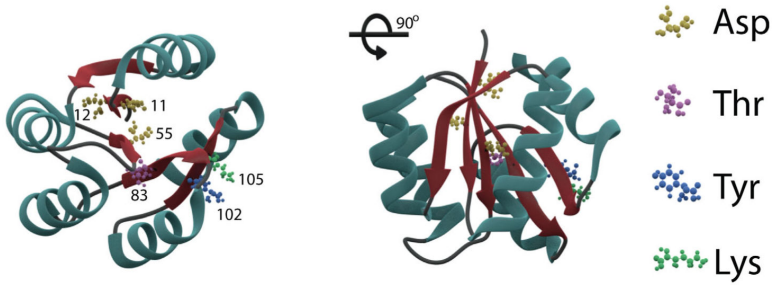

Fig. 1.

Conserved residues required for RR phosphorylation. The N-terminal receiver domain of OmpR is represented modelled on the CheY structure (PDB 4qyw). The side chains of conserved residues are depicted including: aspartate 11 and 12, aspartate 55 (site of phosphorylation), threonine 83, lysine 102 and tyrosine 105. The side chain of tyrosine 105 exists in both outward (depicted here) and inward positions. Alpha helices are illustrated in cyan, beta strands in red and loops are in grey.

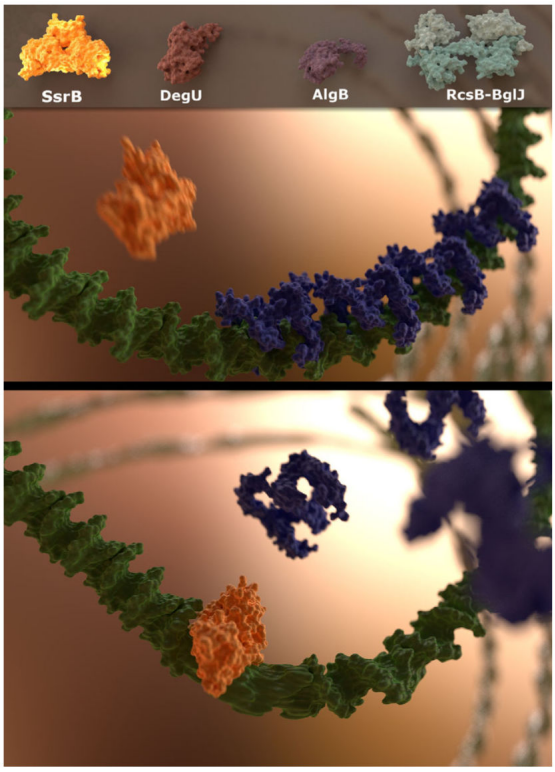

Fig. 2.

Unphosphorylated response regulators bind DNA and bend DNA to de-repress H-NS and relieve gene silencing. The upper panel shows a three dimensional reconstruction of SsrB (yellow) binding to the *csgD* (green) regulatory sequence (for details see Desai *et al.*, 2016) on which the repressor, H-NS (blue), has formed a rigid nucleoprotein complex. Binding of SsrB introduces a bend in the *csgD* regulatory sequence, resulting in destabilization of the H-NS filament and a relief of transcriptional silencing, as shown in the lower panel. We propose that binding of other SsrB homologues (DegU, AlgR and RcsB-BglJ) to upstream gene sequences, may also lead to similar conformational changes and transcriptional activation by anti-silencing.

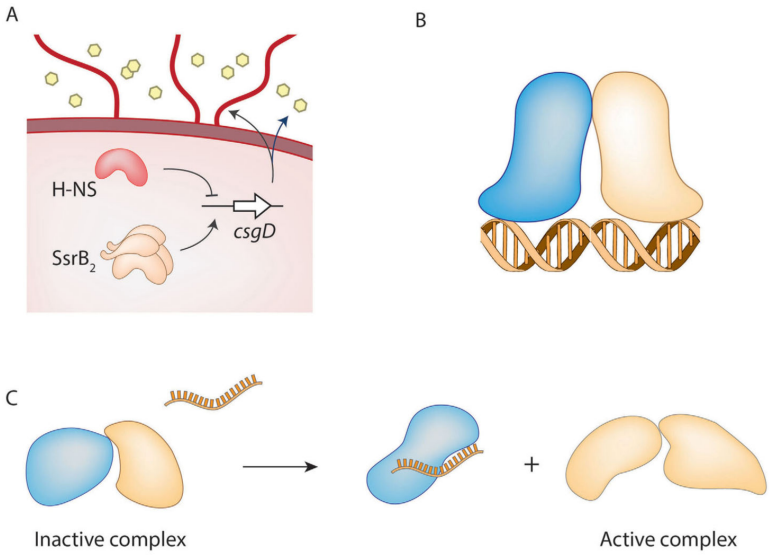

Fig. 3.

Strategies employed by RRs that do not involve phosphorylation.

A. Binding and bending DNA de-represses H-NS by driving it off the DNA or remodelling how it interacts with DNA. For example, unphosphorylated SsrB binds and bends the DNA and that relieves H-NS silencing at *csgD*.

B. Dimerization creates an active interface that is capable of DNA binding. This can involve homo- or hetero-dimerization.

C. RR sequestration via protection of the active surface (preventing dimerization) by small molecule binding or protein binding. A modification of this theme in the case of KaiA/KaiC involves KaiA protein binding to KaiC, stimulating KaiC autophosphorylation. For AmiR, AmiC binds and prevents AmiR interaction with RNA. Peptide binding to AmiC releases AmiR, allowing AmiR dimerization and activation.

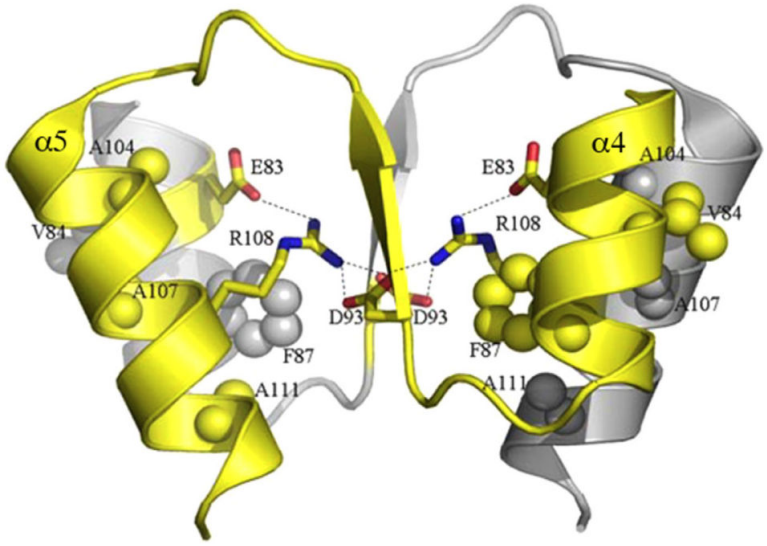

Fig. 4.

Structure of an active HP1043 RR dimer. Electrostatic and hydrophobic interactions of the dimeric interface are shown. A network of ionic interactions is formed between Glu83 (α4), Asp93 (β5) and Arg108 (α5). The core interactions of the hydrophobic patch consisting of Val84 (α4), Phe87 (α4), Ala104 (α5), Ala107 (α5) and Ala111 (α5) are also shown between the dimeric interface. Reprinted with permission from Hong *et al.*, 2007).

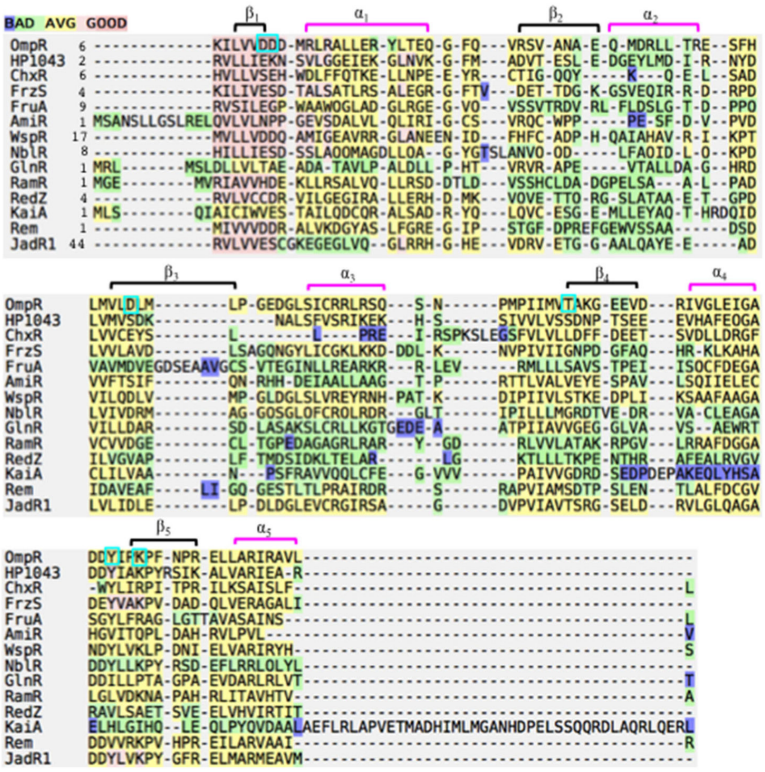

Fig. 5.

Alignment of RRs, highlighting the absence of key catalytic residues. Sequences of the N-terminal receiver domains of RRs or the first 120 amino acids in the case of AmiR, GlnR and RamR, were obtained from the UniProtKB database (www.uniprot.org/uniprot/) or the NCBI Gene database (http://www.ncbi.nlm.nih.gov/). Alignment of these sequences using the software Expresso (tcoffee.crg.cat) is shown. The ‘Expresso’ colour codes indicate the consistency in pairwise structural alignments. The group of conserved residues in the receiver domain of OmpR are boxed in turquoise, while the $(\alpha/\beta)_5$ topology is shown as black brackets for β-strands and magenta brackets for α-helices on top of the OmpR sequence.