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To cite this version:
Felipe Trajtenberg, Daniela Albanesi, Natalia Ruétalo, Horacio Botti, Ariel Mechaly, et al.. Allosteric Activation of Bacterial Response Regulators: the Role of the Cognate Histidine Kinase Beyond Phosphorylation. mBio, American Society for Microbiology, 2014, 5 (6), pp.e02105. 10.1128/mBio.02105-14. pasteur-02554121

HAL Id: pasteur-02554121
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Submitted on 25 Apr 2020

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Allosteric Activation of Bacterial Response Regulators: the Role of the Cognate Histidine Kinase Beyond Phosphorylation

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ABSTRACT Response regulators are proteins that undergo transient phosphorylation, connecting specific signals to adaptive responses. Remarkably, the molecular mechanism of response regulator activation remains elusive, largely because of the scarcity of structural data on multidomain response regulators and histidine kinase/response regulator complexes. We now address this question by using a combination of crystallographic data and functional analyses in vitro and in vivo, studying DesR and its cognate sensor kinase DesK, a two-component system that controls membrane fluidity in Bacillus subtilis. We establish that phosphorylation of the receiver domain of DesR is allosterically coupled to two distinct exposed surfaces of the protein, controlling noncanonical dimerization/tetramerization, cooperative activation, and DesK binding. One of these surfaces is critical for both phosphorylation of the receiver domain of DesR is allosterically coupled to two distinct exposed surfaces of the protein, controlling noncanonical dimerization/tetramerization, cooperative activation, and DesK binding. One of these surfaces is critical for both phosphorylation of the receiver domain of DesR is allosterically coupled to two distinct exposed surfaces of the protein, controlling noncanonical dimerization/tetramerization, cooperative activation, and DesK binding. One of these surfaces is critical for both phosphorylation of the receiver domain of DesR is allosterically coupled to two distinct exposed surfaces of the protein, controlling noncanonical dimerization/tetramerization, cooperative activation, and DesK binding. One of these surfaces is critical for both phosphorylation of the receiver domain of DesR is allosterically coupled to two distinct exposed surfaces of the protein, controlling noncanonical dimerization/tetramerization, cooperative activation, and DesK binding. One of these surfaces is critical for both phosphorylation of the receiver domain of DesR is allosterically coupled to two distinct exposed surfaces of the protein, controlling noncanonical dimerization/tetramerization, cooperative activation, and DesK binding. One of these surfaces is critical for both phosphorylation of the receiver domain of DesR is allosterically coupled to two distinct exposed surfaces of the protein, controlling noncanonical dimerization/tetramerization, cooperative activation, and DesK binding. One of these surfaces is critical for both phosphorylation of

IMPORTANCE The ability to sense and respond to environmental variations is an essential property for cell survival. Two-component systems mediate key signaling pathways that allow bacteria to integrate extra- or intracellular signals. Here we focus on the DesK/DesR system, which acts as a molecular thermometer in B. subtilis, regulating the cell membrane’s fluidity. Using a combination of complementary approaches, including determination of the crystal structures of active and inactive forms of the response regulator DesR, we unveil novel molecular mechanisms of DesR’s activation switch. In particular, we show that the association of the cognate histidine kinase DesK triggers DesR activation beyond the transfer of the phosphoryl group. On the basis of sequence and structural analyses of other two-component systems, this activation mechanism appears to be used in a wide range of sensory systems, contributing a further level of specificity control among different signaling pathways.

Received 8 October 2014 Accepted 20 October 2014 Published 18 November 2014

Citation Trajtenberg F, Albanesi D, Ruétalo N, Botti H, Mechaly AE, Nieves M, Aguilar PS, Cybulski L, Larrieux N, de Mendoza D, Buschiazzo A. 2014. Allosteric activation of bacterial response regulators: the role of the cognate histidine kinase beyond phosphorylation. mBio 5(6):e02105-14. doi:10.1128/mBio.02105-14.

Editor Vanessa Sperandio, University of Texas Southwestern Medical Center, Dallas

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This article is a direct contribution from a Fellow of the American Academy of Microbiology.

Tw o-component systems (TCS) are signaling pathways that respond to extra- and/or intracellular cues by modifying cellular behaviors (1). A phosphotransfer cascade from a sensor histidine kinase (HK) or phosphorelay system eventually results in the phosphorylation of a conserved aspartate of the response regulator (RR), which acts as the effector component of the TCS. RRs include a basic module, the receiver (REC) domain, which, upon phosphorylation, allosterically regulates protein-protein interactions such as homo-oligomerization or specific binding to other proteins or even to other domains in multidomain RRs (2).

Structural and biochemical data have been integrated into a widely accepted model of RR allosteric regulation (2, 3). According to this model, the phosphorylation of the conserved aspartate in the REC domain occurs in and stabilizes a rarely populated preexisting active conformation (4). RR structures corresponding to active and inactive states display shifts in the position of residues around the phosphorylatable Asp residue that are ultimately coupled to a structural rearrangement of the α4β5α5 (named according to the included secondary structural elements) solvent-exposed surface of the REC domain. In many inactive RRs, this surface interacts directly with the output effector domain (5). Therefore, phosphorylation-triggered changes result in the release of the effector domain (6), with greater conformational freedom to eventually select the biologically active conformation (7). The α4β5α5 region is directly involved in phosphorylation-activated dimerization (8) and activation (9) in members of the PhoB/
OmpR family. Recent crystal structures of RRs that belong to the NarL/LuxR family show that a different surface (α1α5) is key in mediating phosphorylation-triggered dimerization (10, 11), pointing to a novel regulatory mechanism of RR activation. This α1α5 surface on RRs seems also to be important in mediating the interaction with their cognate HKs (12).

Apart from their intrinsic autokinase activity, the majority of HKs catalyze two additional reactions in concert with their cognate RRs: phosphotransfer and dephosphorylation. The study of DesK from Bacillus subtilis has shown that HKs adopt distinct and defined three-dimensional (3D) conformations that correlate with their catalytic output status (13, 14), unveiling the molecular bases of those transitions (13). Crystallographic snapshots of HKs in other organisms have recently been reported (15, 16), confirming the ability of sensor HKs to sample a discrete set of 3D conformations, ultimately controlling their functional state.

Although HKs are the most relevant phosphoryl donors for RRs in vitro, RR activation mechanisms have been extensively studied at the molecular level by using small phosphodonorins in vitro (3). Do HKs work just as macromolecular phosphodonors, or do they play additional roles as modulators of their cognate RR’s functional state? Different HK-RR and related phosphorelay pairs (12, 17–21) show large protein-protein interactions among the specific partners, and protein-protein interactions have been shown to be important in RR activation (7), reinforcing the per-tinence of exploring the biological role of HKs beyond phosphorylation.

The model that we have chosen to address these questions is the DesK/DesR TCS from B. subtilis. DesR, a member of the NarL family of RRs, is involved in environmental temperature sensing and membrane fluidity regulation (22) (see Fig. S1 in the supplemental material). Cold shock triggers the sensor HK DesK to switch from a phosphatase to an autokinase competent state through a rotational switch in the helical dimerization and histidine phosphotransfer domain (13, 14). DesK then catalyzes phosphotransfer to Asp54 of DesR, triggering a reorganization of DesR’s quaternary structure, a key event in the physiologic induction of the transcription of the Δ5-desaturase gene (des) (23). The promoter of des (Pdes) has been studied in detail (23, 24), revealing two DNA-binding sites, the first one, farthest away from the transcription start site, displays higher affinity for DesR-P. The two binding sites are separated by a two-nucleotide spacer (lying on the same face of the DNA double helix), and occupation of both is essential for transcription activation. We now report the crystal structures of full-length DesR in the activated conformation, as well as of its REC domain in the active and inactive states. Combining biochemical, biochemical, and in vivo evidence, we show that DesR solvent-exposed surfaces mediate dimerization and tetramerization interactions, extending our current knowledge about the molecular mechanisms of RR activation. The architecture of the DesK-DesR complex is consistent with HK-triggered activity modulation mediated through the RR’s α1α5 surface. Moreover, in vivo data support a model that explains DesR activation by its cognate HK through binding ("preactivation") and subsequent phosphorylation. This model is predicted to reflect a widely used mechanism for TCS activation control, integrating information from several systems.

RESULTS

Crystal structures of DesR reveal a phosphorylation-triggered conformational switch between functional states. DesR is monomeric in solution. However, phosphorylation triggers DesR dimerization, as demonstrated by size exclusion chromatography (SEC) (Fig. 1A), small-angle X-ray scattering (SAXS), and SEC-coupled SAXS (see Table S1 in the supplemental material). To gain mechanistic insights into the process of DesR activation and how oligomerization is regulated, we solved the structures of full-length DesR in complex with BeF$_3^-$/$\mathrm{Mg}^{2+}$, as well as of its REC domain alone, in the presence or absence of BeF$_3^-$/$\mathrm{Mg}^{2+}$ (see Table S2 in the supplemental material). Beryllium fluorides are commonly used as phosphomimetics to study active forms of RRs (25). The crystal structure of full-length DesR has two molecules in the asymmetric unit (Fig. 1B), both showing a molecule of BeF$_3^-$ bound to phosphorylatable Asp54 (see Fig. S2A in the supplemental material). DesR comprises 199 residues showing an N-terminal REC and a C-terminal DNA-binding domain (DBD). The REC domain (residues 1 to 132) is composed of a central, parallel β-sheet (β1 to β6) surrounded by a total of six α-helices distributed on both sides (see Fig. S2B in the supplemental material). This is a variation of the canonical (Be$_3^-$)$_6$ topology present in the vast majority of RRs and also seen in other NarL-like proteins (10, 11). After a short loop (amino acids 133 to 138) that links the two domains, the DBDs (residues 139 to 193) show a helix-turn-helix fold different from those described in other RRs of the NarL/LuxR family. Instead of a tetrahedral motif, DesR displays a trihelical arrangement (Fig. 1B). One DBD is completely free, and the other buries only 357 Å$^2$ at the interface with the REC domain, suggesting that they are in a competent configuration to interact with DNA (Fig. 1B). We posit that the structure of full-length DesR in complex with BeF$_3^-$/$\mathrm{Mg}^{2+}$ represents the activated state, mimicking the phosphorylated form of the regulator.

A structural alignment of the 10 independently refined DesR REC domains shows that they can be clearly grouped into two sets according to pairwise root mean square deviations, indicating two distinct structural states (Fig. 1C; see Fig. S2C in the supplemental material). The structural configurations represented by RECa and DesR most likely correspond to the active state, whereas RECb appears to reflect the inactive state. This is based not only on the presence or absence of BeF$_3^-$/$\mathrm{Mg}^{2+}$ but also on their structural resemblance to previously reported structures (10, 26, 27) (see Fig. S2D).

The phosphorylation of Asp54 induces significant rearrangements of residues Glu8, Asp9, Glu56, Thr80, and Thr81. Even though the residue at position 56 is not well conserved among different RRs, it appears to play a critical role. In the inactive state of DesR, Glu56 makes hydrogen bonds with well-conserved Thr80, Thr81, and Arg84, actually shielding phosphorylatable Asp54 from solvent access. Correlated with phosphorylation, Glu56 moves away and achieves the new stabilized position interacting with the BeF$_3^-$ group and Mg$^{2+}$, the cation being essential to allow for active-state RR phosphorylation. It is worth noting that a conserved aspartate (Asp9 in DesR) within the β1α loop also coordinates the Mg$^{2+}$ cation. This β1α loop follows a dramatically different trace, so that, in the inactive state, the carboxylate group of Asp9 is ~13 Å from the Mg$^{2+}$ site. The shifts of Thr80 and Thr81, already seen in many RRs (27–29), are observed in DesR further spreading to the α4β5α5 region, linked to the
movement of Tyr99, and the rearrangement of the $\beta_4\alpha_4$ loop and hydrophobic core residues Phe88, Val96, Leu101, Tyr123, and Leu127. This overall reorganization ultimately results in a shift in the position, as a well as a decrease in the flexibility, of helix $\alpha_6$.

A feature common to the structures of DesR, RECa, and RECb (which includes both functional states) is that the phosphorylation site is not solvent exposed, being covered either by Glu56 (in the inactive state) or by Phe82 (in the active configuration; Fig. 1D). A third crystal form of the REC domain (RECc) was determined also in the absence of $[^{\text{BeF}_3}]^{-}$ and Mg$^{2+}$, but it unexpectedly showed all four RECc protomers in a configuration that is closer to that of the active state, considering both quantitative figures of structural superposition and local inspection of the phosphorylation site and nearby residues. The structure of RECc shows a different arrangement of the $\beta_4\alpha_4$ loop, Tyr99 does not engage in H bonds, and there is no clear electron density to confidently model the side chains of residues Glu56, Phe82, Ala83, and Arg84. RECc has probably captured the active conformation previously to actual phosphorylation, given that Asp54 is fully exposed to the solvent, and loops $\beta_1\alpha_1$ and $\beta_3\alpha_3$ are in the correct position for Mg$^{2+}$ coordination (Fig. 1D).

DesR dimerizes through the $\alpha_1\alpha_5$ surface and tetramerizes through $\alpha_4\beta_5\alpha_5\alpha_5$ upon DNA binding. Each one of the four crystal structures of DesR displays different packing. Yet, they all reveal two major interaction surfaces between neighboring REC domains (see Fig. S3E in the supplemental material). These interfaces engage the $\alpha_1\alpha_5$ (burying $\sim$800 to 1,030 Å$^2$ of solvent-accessible surface area) and $\alpha_4\beta_5\alpha_5$ ($\sim$430 to 1,000 Å$^2$) surfaces. These interfaces are similar to those found in crystal structures of VraR (10) and spr1814 (11), illustrating high conservation among members of the NarL family. To assess the functional roles of these surfaces, we used a strategy of structure-guided point mutagenesis aiming at the disruption of potential interactions and subsequent biophysical characterization of selected mutant variants.

The $\alpha_4\beta_5\alpha_5$ surface of DesR (Fig. 2A to C) differs from the $\alpha_4\beta_5\alpha_5\alpha_5$ dimerization surface present in many RR (essentially members of the OmpR/PhoB family), with the additional $\beta_6$ and $\alpha_6$ elements (Fig. 2B), burying $\beta_5\alpha_5$ away from bulk solvent. The major protein-protein interactions stabilizing a potential quaternary structure are mainly polar (Fig. 2B and C), including six salt bridges and three hydrogen bonds. Hydrophobic interactions seem to be restricted to the center of this motif (Fig. 2B and C).

The second interaction surface, through the $\alpha_1\alpha_5$ region (including the $\beta_5\alpha_5$ loop) (Fig. 2E), is conserved among NarL/LuxR RR (Fig. 2F) and mediated largely by hydrophobic contacts (Fig. 2F and G). Interestingly, $\alpha_1\alpha_5$-mediated oligomerization brings the phosphorylation sites of each monomer into close proximity, generating a strongly negatively charged cleft. Among the hydrophobic contacts that stabilize the $\alpha_1\alpha_5$-mediated interaction, the N-terminal end of $\alpha_1$ has a solvent-exposed protrusion, notably including the side chain of Met12, which inserts into a hydrophobic cavity delimited by the $\beta_5\alpha_5$ loop and the

![Image](http://mbio.asm.org/). Allosteric Activation of the Response Regulator DesR

FIG 1 Structure of DesR in the active state. (A) Phosphorylation-induced oligomerization of DesRwt analyzed by SEC. The apparent molecular masses of the inactive and phosphorylated species suggest a monomer-dimer transition, according to the indicated standard molecular weight (MW) markers. Abs, absorbance. (B) Structure of the active dimer shown in two orientations. The two chains in the asymmetric unit are depicted in blue and orange. Asp$_{54}$ and BeF$_{3}^{-}$ are displayed as spheres, colored by atom type, to highlight the phosphorylation site. term, terminus. (C) Cartoon representation of the activated REC domain (RECa, blue) compared to the inactive state (RECb, green) (D) Structural alignment of the RECa (active, blue), RECb (inactive, green), and RECc structures (phosphorylation-competent active state, orange). Key residues Asp$_{54}$, Glu$_{56}$, Thr$_{81}$, and Phe$_{82}$ are depicted as sticks, and the Mg$^{2+}$ atom and BEF ligand from RECa structure are shown as spheres and sticks, respectively.
The N-terminal tip of helix α5 (Fig. 2G). The protrusion of one monomer snugly fits into the pocket of the other monomer and vice versa.

To explore the role of these interactions in αβ6α6- and α1α5-mediated oligomerization, we compared wild-type DesR (DesRwt) with variants carrying an Arg121Ala substitution (on helix α4) or Met12Ala and Met12Ala-Ala16Arg substitutions (on helix α1). Following in vitro phosphorylation with acetyl phosphate, the quaternary structure of the different DesR proteins was analyzed by SEC (Fig. 2D and H) and SAXS (see Table S1). They all revealed stable species, consistent with well-folded proteins. While phosphorylation triggered the dimerization of DesR_Arg121Ala to levels comparable to those of DesRwt (Fig. 2D), no dimeric forms of DesR_Met12Ala or DesR_Met12Ala-Ala16Arg were detectable (Fig. 2H) under identical conditions.

Given that the αβ6α6 surface does not appear to affect DesR dimerization, at least by mutating Arg121, we then asked whether the DNA-binding behavior was affected. Interestingly, electrophoretic mobility shift assays (EMSAs) revealed that DesR_Arg121Ala is defective in the ability to occupy the second DNA-binding site on the P_des promoter (Fig. 2I). This site is essential to the generation of a functional complex II on the DNA (23) that is able to trigger transcriptional activation. That DesR_Arg121Ala is able to generate singly occupied complex I with an affinity indistinguishable from that of DesRwt is...
consistent with its being able to dimerize properly, ruling out the implication of the αβ6δα6-mediated interface in dimerization. In contrast, the behavior of the single-mutation variant (DesR_Met12Ala) or the double-mutation variant (DesR_Met12Ala-Ala16Arg) in EMSAs is dramatically different from that of the wild-type protein, revealing no detectable DNA binding whatsoever (Fig. 21). The latter results are consistent with the absence of a stable phosphorylated dimeric species for both α1α5 surface mutant variants (Fig. 2H).

Taking the results together and considering that all mutant variants can be phosphorylated by acetyl phosphate in vitro (see Fig. S4A in the supplemental material), we conclude that DesR dimerizes through the α1α5 surface, while it tetramerizes in a DNA-dependent way through the α4β6δα6 region. Furthermore, direct-coupling analysis (DCA) (30, 31) using a set of 7,691 sequences with the same domain architecture as DesR (see the supplemental material for full details) strongly suggests that α1α5 dimerization is a common trend within the whole NarL family (see Fig. S3).

DesR is activated through a novel allosteric mechanism. Dimerization of the RR PhoB promotes its ability to be phosphorylated by acetyl phosphate (32). Given that PhoB dimerizes though a different interaction surface (α4β5α5) than DesR, we wondered whether α1α5-mediated dimerization in DesR produces a similar outcome. We measured the ratio (ξ) of phosphorylated DesR with respect to the total amount of protein as a function of the total protein concentration by using Phos-tag gels to separate phosphorylated from nonphosphorylated species (33) at a fixed time point. A constant ξ value would rule out a dependence of phosphorylation on the DesR dimerization state because protein concentration is a variable directly linked to oligomerization. On the one hand, we observed sigmoidal positive relationships between ξ and both DesRwt and DesR_Arg121Ala concentrations (Fig. 3; see Fig. S4B). On the other hand, the variants DesR_Met12Ala and DesR_Met12Ala-Ala16Arg, with α1α5 disrupted, abrogated this ξ dependency on protein concentration, strongly supporting the notion that homodimerization favors DesR autophosphorylation. The reduced autophosphorylation capacity observed in the mutant variants affecting the α1α5 interface necessarily implies an allosteric link between this surface and the orthosteric Asp54 carboxylate group, the site of phosphorylation. Note that the mutated residues on α1 are solvent exposed and 10 to 15 Å away.

From the dimeric structures of DesR, we predicted that dimerization could stabilize phospho-Asp by interfering with the correct positioning of a reactive water molecule, as seen in the phosphatase complex CheX-CheY structure (34). Within the β4α4 loop, Phe82 shields the BeF3− moiety and is stabilized by van der Waals contacts with Thr81, Tyr87, and Glu56 (Fig. 1D). Thr81 is in close proximity to Gln10 of the other monomer, a dimerization pair that emerges from DCA (see Fig. S3). To test this prediction, we evaluated the autodephosphorylation of DesR at different protein concentrations and found that, as expected, phospho-DesR was stabilized at higher protein levels (see Fig. S4C). Phospho-PhoB showed no detectable protein concentration dependence of its dephosphorylation (see Fig. S4C), which, together with the DCA data, strongly suggests that dimerization stabilizes the phosphorylated state of many RRs that dimerize through α1α5 within the NarL family. In the monomeric state, the β4α4 loop is expected to be more flexible, allowing for entry of the reactive water molecule.

DesR homodimerization and HK binding occur through a largely overlapping interface that involves the RR α1α5 surface. Structural data on HK-RR complexes (12) and covariance analyses (31, 35) indicate that the RR α1α5 surface is directly involved in the interaction with its cognate HK. Given that the α1α5 surface is allosterically coupled to the phosphorylation site in DesR, we decided to analyze the relationship between DesR dimerization and DesK-DesR interaction. We generated a model by using the crystal structures of the catalytic cytoplasmic region of DesK (DesKC; Protein Data Bank [PDB] code 3GIF) and DesR (Fig. 4A; see Fig. S5A in the supplemental material), assuming that the DesK-DesR complex is similar to HK853-RR468 from Thermotoga maritima (12). We validated the model by studying disulfide cross-linking using engineered Cys mutant forms of both partners (see Fig. S5B). DesK is thus predicted to bind DesR through a surface that significantly, although not completely, overlaps the α1α5 surface engaged in DesR homodimerization. According to the modeled complex, Leu200 of DesK is expected to play a role similar to that of Met12 of DesR, occupying a cavity between helix α1 and the β5α5 loop of the REC domain. The α1 double-mutation variant DesR_Met12Ala-Ala16Arg is predicted to clash with the approaching kinase through the engineered arginine at position 16. We tested these predictions by investigating binding to the catalytic region of DesK. We determined that the single-mutation variant DesR_Met12Ala is able to form a DesK-DesR complex (Fig. 4B), whereas DesR_Met12Ala-Ala16Arg cannot. Further support for our predictions was obtained by exploring the ability of these α1α5 mutant variants to be phosphorylated by DesK and ATP in vitro, which showed that DesR_Met12Ala can be phosphorylated to levels comparable to those of DesRwt, whereas the double-mutation variant cannot (Fig. 4C). The fact that DesK associates with DesR through the RR’s α1α5 surface then opened the question of whether DesR might dimerize differently, depending on the presence or absence of the HK. The DesK-DesR com-

FIG 3 Allosteric coupling between the α1α5 dimerization surface and the phosphorylation site. Autophosphorylation in vitro with acetyl phosphate. The degree of phosphorylation (ξ) is expressed as a function of the logarithm of the protein concentration (molar). ξ was measured after 1 h of incubation with acetyl phosphate. Values from separate experiments are shown with the best-fit sigmoid curves overlaid. A representative Phos-tag SDS-PAGE analysis is shown in Fig. S4B in the supplemental material. WT, wild type.
plex likely leaves the α4β6α6 surface of the RR exposed to the solvent, such that DesK phosphorylation (in contrast to acetyl phosphate) could be thought to drive DesR to dimerize through the α4β6α6 interface. EMSAs performed with DesR phosphorylated by DesK in the presence of ATP (Fig. 4D) revealed a DNA shift with DesR_Arg121Ala, whereas DesR_Met12Ala resulted in no detectable complex. These results are consistent with those obtained previously with acetyl phosphate phosphorylation, confirming that DesR homodimerization always occurs through the α1α5 surface (i.e., the α4β6α6 surface is not involved in dimerization), independently of the source of phosphorylation.

Biological relevance of the dimerization and tetramerization surfaces of DesR. To test the biological relevance of the exposed surfaces of DesR linked to its activation state, we assayed the capacity to regulate des gene transcription in vivo by comparing DesRwt with the different point mutant variants with disrupted α1α5 or α4β6α6 interfaces. des expression was evaluated by complementing B. subtilis strain AKP21 (des desR double knockout) (22) with a plasmid expressing wild-type desK and either wild-type desR or each of the desR surface mutant variants under the control of a xylose-inducible P\_xyl promoter. AKP21 carries a lacZ reporter gene under P\_des promoter regulation. The α4β6α6 DesR_Arg121Ala mutant variant, as well as the α1α5 single-mutation variant DesR_Met12Asp (equivalent to the mutation performed by Leonard et al. [10]) and the double-mutation variant DesR_Met12Ala-Ala16Arg, lost the capacity to upregulate des transcription in response to cold shock (Fig. 5A). These results are consistent with the in vitro EMSA results showing that both surfaces are essential for DesR transcriptional activation function. Somewhat unexpectedly, the single-mutation variant DesR_Met12Ala retained in vivo regulation to extents comparable to those of DesRwt (Fig. 5A), likely because of its milder effect on the α1α5 surface than that of DesR_Met12Asp and DesR_Met12Ala-Ala16Arg.

The cognate HK, in its phosphotransfer state, acts as an allosteric activator of the RR. The observations that α1α5 dimerization is allosterically coupled to DesR activation (Fig. 2 and 3) and that this α1α5 surface also mediates DesK association (Fig. 4) led...
to the question of whether DesK binding might exert an activating effect on the regulator independently of phosphorylation. The use of the nonphosphorylatable variants DesR_Asp54Ala and DesR_Asp54Asn enabled us to differentiate between potential activation and actual phosphorylation. Strikingly, signal-dependent regulation of des was obtained with DesR_Asp54Ala and DesR_Asp54Asn to extents comparable to those obtained with DesRwt and DesR_Met12Ala (Fig. 5A). These results indicate that DesK is also able to activate its cognate RR through a phosphorylation-independent mechanism. Because the results described above were obtained with plasmid-borne genes, we investigated expressions where we inserted the desk/H11001 des/H11001 or desK construct into the thrC locus of the chromosome under the control of the Pxy promoter. As shown in Fig. 5B, the DesR_Asp54Asn mutant variant is able to induce des transcription at low temperatures in a DesK-dependent manner similarly to DesRwt. The strains expressing only the RRs do not exhibit des expression at low temperature, which is direct evidence of a phosphorylation-independent step of activation that requires a signal-triggered change in the conformation of the HK.

**DISCUSSION**

The crystallographic and functional studies, both in vivo and in vitro, of full-length DesR, as well as of its REC domain in the active and inactive configurations, have been instrumental in the discovery of a phosphorylation-stabilized dimeric species. The monomers interact through a “noncanonical” interface defined by helices 1 and 5 and the β5α5 loop. Furthermore, structure-guided mutant variants show that this dimerization is biologically relevant and critical for the RR activation. These results now confirm, in the context of recent reports (10, 11), that this is a widely used mechanism in TCS. A novel feature related to this noncanonical mechanism is now unveiled: the surface located in a position equivalent to that of α4β5α5 (α4β6α6 in DesR), typically involved in the dimerization of PhoB/OmpR RRs, is not engaged in DesR dimerization but is instead critical to the establishment of essential dimer-dimer interactions in DesR, enabling it to fully
bind to its DNA-binding site. An additional and striking finding concerns the cognate kinase DesK, which "preactivates" DesR as a first step toward phosphotransfer, full-blown activation, and response. That homodimerization and DesK binding occur largely through the same DesR surface (α1α5) leads us to propose a shared allosteric pathway coupling this surface with the phosphorelayable aspartate. Further studies are needed to understand the derived geometric constraints during the phosphatase reaction. It can be predicted that the kinase should be able to displace the dimer-monomer equilibrium toward the phosphate-accessible monomorphic species such that dephosphorylation proceeds efficiently. This scenario is likely valid for all RRs that dimerize through their α1α5 surface within an extended subset of NarL-like RRs (10, 11).

**Structural bases of allosteric activation pathways.** The non-linear behavior of DesR phosphorylation versus concentration implies oligomerization-dependent stabilization of its active conformation (Fig. 3), in agreement with recent reports (32). Disruption of the α1α5 interface impairs activation, revealing allosteric control. The comparison of DesR REC domains in the active (BeF$_3$-bound) versus the inactive state (Fig. 1; see Fig. S2C and S7), allows the pinpointing of key residues for this allosteric pathway. Side chains of residues in the β1α1 loop are directly involved in phosphorylation-triggered rearrangements, leading to the significant shift (maximum, ~1.8 Å) and rotation (maximum, ~30 to 40°) of helix α1, with the effect being more pronounced in the N-terminal half. The direct physical link between the α1 helix position and the preceding β1α1 loop immediately suggests a mechanistic link for α1α5-triggered activation. DesR homodimerization induces a shift of α1 (Fig. 6A) affecting the position of a conserved carboxylate side chain within the β1α1 loop (Asp9 in DesR), which is critical for cation coordination and activity. This feature, wherein the carboxylate O atoms of the conserved Asp are located too far away to coordinate the Mg$^{2+}$ with a phosphorylation-competent geometry (Fig. 6B), is conserved in the inactive conformation of several RRs from different families (5, 10, 12).

Our structures also indicate that when DesR is in the inactive state, Glu56 (in the β3α3 loop) is shielding phosphorylatable Asp54, stabilized by contacts with Thr80, Thr81, and Arg84. Activation of DesR implies a significant shift of Glu56, making Asp54 solvent accessible. Similar conformational changes can be identified in other RRs, such as Spo0F (36), CheY (37), NtrC (38), and RR468 (12). The residue equivalent to Glu56 in NarL (Asn61) also covers the phosphorylation site (5, 26), suggesting a common property of the inactive state. In the case of VraR, the nonphosphorylated structure does not adopt this inactive configuration, possibly because the β3α3 loop is part of the crystal packing (10).

**DesR homodimerization and binding of DesK induce allosteric activation of DesR.** The association geometry of DesK and DesR evokes an activation mechanism similar to the one triggered by DesK homodimerization, with the first α helix of the HK DHp domain fulfilling the role of the RR’s α1 (Fig. 6C). DesK is thus well positioned to induce α1α5-mediated DesR activation. Alternative mechanisms cannot be ruled out. Experimental data revealing the 3D structure of DesK-DesR complexes in different functional configurations will be instrumental in this direction. Available crystallographic data from other HK-RR (12, 39) and phosphorelay (20) complexes do show a similar set of rearrangements, especially at the β1α1 and β3α3 loops and helix α1 of the RR, in support of this mechanism and its wide use among TCS. The RR’s α1α5 surface is implicated in different complexes involving RRs as partners (17–21), and this is consistent with mutual information analyses (31, 35, 40). This interface not only seems critical for specific RR recognition but, as we now show, is also key for RR activation.

We have now established that DesR’s cognate HK plays an allosteric activation effect similar to that of the one resulting from DesR dimerization, as DesK can activate the pathway independently of DesR phosphorylation. Importantly, this effect is regulated by temperature, the physiological signal in the DesK/DesR system (Fig. 6D; see Fig. S1). That $P_{\text{dimer}}$ activation by nonphosphorylatable DesR mutant variants occurs only after cold shock means that the functional state of DesK (in this case, its phosphotransferase-active form) is relevant for the activation mechanism. In the high-temperature regimen, DesK is mainly in its kinase-off/phosphatase-on state and the 3D structure of its cytoplasmic domain is significantly different (13), which not only explains the differential catalytic output but also predicts differences in the way it interacts with DesR. We posit that HK-RR interactions are different between the phosphatase (12) and phosphotransfer states, yet conclusive evidence awaits a high-resolution structural snapshot of an HK-RR phosphotransfer complex. We obtained a model of such a complex by using separate DesK and DesR crystal structures, resulting in an overall geometry similar to the available crystal structure (12).

A phosphorylation-independent activation mechanism of DesR can be envisaged that, although signal and DesK dependent, likely depends on DesR reaching high enough concentrations that sufficient dimer is built in the cell (see Fig. S6 and S7C in the supplemental material). At physiological DesK-DesR concentrations and ratios, DesR phosphorylation seems otherwise essential to increase the half-life of enough dimeric species that response activation stays tightly linked to signal sensing (Fig. 6D). Conclusive validation of this hypothesis awaits the quantitative determination of intracellular DesK and DesR concentrations. A working model consistent with all of the data leads us to propose a “preactivation” role for DesK, via DesR monomer binding, able to raise the concentration of active-state DesR (4, 41), a dimerization-prone state (10, 42). Enough dimeric DesR species is sufficient to exert the effector transcriptional response, and a slow active-to-inactive-state transition (43, 44) would allow sequential DesK-DesR dissociation and DesR homodimer formation. The HK-dependent allosteric activation mechanism now provides a molecular insight into reported observations such as the differential phosphorylation efficiencies of small phosphoryl donor molecules versus the cognate HK observed with the PhoB-like RR PrrA (7) or FixL-dependent in vivo signaling using nonphosphorylatable variant FixJ proteins (45). With a broader perspective, this activation role is relevant to explain why small phosphoryl donor molecules are inefficient at phosphorylating the vast majority of RRs in vivo (46), even when these phospho donors reach high intracellular concentrations (47) (Fig. 6D).

Phosphorylation-independent mechanisms have been described for a few TCS (48–52), but the regulatory mechanisms are still not fully understood. Interestingly, HKs can also be allosterically regulated by RRs: the RR DivK binds and activates the autokinase activity of HKs DivJ and PleC (53), although the mechanism is still elusive. Our evidence that DesK is able to modulate the functional state of DesR by binding through an interface expected
FIG 6 Working model of DesK−DesR allosteric regulation. (A) Superposition of the active (blue) and inactive (yellow) conformations of the REC domain of DesR, illustrated in cartoon representations. BeF$_3^-$ is bound to Asp54. Mg$^{2+}$ is shown as a purple sphere. The direct link between the α1 shift and the competent position of Mg-coordinating Asp9 is highlighted. (B) Same orientation as in panel A, showing in solid colors the superposition between the active (cyan) and inactive (green) conformations of VraR (10). Note the side chain of Asp10, which, in concert with the movement of helix α1, shifts its side chain away from the cation coordination sphere. In a transparent representation (red), the equivalent Asp14 of inactive NarL (5) is highlighted adopting a similar distant position. RR468 (12) displays the same Asp10 rearrangement (not shown for clarity). (C) The calculated model of DesK−DesR complex is shown in the same orientation as in the previous panels. DesK is green, and the color code for the active and inactive forms of DesR are identical to those in panel A. Note the predicted favorable interactions between the active state of DesR and the kinase with contacts between the α1 helices of both partners (DesK Leu200 is predicted to occupy a position similar to that of DesR Met12 in the DesR homodimer). Phosphorylatable His188 is shown as a reference. (D) The cell membrane is shown at the top. Depicted is the three-state conformational transition of DesK based on the structural data we have previously reported (13); the 3D structure of its transmembrane domain is still not known. The asymmetric structure adopted by P−DesK forms a phosphotransfer complex with DesR, shifting the conformational equilibrium of the RR population toward the active state (preactivation) and transferring the phosphate. P−DesR increases the half-life of the active conformation, allowing for functional dimers to form. In the absence of phosphorylation (e.g., nonphosphorylatable DesR mutant variants), a higher DesR concentration is essential for stabilization of the activated conformation (see Fig. S6 in the supplemental material). The pathway is shut down by DesK in its phosphatase state, forming a complex with monomeric P~DesR. The DesR dimer binds first the high-affinity (RA) and then the lower-affinity (RB) DNA-binding site (23). DesR dimer−dimer interactions through the α4β6α6 interface seem crucial to the achievement of full RB occupancy. RNAP, RNA polymerase.
to be used in most HK-RR pairs suggests that this allosteric mechanism is widely used, promoting RR activation in the presence of the signal and adding a further level of specificity, avoiding uncontrolled pathway activation.

**MATERIALS AND METHODS**

Plasmid construction and site-directed mutagenesis. Detailed protocols used for plasmid construction and site-directed mutagenesis are included in the supplemental material (see Text S1). Lists of the primers and plasmids used are available at http://intranet.pasteur.edu/pxf/Supplemental_plasmids.pdf http://intranet.pasteur.edu/pxf/Supplemental_plasmids.pdf, respectively.

Protein expression and purification. Recombinant proteins were expressed as N-terminally His$_6$-tagged fusions in *Escherichia coli* strain TOP10F’ (Invitrogen). Protein purifications were performed as previously described (13), except that the last SEC (HiLoad 16/60 Superdex 75 preparation grade column; GE Healthcare) for DesR (full-length construct) or DesRREC (REC domain) was pre-equilibrated with a mixture of 50 mM Tris-HCl (pH 8), 300 mM NaCl, 10 mM MgCl$_2$, and 0.5 mM dithiothreitol (DTT). After gel filtration, full-length DesRwt and DesRREC were concentrated to 20 and 80 mg/ml, respectively, and stored at −80°C.

Protein crystallization and structure determination. Single crystals were grown at 20°C by using vapor diffusion techniques. Full details of the crystallization conditions and data collection protocols used are described in the supplemental material (see Table S2). Initial phases for full-length DesR were obtained by molecular replacement (54) using an RR from *Staphylococcus aureus* (PDB code 3B2N). The structure was rebuilt by using Coot (55) and refined with BUSTER (56). The three structures of the REC domain were solved by MR using the refined full-length model as the search probe.

**SAXS data collection and analysis.** SAXS data from different DesR variants were collected at the BM29 (ESRF) and SWING (Soleil) synchrotron beamlines. At BM29, all samples were measured in dilution series at four different concentrations (20 to 200 μM) at 20°C (for full details, see the supplemental material).

**EMSAs.** EMSAs were performed as described previously (22). Briefly, different concentrations of phosphorylated DesR or point mutant variants (previously phosphorylated at 100 μM in the presence of 50 mM acetyl phosphate) were incubated for 20 min at room temperature in a mixture of 50 mM Tris-HCl (pH 8), 5 mM MgCl$_2$, 0.5 mM EDTA, 1.25 mM DTT, 10% glycerol, 50 mM acetyl phosphate, 5 μg/ml heparin, and 1.8 μM DNA probe. After the incubation period, glycerol was added to a final concentration of 15% and the mixture was applied to a 5% polyacrylamide gel that had been pre-run for 1 h in a mixture of 45 mM Tris-borate (pH 8), 1 mM EDTA, and 4 mM MgCl$_2$. A similar procedure was followed with DesKC instead and with 5 mM ATP as the phosphoryl donor.

Effect of protein concentration on DesR autophosphorylation. DesR autophosphorylation reactions were performed with protein concentrations ranging from 0.2 to 500 μM at 25°C and pH 8 and incubation with a mixture of 50 mM acetyl phosphate, 300 mM NaCl, 50 mM MgCl$_2$, and 20 mM Tris-HCl. The reaction volumes varied between 20 and 100 μl. Addition of acetyl phosphate was immediately followed by warming from 80°C.

β-Galactosidase assays in liquid cultures. Replicative plasmids pACRK1 (DesRwt), pACRK2 (desR$_{M12A}$), pACRK3 (desR$_{M12A,A16R}$), pACRK4 (desR$_{R121A}$), pACRK5 (desR$_{R121}$), pACRK6 (desR$_{M12A,A16R}$), and pACRK7 (desR$_{R121A}$) were transformed into *B. subtilis* strain AKP21 (JH642 desKR:Km$^r$ amyE::P$_{phoP}$-lacZ) (22). The resulting strains were grown overnight in Spizizen minimal-salts medium supplemented with 0.5% glycerol, 0.01% tryptophan, 0.01% phenylalanine, trace elements, and 0.05% casein hydrolysate in the absence of xylose. This medium was named SMM-CAA. The cells were collected by centrifugation and diluted in SMM-CAA containing 0.1% xylose to an optical density at 525 nm (OD$_{525}$) of 0.12. The xylose concentration was a critical parameter to optimize. Cultures were incubated with shaking at 37°C until they reached an OD$_{525}$ of 0.3 and divided in two. One half was kept at 37°C, and the other was transferred to 25°C. Samples were taken at the times indicated and assayed for β-galactosidase activity as previously described (24). The specific activity was expressed in Miller units.

β-Galactosidase assays in plates. Strains AKP21_ Rwt, AKP21_R5D4AN, AKP21_Rwt_K, and AKP21_R5D4AN_K carry a single copy of the *desR* gene (wild type or with the single point mutation D54N) integrated into the thrC locus under the control of the P$_{phoP}$ (xylose-inducible) promoter. In addition, AKP21_Rwt_K and AKP21_R5D4AN_K contained the desK gene integrated into the same locus under the control of a second P$_{phoP}$ promoter (for further details of the construction of the strains, see the supplemental material). The four strains, together with AKP3 and AKP21 (22), were streaked onto SPI-CAA medium plates containing 80 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Sigma) and different glucose (Sigma) or r-t xylose (Sigma) concentrations, as indicated, and incubated at 37°C for 12 h with the corresponding antibiotics. A set of plates was kept at 37°C for an additional 48 h, and an identical set was induced by transfer to 25°C for 48 h before photography. Strains were streaked onto SPI-CAA medium plates containing 80 μg/ml X-Gal (Sigma) and different r-xylose (Sigma) concentrations, as indicated, and incubated at 37°C for 12 h. Cold shock was induced by transfer to 25°C for 48 to 72 h before were photographs taken.

**Protein structure accession numbers.** The protein structures presented here have been deposited in the PDB under accession codes 4LDZ (full-length DesR in complex with BeF$_3$–Mg$^{2+}$, activated state), 4LE0 (RECa, DesR REC domain in complex with BeF$_3$–Mg$^{2+}$, activated state), 4LE1 (RECb, DesR apo REC domain, inactive state), and 4LE2 (RECc, DesR apo REC domain, active-state-like configuration).

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02105-14/-/DCSupplemental.

Figure S1, JPG file, 0.9 MB.
Figure S2, JPG file, 1.7 MB.
Figure S3, JPG file, 0.7 MB.
Figure S4, JPG file, 0.7 MB.
Figure S5, JPG file, 0.9 MB.
Figure S6, JPG file, 1.5 MB.
Figure S7, JPG file, 1.1 MB.
Table S1, DOCX file, 0.1 MB.
Table S2, DOCX file, 0.2 MB.
Text S1, DOCX file, 0.1 MB.

**ACKNOWLEDGMENTS**

We thank the staffs at synchrotron beamlines SWING (Soleil, Gif-sur-Yvette, France) and BM29 (ESRF, Grenoble, France) for assistance with SEC-coupled SAXS and SAXS, respectively. We are grateful to Pedro Alzari (Institut Pasteur) and Eduardo Groisman (Yale University) for helpful discussions. At the Institut Pasteur de Montevideo, special thanks to Mathias Machado for useful input about scripting for Rosetta and Analía Lima for her assistance with gel densitometry analyses.

This work was supported by grants from the Agencia Nacional de
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