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## Review

# The molecular puzzle of two-component signaling cascades

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**ABSTRACT** – Two-component systems constitute prevalent signaling pathways in bacteria and mediate a large variety of adaptative cellular responses. Signaling proceeds through His-Asp phosphorelay cascades that involve two central partners, the histidine protein kinase and the response regulator protein. Structural studies have provided insights into some design principles and activation mechanisms of these multi-domain proteins implicated in the control of virulence gene expression in several pathogens. © 2001 Éditions scientifiques et médicales Elsevier SAS

two-component systems / signal transduction / functional domains

## 1. Introduction

Efficient signaling cascades are necessary for microorganisms in order to face limited resources and survive in any local environment. Microorganisms must be masters at accommodating potential sources of carbon, nitrogen or energy, at resisting poisons of their metabolic and regulatory processes and at establishing intra- and inter-species communications. This adaptation requires that the organism senses the multitude of extracellular signals and responds, in most instances, by controlling the expression of an adequate repertoire of genes.

The study of sensory–response systems has firmly established the ‘two-component paradigm’ as prevalent signaling cascades in bacteria [1]. These pathways involve phosphorylation of two key effector proteins. The modular sensor histidine-kinase is the primary signal transduction protein, and detection of the signal via the input domain(s) controls either the inhibition or the activation of the kinase. Active kinases hydrolyse ATP and autophosphorylate on a histidine residue (*figure 1*). Each sensor kinase has a cognate response regulator (*figure 2*). Phosphotransfer from the phospho-histidine to an aspartate residue in the receiver domain of the response regulator activates the protein and the cellular responses.

The modular organization of the sensors and of the response regulators is a key feature of two-component systems. The variety of input and output functional domains and their arrangements in different configura-

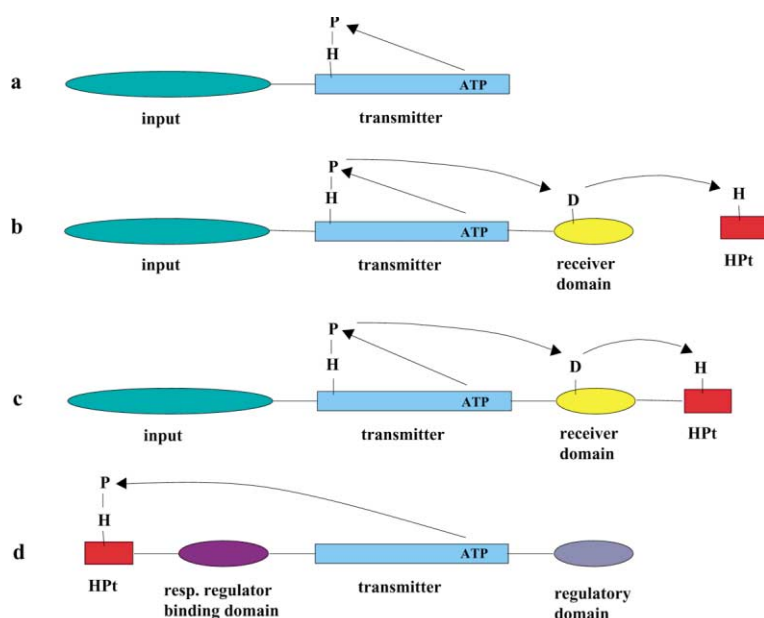
tions built many types of phosphorelay circuits. In the most sophisticated cases, activation occurs through multistep His-Asp phosphorelay cascades [2–4]. In several instances, additional protein partners are involved that contribute to the control of the phosphorylation state of the response regulator, the ‘on-off’ switch of the biological response. This diversity reflects the specific localizations, functions and regulatory mechanisms of two-component systems in the cell.

The understanding of the signaling cascades raises challenging questions. How do essential pathways have common mechanisms of chemical activation and avoid cross-talk? Could the diversity of phosphorelay circuits be rationalized in terms of a puzzle of functional modules? How are individual functions modulated by protein–protein interactions? What does activation by phosphorylation mean? Recent structural approaches provide some insights into these questions.

## 2. Organization and functional modules in histidine protein kinases

Kinases operate as homodimers, and two classes of enzymes have been defined, based on the organization of their functional domains (*figure 1*). Class I histidine kinases are the most commonly encountered. Canonical constructs typically contain a variable N-terminal sensing region which can be more than 500 residues long and a C-terminal core region, also called the transmitter domain. This 250-residue long region exhibits sequence fingerprints that are generally conserved in the histidine protein kinase superfamily. These homology boxes have been

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**Figure 1.** Modular organization of the histidine kinases. In class I enzymes (a, b, c), input domains are of variable length and sense a variety of signals. In most cases, they display putative transmembrane regions. The transmitter core-domain is conserved in the superfamily of histidine kinases. Hybrid kinases (b, c) contain a phosphorylatable receiver module and in some cases, a phosphotransfer unit. Class II (CheA) histidine kinases (d) are involved in chemotaxis and coupled to membrane receptors.

termed the H, N, G1, F and G2 boxes. Hybrid kinases derive from this canonical type and display a phospho-accepting domain and a histidine phosphotransfer (HPT) domain fused at the C-terminal end. In some cascades, this HPT domain is distinct from the kinase and constitutes an isolated module.

Class II (CheA) histidine kinases are specialized in chemotaxis responses (*figure 1*). They contain well-characterized domains linked by flexible polypeptides of variable lengths. In these proteins, the phospho-accepting

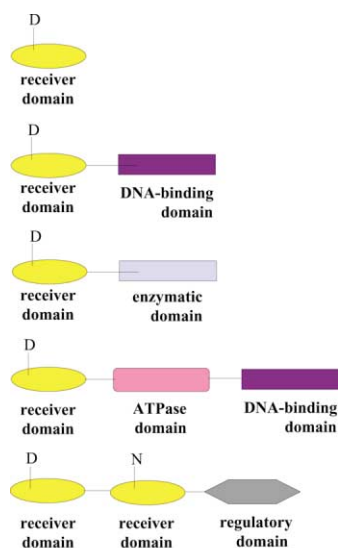
histidine residue (H box) is located at the N terminus of the protein, in the so-called P1 domain. The next module in these kinases, the P2 domain, is dedicated to the recognition of the cognate response regulators. It is followed by the transmitter domain that only carries the N, G1, F and G2 boxes. The C-terminal region of these kinases is involved in the regulation of the autokinase activity through the coupling to the transmembrane receptor-transducer proteins and to CheW [5, 6].

### 2.1. The H box

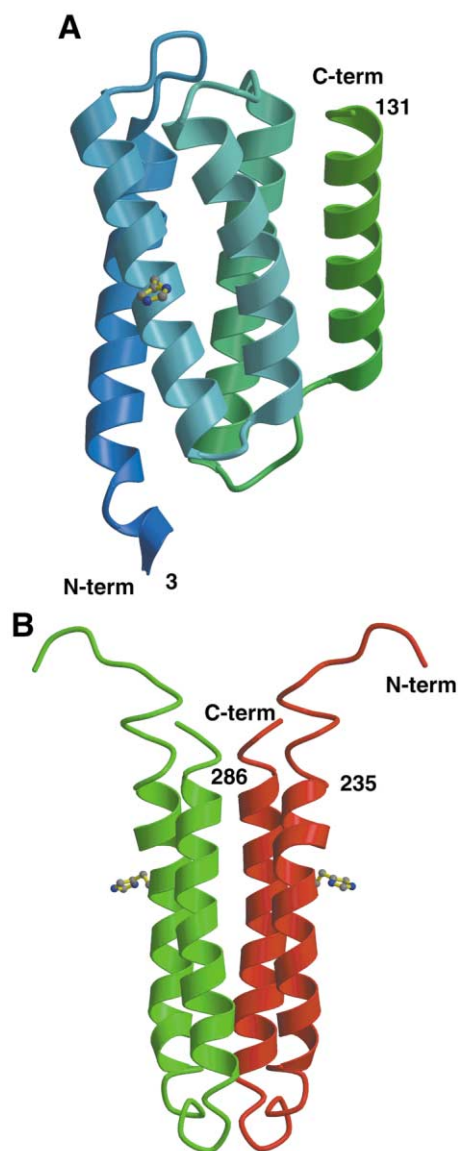
As illustrated in *figure 1*, the phospho-accepting histidine residues are found in two distinct locations: the N-terminal region of the transmitter core-domain and dedicated protein modules. However, the study of several two-component proteins showed that the H box always belongs to a similar structural motif, a finding that may be considered as a design principle in His-Asp phosphorelay cascades.

Nuclear magnetic resonance (NMR) investigation [7] and X-ray structure determination in our laboratory of the P1 domain from the chemotactic class II CheA kinase revealed that the phosphorylatable histidine residue belongs to a four-helix bundle motif (*figure 3A*). A similar architecture was found for the HPT domain fused at the C terminus of the hybrid class I kinase ArcB [8] and for YPD1 [9, 10], the isolated HPT domain in the two-component system that controls the HOG1-dependent MAP kinase in *Saccharomyces cerevisiae* [11]. In these three cases, the four-helix bundles are built from continuous polypeptide stretches that share less than 10% sequence homology.

From a topological point of view, a common way to design a four-helix bundle is by protein dimerization. It is precisely how HPT domains are designed in class I histidine kinases where each transmitter core-domain contributes two N-terminal helices. This finding was first revealed by NMR studies on EnvZ (*figure 3B*), the osmosensor



**Figure 2.** Modular organization of the response regulators. These proteins share a common phosphorylatable receiver domain. Response regulators usually carry additional functional modules whose activity may be regulated by the receiver domain.



**Figure 3.** Four-helix bundle topology of HPT domains. (A) The P1 domain in the *S. typhimurium* CheA kinase. The phosphorylatable histidine residue is carried by the second helix of the bundle. (B) The four-helix bundle generated by dimerization in EnvZ, a class I histidine kinase.

kinase from *Escherichia coli* [12]. The H box is evidently present on each subunit, and the four-helix bundle therefore carries two phosphorylatable histidine residues. This setting satisfies *trans*-phosphorylation, since histidine kinases phosphorylate the histidine residue on the partner subunit within the dimer.

As already mentioned, CheA kinases do not display the H box in the N-terminal region of the transmitter core-domain. Nevertheless, the X-ray structure of the homodimer of CheA revealed that this region also folds as two helices that mediate association of the two subunits through a four-helix bundle [13]. The finding that dimerization of class I and CheA histidine kinases is mediated by

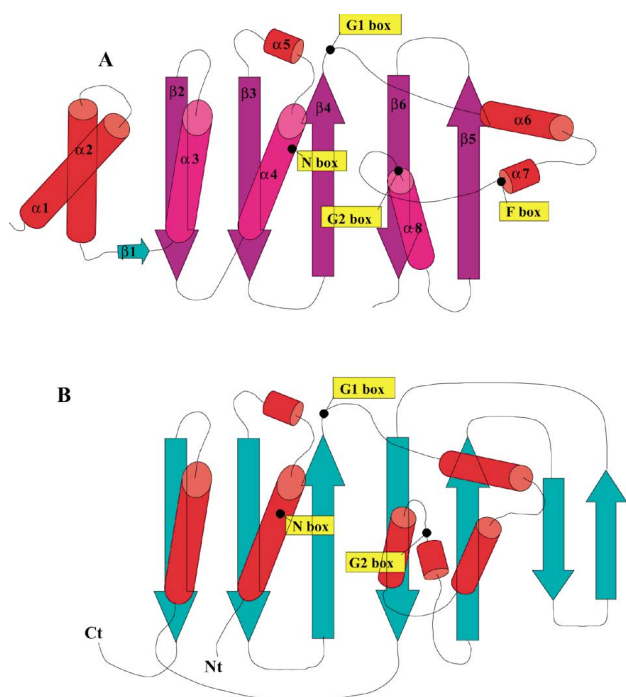
this structural motif appears to be a second design principle in this protein superfamily. These two principles provide a first insight into the function of histidine kinases. The four-helix bundle motif mediates dimer formation of class I and CheA histidine kinases. The bundle becomes a phosphotransfer (HPT) unit when it carries the H box (class I kinases). The phosphotransfer unit has a similar fold when it is made from a continuous polypeptide stretch (CheA kinases and hybrid kinases). These HPT domains can either be fused at the N- or C-termini in proteins or can be isolated modules. In multistep His-Asp phosphorelay cascades, HPT domains may allow further control of the activation cascade and/or may collect the signals from independent upstream activation pathways.

Interestingly, these principles hold for other two-component kinases and for partners of the signaling cascades. Sequence analysis of genome databases indicates that in some proteins the H box has been replaced by other residues including serine or tyrosine. In the two-component kinase DivL of *Caulobacter crescentus*, it was shown that the tyrosine residue is phosphorylated [14]. Spo0B is a phosphotransferase, and relays the phosphoryl group between two response regulators (Spo0F and Spo0A) in the cascade that controls sporulation in bacilli [2]. As shown by the X-ray structure [15], dimerization of the protein is achieved through the formation of a four-helix bundle that carries two phosphorylatable histidine residues.

## 2.2. The N, G1, F and G2 boxes of the catalytic domain

In all histidine kinases, the catalytic ATP-binding domain follows in sequence the dimerization four-helix bundle structural unit. The three-dimensional structure of this domain has been revealed by X-ray crystallography for CheA [13] and by NMR spectroscopy for EnvZ [16]. Its topology (figure 4A) is completely unrelated to that of Ser/Thr or Tyr kinases but strikingly similar to that of heat-shock protein 90 (figure 4B) [17], to the DNA mismatch repair protein MutL [18] and to gyrase B [19] showing that the catalytic domain of histidine kinases belongs to a superfamily of ATPases. From the structure-based sequence alignment (figure 5), it is apparent that the N, G1, F and G2 boxes are among the very few invariant residues in all sequences. These boxes delineate a cavity where ATP binds [20, 21]. The fingerprints of these homology boxes were thoroughly analyzed in 348 histidine protein kinases [22] and led to the categorization of these proteins into 11 subfamilies. This study pointed out that in some bacterial strains most of the histidine protein kinases belong to a single subfamily, suggesting a single lateral gene transfer event. In other cases, such as *Bacillus subtilis*, the 31 histidine protein kinases are nearly evenly distributed over eight subgroups.

The sequence alignment (figure 5) also shows that the number of residues between the G1 and G2 boxes, located at the end of  $\beta 4$  and before  $\alpha 8$ , respectively (figure 4), varies among procaryotic histidine kinases, and between kinases and Hsp90. This variability is also a major difference between procaryotic histidine kinases and the Sln1 histidine kinase from the yeast *S. cerevisiae* [23], in which 120 amino acids are inserted in this region. In contrast,



**Figure 4.** The similar topology of the ATP-binding domain in (A) the transmitter core-domain of the CheA histidine kinase (B) Hsp90 ATPase. The location of the conserved boxes in histidine kinases is indicated. The two N-terminal helices in CheA mediate dimerization of the protein through formation of a four-helix bundle but do not carry the H box. It could be noticed that the first strand and helix ( $\beta 2$  and  $\alpha 3$ ) of the ATP-binding domain in CheA is provided by the C-terminal part of the protein in Hsp90.

deletion of 50 residues in the region that includes the F and G2 boxes, occurs in the C-terminal domain of the phosphotransferase Spo0B. This domain displays the fold of the catalytic domain of the kinases but is devoid of ATPase activity, and one may speculate that these proteins have evolved from a common ancestor. This latter hypothesis may also hold for SpoIIAB, an anti-sigma F factor from *B. subtilis*, which exists independently in nature [24, 25]. This phosphokinase contains all homology boxes of the catalytic domain but acts as a serine protein kinase on the anti-anti-sigma factor spoIIAA [26].

The conservation of the fold and of the invariant residues in the catalytic domains of histidine kinases and gyrases now raises the need for better insight into what determines the kinase versus the ATPase activities in these proteins.

In all two-component histidine kinases, the four-helix bundle is the substrate of the ATP-binding catalytic domain. The modular and respective locations of these functional units (figures 1 and 4) imply movements of these two domains with respect to each other, and a highly specific molecular recognition to prevent cross-talk. These two aspects remain to be documented, but some evidence suggests that the hinge regions between domains may play an important role in the modulation of signal transduction and in the properties of the kinases [27].

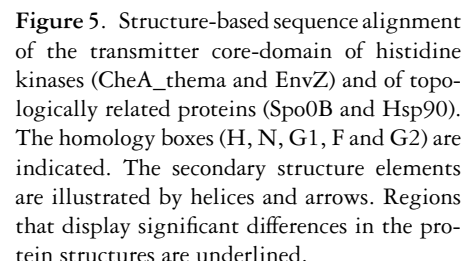
### 3. Modules and functions in response regulator proteins

Response regulators may contain one, two or three domains (figure 2). They all contain a conserved amino-terminal receiver module of about 125 residues that carries the phosphorylatable and invariant aspartate amino acid. A significant number of response regulators only consist of this receiver domain, but the majority of response regulators carry carboxy-terminal output domains. In most instances, one of these domains binds promoter sequences in DNA to negatively or positively control transcription. The phosphorylation state of the receiver domain is, in most cases, the 'on-off' switch of the cellular response, although transcriptional regulation of an alternate set of genes by the unphosphorylated response regulator has been reported [28].

The receiver domains in response regulators display a doubly wound five-stranded  $\alpha/\beta$  fold (figure 6). The active site is a conserved acidic pocket and contains the phosphorylatable aspartate residue, which argues for a common  $Mg^{2+}$ -dependent mechanism of phosphorylation for all members of this superfamily. Phosphorylation may, in most cases, be achieved in vitro, by small phosphodonors such as acetyl phosphate or phosphoramidate, suggesting that the active site of the response regulators catalyses phosphoryl transfer. In vivo, the receiver modules are the substrates of the cognate phosphorylated HPT domains. The half-life of the phosphorylated response regulators ranges from a few seconds to over 1 h, a property related to the type of cellular response that must be achieved. This autophosphatase activity of the receiver domain may be stimulated by specific phosphatases [29, 30] or by the kinase itself. Complex reversal of phosphotransfer was demonstrated in the ArcA/ArcB system [31], and in the regulation of chemotaxis in *Sinorhizobium meliloti*. In this system, a second response regulator, CheY1, assumes the role of a phosphate sink whenever phosphorylated CheY2, the master switch for chemotaxis, and unphosphorylated kinase are present. The kinase in this system may also act as a phosphorelay protein between the two response regulators [32].

Receiver domains mediate various protein-protein interactions, both in their unphosphorylated and phosphorylated states. The functional significance of these interactions has been revealed in a few cases. Unphosphorylated receiver domains may act negatively [33–35] and inhibit the function of the output domains. X-ray structure determinations of NarL and CheB revealed that in each response regulator the interface between the two protein modules sterically prevents DNA binding and methyl-esterase activity, respectively [36, 37]. From biochemical and genetic studies it was shown that phosphorylation disrupts previous interactions and fosters new ones that are required for optimal biological function [38]. This modulation of the function by the phosphorylation state of the regulatory domain has been a central question in the field. It was postulated that phosphorylation induces conformational changes in the receiver domain, and recent advances have provided new insights into the events that drive signal transduction.

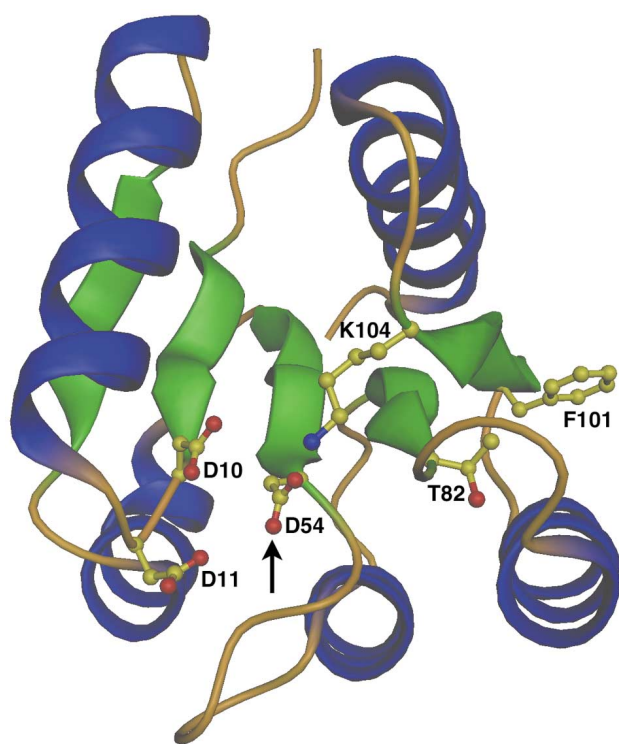




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oligomeric state for FixJ is an essential feature for binding to the target DNA and thus for regulation of the transcription [43].

Activated proteins intriguingly display significantly variable extents of conformational changes. This finding raised the question of why receiver domains use the aspartyl-phosphate, a high-energy bond just like the anhydride linkage in ATP, and not phosphotransfer from ATP to serine or threonine that would generate enough energy to account for the observed conformational changes of the proteins. One hypothesis is that the change in structure that decreases the free energy of hydrolysis of the acylphos-



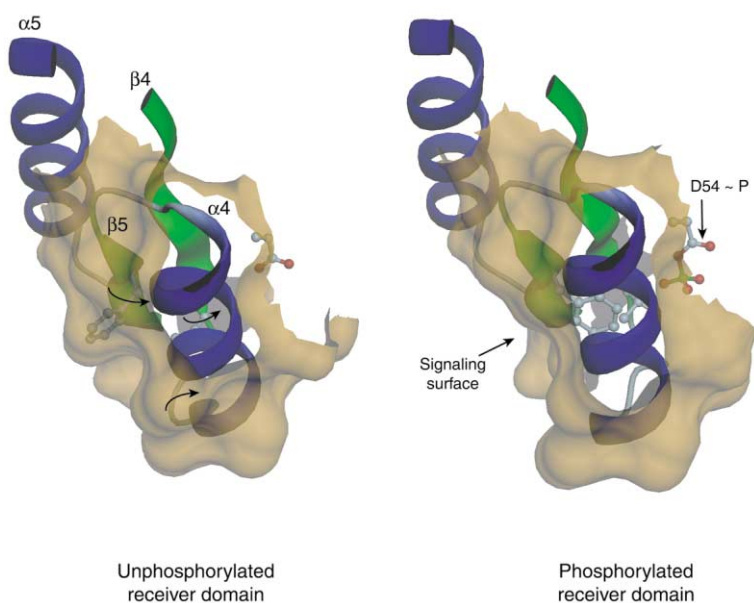
**Figure 6.** The common fold of receiver domains. The highly conserved residues are shown. An arrow indicates the phosphorylatable aspartate in the active site.

phate only occurs during complex formation between the response regulators and their targets [48]. This proposal provides a possible explanation for the small structural changes observed in phosphorylated Spo0A or activated CheY, which contrast with the drastically altered phosphorylated FixJ structure. Indeed, the target for FixJ is FixJ itself,

and dimerization constitutes FixJ activation. Isolated phosphorylated Spo0A which targets DNA, and activated CheY which targets FlhM, would only be high-energy species expected to promote and/or undergo significant conformational changes upon binding to their respective targets.

The structures of all conserved pieces of the puzzle in two-component systems have revealed some key functional features and design principles. However, the complexity and the fine tuning of the activation of the cellular response remain to be documented. The next challenges concern the communication between domains and the characterization of complexes with downstream targets of the response regulators. The variety of signal transduction input domains and the regulation of the histidine kinase activity that determines transmission of the information also represent a formidable task. The growing amount of data from genomic sequencing, proteomic and transcriptome analysis, structural and biocomputing approaches will likely reveal new and more complete pictures of the regulatory aspects of two-component systems.

These signaling cascades have been, in several instances, implicated in virulence and pathogenicity. For example, in *Streptococcus pneumoniae*, loss of function of the VncRS system produced tolerance of the bacteria to vancomycin and other classes of antibiotics [49], and a systematic program of mutagenesis identified two-component systems that are essential for viability and pathogenicity [50]. Interestingly, it was also found that systems which apparently play a role in the maintenance of the respiratory tract infection are conserved in other Gram-positive bacteria. The PhoPQ two-component system in *Salmonella typhimurium* modulates expression of a suite of genes in response to external  $Mg^{2+}$  concentration. A complex regulatory cascade involving the PhoPQ and the PmrAB systems allows *S. typhimurium* to integrate multiple signals for macrophage survival and resistance to antimicrobial peptides [51, 52]. *Clostridium perfringens* is



**Figure 7.** Illustration of the  $\alpha 4$ - $\beta 5$ - $\alpha 5$  surface of the receiver domain in FixJ according to the structures of the unphosphorylated and phosphorylated species. The arrows indicate the phosphorylation-induced displacements of the highly conserved Thr82 and Phe104 side chains and of the  $\beta 4$ - $\alpha 4$  loop. These changes affect the signaling surface of receiver domains and provide the protein-protein interface for dimerization in the case of phosphorylated FixJ.

characterized by its ability to produce numerous extracellular toxins and to cause human gas gangrene and food poisoning. The expression of many of these toxins is regulated at the transcription level by the VirRS two-component signal transduction system [53].

It could be anticipated that an improved and detailed understanding of these signaling mechanisms will help the design of new antibacterial drugs.

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