

Altered recognition mutants of the response regulator PhoB: A new genetic strategy for studying protein–protein interactions

(P1-Int-Xis cloning/protein phosphorylation/two-component regulatory systems/vancomycin resistance)

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ABSTRACT Two-component regulatory systems require highly specific interactions between histidine kinase (transmitter) and response regulator (receiver) proteins. We have developed a novel genetic strategy that is based on tightly regulated synthesis of a given protein to identify domains and residues of an interacting protein that are critical for interactions between them. Using a reporter strain synthesizing the nonpartner kinase VanS under tight arabinose control and carrying a promoter-*lacZ* fusion activated by phospho-PhoB, we isolated altered recognition (AR) mutants of PhoB showing enhanced activation (phosphorylation) by VanS as arabinose-dependent Lac⁺ mutants. Changes in the PhoB^{AR} mutants cluster in a “patch” near the proposed helix 4 of PhoB based on the CheY crystal structure (a homolog of the PhoB receiver domain) providing further evidence that helix 4 lies in the kinase-regulator interface. Based on the CheY structure, one mutant has an additional change in a region that may propagate a conformational change to helix 4. The overall genetic strategy described here may also be useful for studying interactions of other components of the vancomycin resistance and P_i signal transduction pathways, other two-component regulatory systems, and other interacting proteins. Conditionally replicative *oriR_{6Kγ} attP* “genome targeting” suicide plasmids carrying mutagenized *phoB* coding regions were integrated into the chromosome of a reporter strain to create mutant libraries; plasmids encoding mutant PhoB proteins were subsequently retrieved by P1-Int-Xis cloning. Finally, the use of similar genome targeting plasmids and P1-Int-Xis cloning should be generally useful for constructing genomic libraries from a wide array of organisms.

Protein–protein interactions are important in genetic regulatory responses in all cells. Identifying domains and amino acid residues that determine how a given protein recognizes an interacting partner protein(s) is therefore a fundamental problem. Many interacting proteins are members of gene families and yet interact only with specific proteins that are not (or only poorly) recognized by other members of the same family. Determination of the molecular basis of recognition between such proteins would provide new insights into the specific interactions that are critical in innumerable cellular signaling pathways.

Two-component regulatory systems are crucial in a variety of signal transduction pathways in bacteria (1). Most of these systems share a common biochemical signaling mechanism in which a histidine kinase (usually a membrane-associated sensor protein) autophosphorylates on a conserved histidine residue in response to an environmental stimulus and then transfers the phosphate group to a conserved aspartate residue on its partner response regulator (commonly a transcriptional

activator). As many as 50 pairs of these kinase (transmitter) and response regulator (receiver) proteins may exist in a single bacterium such as *Escherichia coli*. Thus, interactions among these partner proteins must be highly specific to prevent undesirable interactions, or nonspecific cross talk (noise), between nonpartner proteins. Analogous signaling systems exist in eukaryotic cells including *Saccharomyces cerevisiae* (2), *Arabidopsis thaliana* (3), and others (4). In yeast, a two-component system (SLN1 and SSK1) provides signaling input to a mitogen-activating protein kinase cascade that is responsive to small molecules (5). Mitogen-activating protein kinase cascades are key integrators of convergent signal transduction pathways in mammalian cells (6).

The PhoR–PhoB two-component regulatory system is required for transcriptional activation of the phosphate (Pho) regulon of *E. coli* in response to environmental P_i levels (7). P_i control of the Pho regulon is a paradigm of a signal transduction pathway in which occupancy of a cell surface receptor(s) regulates gene expression in the cytoplasm. In response to P_i limitation, the sensor kinase PhoR is autophosphorylated by ATP, phospho-PhoR transfers the phosphate group to the response regulator PhoB, and phospho-PhoB activates transcription at Pho regulon promoters (the P_i signaling response). Deactivation (dephosphorylation) occurs upon growth shift to P_i excess conditions and requires in addition to PhoR, an intact Pst (P_i-specific transport) system and a protein called PhoU. Inhibition (prevention of phosphorylation) also requires PhoR, the Pst system, PhoU, and excess P_i. In the absence of PhoR, cross regulation of PhoB by two other signaling pathways—involving the catabolite sensor histidine kinase CreC (formerly called PhoM) or acetyl phosphate—result in high activation (phosphorylation) of PhoB in response to carbon sources (8–10).

The VanS–VanR two-component regulatory system is required for high-level inducible vancomycin resistance in the clinical isolate *Enterococcus faecium* BM4142 (11). The sensor kinase VanS is autophosphorylated in the presence of vancomycin (possibly due to accumulation of a cell wall precursor), phospho-VanS transfers the phosphate group to the response regulator VanR, and phospho-VanR activates transcription of the *vanRSHAXYZ* gene cluster required for vancomycin resistance (12).

VanS is also capable of high activation (phosphorylation) of PhoB due to cross talk when VanS is produced from a multicopy plasmid (13). Using cross talk as an *in vivo* assay, we previously identified VanS peptides capable of interfering with activation of PhoB by VanS; the peptides may correspond to a VanS domain for interaction with a response regulator. A

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Abbreviations: AR, altered recognition; CA, constitutively active; Kan^R, kanamycin resistant; Int, integrase; Xis, excisionase; PIX, P1-Int-Xis; wt, wild type; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; MBP, maltose-binding protein.

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similar approach had been used earlier to identify the phosphotransfer region of the kinase CheA for its partner response regulator CheY (14). We now describe a new strategy for studying sensor kinase-response regulator interactions; we used our new approach to isolate altered recognition (AR) PhoB mutants as gain-of-function mutants showing enhanced activation by VanS. We expect that methods developed in this study should be generally useful for studying other protein-protein interactions *in vivo*.

MATERIALS AND METHODS

Media and Reagents. Luria-Bertani broth, MacConkey, 4-morpholinepropanesulfonic acid (Mops), and TYE media are described elsewhere (15). 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-Gal) was used at 40 μ g per ml for detection of β -galactosidase. Ampicillin was added at 100 μ g per ml; kanamycin, at 12.5 or 50 μ g per ml to select kanamycin-resistant (Kan^R) integrants or maintain plasmids, respectively; and L-arabinose, at 0.13 mM for induction. Integrants were routinely grown without an antibiotic. *Taq* DNA polymerase (Promega) was used for polymerase chain reaction (PCR) mutagenesis and testing of single copy integration. Vent DNA polymerase (New England Biolabs) was used to generate DNAs for cloning.

Bacteria. Unless noted otherwise, bacteria are derivatives of *E. coli* K-12 BW13711, which were constructed by P1*k*c transduction (15). Reporter strains have a *P_{phnC}-lacZ* transcriptional fusion in single copy at the *lac* locus; they synthesize also the C-terminal cytoplasmic domain of PhoR (residues M63 to D431; denoted 'PhoR) or of VanS (residues M95 to S384; denoted 'VanS) under arabinose control from a *P_{araB}-'phoR* or *P_{araB}-'vanS* fusion in single copy at the *araCBAD* locus. These *-lacZ* and *P_{araB}-* fusions were constructed using methods similar to ones described elsewhere (16). In brief, the appropriate DNAs were made with the PCR, cloned, sequenced, and recombined onto the chromosome by allele replacement. The *P_{araB}-'phoR* strain BW23423 [*lacI*^q *P_{phnC}-lacZ*_{WJ19} Δ *phoBR580* Δ *creBCD153* Δ (*pta ackA hisQ hisP*)_{TA3516} *phn*(*EcoB*) Δ *araBAD*_{AH37::P_{araB}-NFLAG-'phoR}_{AH41}] was used to test for activation of PhoB by 'PhoR; the *P_{araB}-'vanS* strains BW23425 (like BW23423, except Δ *araBAD*_{AH37::P_{araB}-'vanS}_{AH49}), BW23042 (like BW23425, except Δ *phoR574*), and BW23427 (like BW23425, except with the 'vanS_{H164Q} allele), for activation by 'VanS or 'VanS_{H164Q} (an inactive protein), and the *P_{araB}-'vanS* strain BW23562 (like BW23425, except Δ *phoB578-phoR*⁺), for inhibition by PhoR.

Plasmids. A Kan^R, *attP* "genome targeting" suicide vector with the plasmid R6K γ replication origin (*oriR_{R6K}*); unpublished data cited in ref. 17) and pINT-ts were from M. Koob (University of Minnesota School of Medicine). An *oriR_{R6K}* γ plasmid requires the transacting π protein (the *pir* gene product) for DNA replication. pINT-ts is an ampicillin-resistant, low copy, temperature-sensitive (Ts⁻) replicon plasmid synthesizing λ integrase (Int) under control of *lcI857* (17). The same system for integration of similar plasmids into *att* λ has been used elsewhere (18). pAH57 is a derivative of pINT-ts that synthesizes both Int and excisionase (Xis); it was made by joining the *EcoRI* to *BamHI* backbone of pINT-ts with a 1.1-kbp *EcoRI* to *BglII* PCR fragment encoding Xis and N terminus of Int (restoring *int*⁺), which was PCR-generated using λ DNA (from R. Somerville, Purdue University) as template. Our integration plasmids (Fig. 1) have an *attP* region in which a nearby *NdeI* site was destroyed; ones with *P_{tac}* contain the *PstI* to *NdeI* *lacI*^q and *P_{tac}* segment of pASLR2 [from A. S. Lynch (Harvard Medical School) (18)]. *oriR_{R6K}* γ plasmids were maintained at different copy numbers in the *pir*⁺ host BW23473 (Δ *lac-169 robA1 creC510 hsdR514 endA recA1* Δ *uidA::pir*⁺), the isogenic *pir-116* host BW23474, or similar hosts (16).

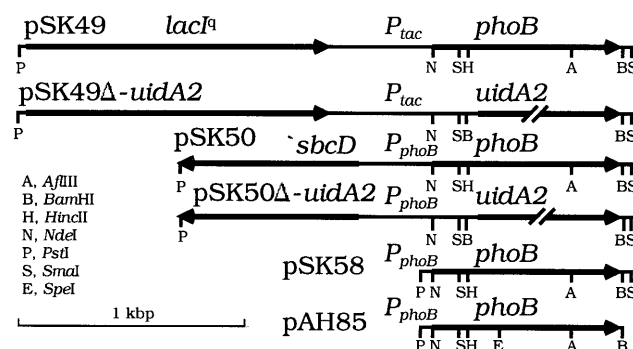


FIG. 1. Plasmids. pSK49 and pSK49 Δ -*uidA2* express *phoB* or *uidA* from *P_{tac}*. pSK50 and pSK50 Δ -*uidA2* express *phoB* or *uidA* from *P_{phoB}*. A 189-bp *P_{phoB}* fragment in pSK58 and pAH85 was generated using PCR. An *NdeI* site coinciding with the *phoB* start codon and *BamHI* site immediately beyond the *phoB* stop codon were introduced by site-directed mutagenesis. pAH85 has a silent *SpeI* site at codon 112 of *phoB* that was introduced with PCR. Mutations were separated by subcloning the respective fragments into pSK58 or pAH85. Only relevant restriction sites are shown. All sites shown for pAH85 are unique. The 2.5-kbp vector backbone is not shown.

Mutagenesis, *attP* Plasmid Integration, and Mutant Selection. Plasmid libraries were made by PCR mutagenesis (19) of *phoB* using pSK5 (20) as template, after which gel-purified *NdeI* to *BamHI* fragments were cloned into pSK49 Δ -*uidA2* or pSK50 Δ -*uidA2* (Fig. 1); plasmids were propagated in a *pir*⁺ host and then integrated into the chromosome of BW23042 or BW23425 to construct mutant libraries with *phoB* in single copy. Cells carrying pINT-ts were grown in Luria-Bertani broth with ampicillin at 30°C, transformed by electroporation, incubated at 37°C for 1 hr, 42°C for 30 min, spread onto glycerol Mops agar containing 2 mM P_i, arabinose, kanamycin, and X-Gal, and incubated at 37°C.

PCR Testing for Copy Number. Two primers specific for the vector *attP* region and two specific for the chromosomal *att* λ region were used to verify strains with a single integrated plasmid. Details will be described elsewhere.

P1-Int-Xis (PIX) Cloning and DNA Sequencing. We reasoned that we may be able to retrieve integrated *oriR_{R6K}* γ *attP* plasmids using a helper plasmid encoding both Int and Xis, which catalyze excision and recircularization of phage λ (21). Indeed, we showed that such plasmids are retrievable by P1*k*c transduction of a *pir*⁺ (or *pir-116*) host synthesizing Int and Xis, a procedure we named "PIX cloning" for P1-Int-Xis cloning. Accordingly, BW23473 carrying pAH57 was grown in Luria-Bertani broth with ampicillin at 30°C and, following infection and 2–3 hr incubation at 37°C, Kan^R colonies were selected. These were inoculated into Luria-Bertani–kanamycin broth to prepare plasmid DNAs, which were used for DNA sequencing and integration into new hosts. Both DNA strands were sequenced using an Applied Biosystems model 373A automated sequencer at the Dana-Farber Cancer Institute (Harvard Medical School) Core facility. Mutations were separated using a combination of cloning and PCR strategies with the plasmids shown in Fig. 1.

Enzyme Assays. Measurements of bacterial alkaline phosphatase and β -galactosidase activities have been described (15).

RESULTS

Genetic Strategy for Identifying Mutants. We considered that gain-of-function PhoB mutants isolated in a strain synthesizing the nonpartner kinase 'VanS may include altered recognition PhoB^{AR} mutants that would be recognizable as ones requiring 'VanS for activation. We expected other gain-of-function mutants to include PhoB^{AR} mutants requiring an

endogenous kinase(s), as well as constitutively active (CA) mutants. We therefore developed reporter strains to facilitate the identification and characterization of 'VanS-activated PhoB^{AR} mutants. With one exception these strains are Δ *phoBR*, Δ *creC*, and Δ (*pta ackA*), so that production of activated PhoB depends upon the introduction of a *phoB* gene; activation of wild-type (wt) PhoB is disabled due to the combined absence of PhoR, the alternative kinase CreC, and acetyl phosphate (22). One otherwise isogenic Δ *phoB-phoR*⁺ strain was used to test the effects of PhoR^{wt}. Many strains encode a mutant kinase ('VanS or 'PhoR) that behaves like a "constitutive kinase" because its N-terminal domain is absent. 'VanS (or 'PhoR) is synthesized from the tightly regulated *P*_{araB} promoter (23); this is done so that we can identify PhoB^{AR} mutants activated by 'VanS as ones showing activation only in the presence of arabinose. Strains with a single copy *P*_{araB}-*vanS* (or -*phoR*) fusion were used because preliminary experiments showed that only strains with a chromosomal (but not ones with a plasmid) *P*_{araB}-*phoR* fusion provided sufficiently tight arabinose control for activation of PhoB^{wt} by PhoR^{wt} (data not shown). In addition, strains carry two reporters for activation of PhoB, the PhoB-dependent *P*_{phnC-lacZ} fusion (encoding β -galactosidase) and the *phoA* gene (encoding bacterial alkaline phosphatase). Thus, mutants are easily identified on indicator agar.

Mutant Isolation. Our genetic strategy is illustrated in Fig. 2. We prepared several *phoB* plasmid libraries by random PCR mutagenesis followed by cloning the *phoB* coding region into a conditionally replicative *ori*R_{R6K}*γ attP* plasmid that requires the π replication protein (the *pir* gene product of the R6K plasmid) for DNA replication (Fig. 1). After propagation in a *pir*⁺ host, the plasmids were transformed into a (non-*pir*) *P*_{phnC-lacZ}, *P*_{araB}-*vanS* reporter strain carrying pINT-ts. Integrants resulting from site-specific recombination at *att* λ were selected on X-Gal agar containing kanamycin and arabinose. Lac⁺ colonies representing 0.1% or more of the total were screened for ones showing an arabinose-dependent Lac⁺ phenotype. Colonies showing arabinose dependence were candidates for VanS-activated PhoB^{AR} mutants; others were candidates for PhoB^{CA} mutants.

Mutant Characterization. We characterized 11 mutants, including 6 candidates for PhoB^{AR} mutants, 2 candidates for PhoB^{CA} mutants, and 3 colonies showing no activation above background (PhoB^{wt} or null mutants). PIX cloning was used to retrieve each integrated plasmid; the retrieved plasmids were again integrated into the same and different reporter strains to test each PhoB protein for activation by 'PhoR, 'VanS, 'VanS_{H164Q}, or PhoR^{wt} (under conditions of P_i limitation) and

to test for deactivation or inhibition by PhoR^{wt}. All PhoB^{AR} mutants were activated by 'PhoR, 'VanS, and PhoR^{wt}; none was activated by 'VanS_{H164Q}, in which the histidine autophosphorylation site (residue 164) has been changed to a glutamine. In the presence of excess P_i, activation of all six PhoB^{AR} mutants by 'VanS was inhibited by PhoR^{wt}; in contrast, the two PhoB^{CA} mutants were not deactivated by PhoR^{wt}. Two PhoB^{wt} plasmids served as controls because they showed normal regulation by 'PhoR and PhoR^{wt} and no effect due to 'VanS. A single plasmid encoding a null PhoB protein that was not activated by any kinase served as another control.

DNA sequencing revealed that all 11 *phoB* coding regions had between 2 and 7 nucleotide changes, including several silent ones. Four PhoB^{AR} mutants had the T97A amino acid change and different silent changes; one PhoB^{AR} mutant had two residue changes (M17V and E87D); the sixth had three residue changes (Q28L, T83S, and S138R). The PhoB^{CA} mutants had two (N40K and T76A) or five (M55V, T83S, E159G, T168S, and F229Y) residue changes. One PhoB^{wt} had no amino acid change; the other had a single residue change (S175C). The PhoB null mutant had three changes (M17L, L95P, and D196G). The PhoB_{T97A} and PhoB_{M17V,E87D} PhoB^{AR} mutants were isolated from pSK50 Δ -*uidA2* libraries; the PhoB_{Q28L,T83S,S138R} PhoB^{AR} mutant was isolated from a pSK49 Δ -*uidA2* library (Fig. 1).

Activation of PhoB^{AR} Mutants. In our reporter strains, activation of PhoB^{wt} by 'PhoR occurs only in the presence of arabinose (Table 1). The low basal level seen in the absence of arabinose is similar to that seen in the absence of a *phoR* or *phoB* gene (data not shown). Also, in these reporter strains, no activation of PhoB^{wt} by 'VanS is evident. In contrast, 'VanS activates PhoB_{M17V,E87D} and PhoB_{T97A} >200-fold and >400-fold, respectively. Furthermore, activation of these PhoB proteins by 'VanS occurs to levels similar to those when PhoB^{wt} is activated by 'PhoR. Activation levels of PhoB_{T97A} and PhoB^{wt} by 'PhoR are nearly the same; activation of PhoB_{M17V,E87D} by 'PhoR is enhanced \approx 3- to 5-fold when compared with activation of PhoB^{wt} by 'PhoR. Similar effects are seen when activation is compared using *phoA* or *P*_{phnC-lacZ} as reporter.

Effects of Individual Residue Changes on Activation. We assessed the contributions of individual changes by constructing integration plasmids and strains with each mutation alone. Our results showed that both M17V and E87D are required for the 200-fold activation of PhoB_{M17V,E87D} by 'VanS, although a 5-fold activation of PhoB_{E87D} is also apparent (Table 2). However, the M17V change appears to be solely responsible for the enhanced activation of PhoB_{M17V,E87D} by 'PhoR. In contrast, T83S alone is sufficient for the 400-fold activation of PhoB_{Q28L,T83S,S138R} by 'VanS. None of these residue changes alone showed a substantial effect on activation by 'PhoR (Table 2) or activation, deactivation, or inhibition by PhoR^{wt} (data not shown).

DISCUSSION

We used a new strategy to identify the protein interface of PhoB that appears to be critical for interactions between kinase (transmitter) and response regulator (receiver) domains of two-component signal transduction proteins. It involves (i) tightly regulated synthesis of one component (the nonpartner 'VanS under tight arabinose control) and (ii) an *in vivo* assay system (a reporter strain with a PhoB-regulated promoter-*lacZ* fusion) to recognize PhoB^{AR} mutants that can be activated by 'VanS. In preliminary experiments, we showed that strains with the homologous kinase PhoR under the same tight regulation reveal arabinose-dependent activation of PhoB^{wt}. We therefore isolated gain-of-function mutants as Lac⁺ colonies in the presence of arabinose (for induction of 'VanS synthesis) and recognized PhoB^{AR} mutants as ones

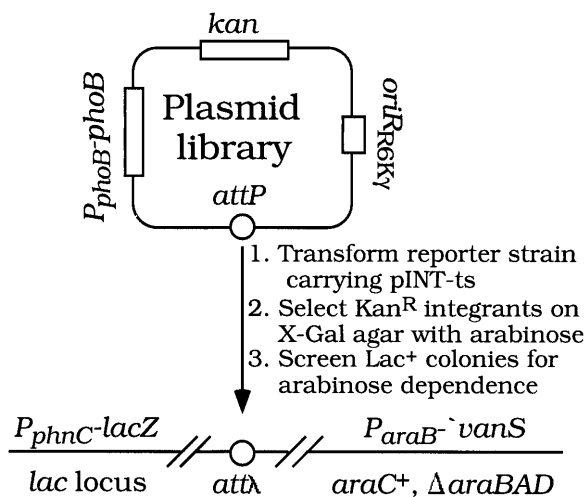


FIG. 2. Genetic strategy for isolating PhoB^{AR} mutants.

Table 1. *In vivo* activation of PhoB proteins by 'PhoR and 'VanS kinases

Regulator	Kinase [†]	Bap sp act*		β -galactosidase sp act [†]	
		No arabinose	With arabinose	No arabinose	With arabinose
PhoB ^{wt}	None	0.3 \pm 0.1	0.3 \pm 0.0	6.1 \pm 0.2	6.5 \pm 0.7
PhoB ^{wt}	'PhoR	0.4 \pm 0.1	185 \pm 3	6.4 \pm 0.2	194 \pm 7
PhoB ^{wt}	'VanS	0.4 \pm 0.2	0.3 \pm 0.0	6.1 \pm 0.4	6.4 \pm 0.1
PhoB _{M17V,E87D}	'PhoR	1.2 \pm 1.5	516 \pm 36	7.5 \pm 2.2	968 \pm 8
PhoB _{M17V,E87D}	'VanS	0.4 \pm 0.2	63.5 \pm 1.3	5.7 \pm 0.5	53.1 \pm 4.4
PhoB _{M17V,E87D}	'VanS _{H164Q}	0.3 \pm 0.0	0.7 \pm 0.2	6.7 \pm 0.2	4.7 \pm 0.1
PhoB _{T97A}	'PhoR	0.6 \pm 0.0	174 \pm 8	6.7 \pm 0.1	204 \pm 10
PhoB _{T97A}	'VanS	0.4 \pm 0.0	125 \pm 9	6.4 \pm 0.3	127 \pm 7

Cells were assayed after growth in 0.06% glycerol Mops 2 mM P_i media without or with arabinose. Bap, bacterial alkaline phosphatase.

*Specific activity (sp act) units are nanomoles of product formed per minute per cell optical density at 420 nm. Means of triplicate determinations with standard deviations are given.

[†]Strains are integrants of BW23316 [like BW23423, except Δ (*araBAD*)_{AH37}], BW23423, BW23425, or BW23427 with a pSK50 derivative encoding the respective PhoB protein.

requiring arabinose for the Lac⁺ phenotype. We anticipated being able to isolate PhoB^{AR} mutants showing enhanced activation by the heterologous 'VanS kinase because of the initially weak interaction of 'VanS with PhoB^{wt}; the reactivity between a maltose-binding protein-'VanS fusion protein (MBP-'VanS) and PhoB^{wt} is 10⁴-fold weaker than between MBP-'VanS and its partner response regulator VanR (20). Our use of a heterologous kinase to isolate PhoB^{AR} mutants is in principle similar to the isolation of suppressor mutations as extragenic compensatory mutants. In similar ways, it should be possible to isolate response regulator or kinase mutants showing increased interaction with other heterologous partners.

The N-terminal receiver domain of PhoB is expected to share structural conservation with the chemotaxis response regulator CheY (24), the sporulation phosphorelay protein SpoOF (25), and the N-terminal domain of *E. coli* NarL (26) whose structures have been solved by x-ray crystallography. Based on the CheY structure, all six PhoB^{AR} mutants have one residue changed (T83, E87, or T97) in the loop preceding helix 4 or in helix 4 (Fig. 3). The helix 4 region of CheY is thought to interact with the kinase CheA as changes in this region reduce affinity for CheA (30, 31). CheY changes reducing affinity are generally nonconservative ones, however. In contrast, PhoB changes showing enhanced activation by 'VanS are usually conservative ones (T83S, E87D, T97A). Conservative changes among closely related proteins may therefore be more significant than nonconserved residues for determining specificity of kinase-regulator interactions. Our results provide further evidence that the helix 4 region may be critical for interactions of response regulators and histidine kinases.

The PhoB^{AR} mutants described here show nearly as high activation by 'VanS as by 'PhoR. Different classes of PhoB^{AR} mutants may be revealed by using plasmids with more strin-

gently controlled PhoB synthesis. No change leading to enhanced activation by 'VanS abolished activation by 'PhoR. Multiple changes may be necessary to gain recognition by 'VanS and to lose recognition by PhoR simultaneously. Mutants showing enhanced activation by 'VanS and reduced activation by 'PhoR may be found in a more exhaustive search. It is notable that no PhoB^{AR} change makes PhoB more like the natural VanR sequence. On the contrary, T83 in PhoB and T81 in VanR are equivalent. Yet, the T83S change results in enhanced activation of PhoB by 'VanS. Clearly, the context of these residues is especially important for defining the recognition interface. In addition, the respective residue in SpoOF (S87) is structurally linked to the phosphorylation site (D59) via an intermediate residue (N61; ref. 25).

Our finding of one mutant with two distantly located changes (M17V and E87D), which are both required for activation by 'VanS, was at first unexpected. However, it has been recently shown that changes in helix 1 of CheY result in a conformational change (among others) in helix 4 that may mimic its molecular activation mechanism (32). Hence, M17V and E87D together may alter the helix 4 region resulting in enhanced activation by 'VanS. Changes in helix 1 of CheY and SpoOF also confer resistance to the phosphatase activity of CheZ (33) and Rap (25), respectively. Accordingly, M17V may act by abolishing interaction with a phospho-PhoB phosphatase, allowing more efficient activation of the PhoB_{M17V,E87D} protein by 'VanS. In this regard, deactivation (dephosphorylation) of phospho-PhoB *in vivo* requires the Pst system and PhoU. While the Pst system and PhoU were always present in this study, no evidence indicates that a Pst component or PhoU acts on PhoB in the absence of PhoR (7).

The PhoB_{T97A} and PhoB_{M17V,E87D} proteins have now been purified to homogeneity. Preliminary kinetic studies of these PhoB^{AR} proteins show that phosphotransfer from phospho-

Table 2. *In vivo* activation of wild-type and mutant PhoB proteins

Regulator	By 'PhoR*		By 'VanS [†]	
	Bap sp act	Relative to PhoB ^{wt}	Bap sp act	Relative to PhoB ^{wt}
PhoB ^{wt}	186 \pm 13	1.0	0.3 \pm 0.0	1.0
PhoB _{M17V,E87D}	469 \pm 10	2.5	55.0 \pm 15.9	167
PhoB _{E87D}	207 \pm 25	1.1	1.7 \pm 0.2	5.2
PhoB _{M17V}	410 \pm 20	2.2	0.5 \pm 0.1	1.6
PhoB _{T97A}	182 \pm 33	1.0	142 \pm 4	432
PhoB _{O28L,T83S,S138R}	236 \pm 2	1.3	128 \pm 11	389
PhoB _{T83S}	217 \pm 21	1.2	137 \pm 6	416

Cells were grown with arabinose and assayed as in Table 1. Each *phoB* allele is in single copy in a pSK58 or pAH85 derivative. Bap, bacterial alkaline phosphatase; sp act, specific activity.

*Integrants of BW23423 were assayed. No arabinose values were 0.3 \pm 0.0 or less.

[†]Integrants of BW23425 were assayed. No arabinose values were 0.6 \pm 0.0 or less.

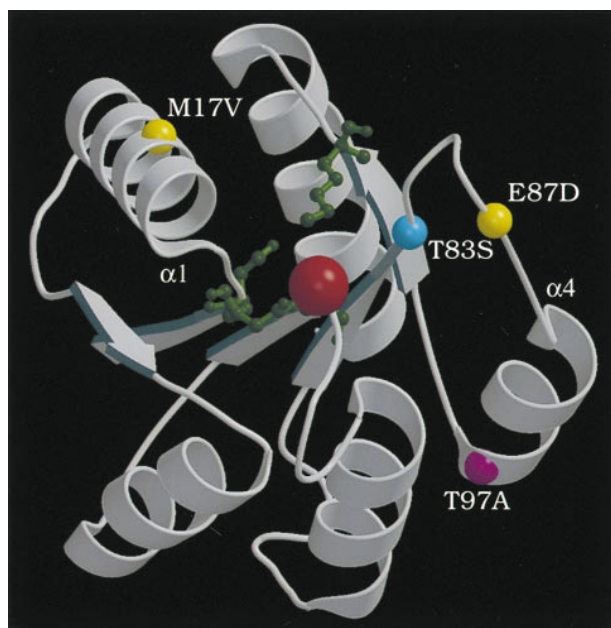


FIG. 3. Ribbon diagram of Mg^{2+} -bound CheY crystal structure from coordinates (27), drawn with MOLSCRIPT (28) and RASTER3D (29). CheY active site residues D12, D13, D57, and K109 are depicted as green ball-and-stick forms; a red ball shows Mg^{2+} . The locations of the equivalent PhoB residues are shown as colored balls: T83 (cyan), M17 and E87 (yellow), and T97 (purple). The amino termini of helix 1 and helix 4 are labeled $\alpha 1$ and $\alpha 4$, respectively.

MBP-'VanS to PhoB_{T97A} and PhoB_{M17V,E87D} is 20-fold and 15-fold more rapid, respectively, than phosphotransfer to PhoB^{wt} (20). The increased rates of phosphotransfer appear to be primarily due to changes in k_{cat} without appreciable effects on K_M values (unpublished data). An alteration in the PhoB interface may result in a different orientation of MBP-'VanS binding and thereby be responsible for such kinetic effects. More detailed studies are required to understand the biochemical basis of the enhanced activation of PhoB^{AR} proteins by 'VanS.

Our approach for studying protein-protein interactions may have widespread application, not only for studying interactions between components of the vancomycin resistance and the P_i signal transduction pathways, but also for analyzing interactions between partner proteins of other two-component regulatory systems, as well as other interacting proteins involved in many diverse intracellular processes. For example, searching for mutants of PhoR capable of activating a nonpartner response regulator should allow one to define a domain(s) and residues of a signaling kinase for recognition of response regulators. Furthermore, when the activity of an expressed (given) protein depends on the presence (or absence) of a second protein, this indicates an interaction between the respective proteins. Therefore tightly regulated synthesis of other interacting proteins can be used to identify mutants affecting interactions with the partner protein(s) or to identify new interacting proteins.

Our searches for PhoB^{AR} mutants involved use of a new cloning strategy that may be generally useful, not only for the isolation of mutants but also for the construction of genomic libraries. We made mutant libraries by cloning mutagenized *phoB* genes into conditionally replicative *oriR_{6K γ} attP* plasmids, which were then integrated into the chromosome of a reporter strain by site-specific recombination. We were therefore able to screen mutants while avoiding problems often inherent in the use of multicopy plasmids. We subsequently retrieved the mutant plasmids by PIX cloning, a remarkably efficient process. Similar conditionally replica-

tive *oriR_{6K γ} attP* plasmids should also be useful as general cloning vectors for preparation of genomic libraries that can be maintained more stably as integrated plasmids and later retrieved individually or en masse by PIX cloning. Furthermore, the plasmid size of such genomic libraries should be limited only by the amount of DNA packageable by phage P1 (≈ 100 kbp).

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