Interdomain Linkers of Homologous Response Regulators Determine Their Mechanism of Action

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OmpR and PhoB are response regulators that contain an N-terminal phosphorylation domain and a C-terminal DNA binding effector domain connected by a flexible interdomain linker. Phosphorylation of the N terminus results in an increase in affinity for specific DNA and the subsequent regulation of gene expression. Despite their sequence and structural similarity, OmpR and PhoB employ different mechanisms to regulate their effector domains. Phosphorylation of OmpR in the N terminus stimulates the DNA binding affinity of the C terminus, whereas phosphorylation of the PhoB N terminus relieves inhibition of the C terminus, enabling it to bind to DNA. Chimeras between OmpR and PhoB containing either interdomain linker were constructed to explore the basis of the differences in their activation mechanisms. Our results indicate that effector domain regulation by either N terminus requires its cognate interdomain linker. In addition, our findings suggest that the isolated C terminus of OmpR is not sufficient for a productive interaction with RNA polymerase.

Escherichia coli contains 28 histidine kinases and 32 response regulators (43) that couple changes in the environment to changes in gene expression or protein function (for a review, see reference 56). In their simplest form, two-component systems are comprised of a sensor kinase and a response regulator. The sensor kinase, typically a membrane-bound protein, responds to specific environmental signals by modulating its ability to phosphorylate (and in some cases dephosphorylate) its cognate response regulator. Response regulators are usually DNA binding proteins containing two domains separated by a flexible linker. The N-terminal receiver domain modulates the activity of the C-terminal effector domain. Most often, phosphorylation of the N terminus enhances the affinity of its C terminus for specific DNA, resulting in a subsequent change in gene expression.

In *E. coli* and related enteric bacteria, EnvZ is a membrane-bound histidine kinase (12) that undergoes autophosphorylation by intracellular ATP (EnvZ-P) (1, 13, 20). EnvZ-P transfers the phosphate to its cognate response regulator, OmpR (1, 2, 13, 21, 22). OmpR reciprocally regulates transcription of the outer membrane porin genes *ompF* and *ompC* in response to changes in medium osmolarity (14, 18, 32, 47, 53, 54). OmpF is the predominant porin at low osmolarity, whereas OmpC predominates at high osmolarity (4). PhoB is a response regulator in the same subfamily and is highly homologous to OmpR. Under conditions of limiting inorganic phosphate, PhoB activates the transcription of a regulon involved in the metabolism and uptake of phosphorus-containing compounds (33, 51, 63, 64; for a review, see reference 65). Phosphorylation by the inner-membrane-bound histidine kinase PhoR controls the levels of phosphorylated PhoB in response to extracellular inorganic phosphate levels (34, 37, 66).

Response regulators share a conserved, doubly wound α/β fold and phosphorylation site geometry in their N terminus but are grouped into subfamilies based on the structure of their C-terminal domains (31, 55, 62). OmpR and PhoB are members of the winged-helix-turn-helix family of DNA binding proteins (6; for reviews, see references 23, 26, and 38). The major differences between OmpR and PhoB reside in the interdomain linker (15 residues versus 6 residues, respectively) and in the transactivation loop that precedes the DNA recognition helix (45). Apart from these differences, the C-terminal structures of OmpR (39) and PhoB (45) are readily superimposed (23). Despite their significant structural homology, OmpR and PhoB employ different mechanisms to regulate their DNA binding domains and activate transcription. Phosphorylation of OmpR enhances its affinity for specific DNA (2, 16, 17), and substitution of aspartate 55, the site of phosphorylation (10), with glutamine (24) or alanine (V. K. Tran and L. J. Kenney, unpublished data) renders OmpR unable to activate transcription. The isolated C terminus of OmpR (OmpR$_C$) binds to DNA only weakly (25, 57) and is unable to activate transcription (reference 60 and this work). These findings indicate that OmpR$_C$ requires its phosphorylated N-terminal domain for activation and suggest that the N terminus of OmpR (OmpR$_N$) may be required for proper interactions with RNA polymerase (RNAP). In contrast, the isolated C terminus of PhoB (PhoB$_C$) is constitutively active for transcription (36) and has a higher affinity for specific DNA than full-length, unphosphorylated PhoB (11). The unphosphorylated N terminus of PhoB (PhoB$_N$) inhibits its DNA binding domain, and genetic evidence suggests that the $\alpha5$ helix of the N terminus is responsible for the inhibition of PhoB$_C$ (3). In summary, phosphorylation of OmpR enhances DNA binding affinity whereas phosphorylation of PhoB results in a relief of the inhibition of DNA binding.

We are interested in the structural basis of the differences with which the highly homologous response regulators OmpR and PhoB regulate their DNA binding domains. Evidence

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TABLE 1. Oligonucleotides used in plasmid constructions[b]

Oligo-nucleotide	Sequence from 5′ to 3′[a]
OmoR-S	GCGGTGCTGC<u>GTCGAC</u>AGGCGAAGGAACTGC
PhoB-S	GCGGTAATGC<u>GTCGAC</u>TTTCGCCAATGGCG
OmpR-X	CCGTCACAGGAAGAGGCG<u>CTCGAG</u>GCTTTCGGTAAGTTC
PhoB-X	CCAATGGCGGTGGAAGAG<u>CTCGAG</u>GAGATGCAGGG
BBR	CCAATGGCGGTGGAAGAGGTCATTGCATTCGGTAAGTTC
BRR	CCGTCACAGGAAGAGGCGGTAATTGCTTTCGGTAAGT
OmpR5	<u>GAATTC</u>ACCATGCAAGAGAACTACAAGATTCTG
PhoB3	CTGCAG<u>AAGCTT</u>TTAAAAGCGGGTTGAAAAACGA

[a] Only top-strand sequences are shown.
[b] Engineered restriction sites are underlined.

from our laboratory indicates that the OmpR interdomain linker is important for signaling to the C terminus (5, 27, 41). To better understand the functions of the N-terminal domain and interdomain linker in effector domain regulation, we constructed chimeras between OmpR and PhoB that contained either interdomain linker (Fig. 1). In the present work, we emphasize the distinct role of the interdomain linker in the communication between the N terminus and C terminus of response regulators. From the results presented here, it is apparent that structural homology does not always infer functional homology, even among closely related members of the same response regulator subfamily.

MATERIALS AND METHODS

Construction of OmpR-PhoB chimeras. The *ompR* and *phoB* genes were subcloned into a Bluescript KS vector (Novagen) by using the *Xba*I and *Hin*dIII restriction sites to create templates for subsequent constructions (pOmpR and pPhoB, respectively). The following oligonucleotide sequences are listed in Table 1. Silent *Sal*I sites were engineered at the 5′ end of the *ompR* and *phoB* linkers by site-directed mutagenesis using oligonucleotides OmpR-S and PhoB-S to create pOmpR-Sal and pPhoB-Sal, respectively, following a method described previously (58). *Xho*I sites were engineered at the 3′ end of the *ompR* and *phoB* linkers by using oligonucleotides OmpR-X and PhoB-X to create pOmpR-Xho and pPhoB-Xho, respectively. Domain swapping was carried out by cutting recipient and donor plasmids with the appropriate restriction enzymes and ligating the relevant fragments with T4 DNA ligase. For example, the BBR chimera was constructed by replacing the *Xho*I-*Hin*dIII fragment of pPhoB-Xho with that from pOmpR-Xho. The remaining chimeric genes were constructed in a similar manner. The *Xho*I sites were removed from the BBR and RRB chimeric genes by using oligonucleotides BBR and RRB, respectively. For the alkaline phosphatase assays, the RBB and RRB chimeras were PCR amplified with primers OmpR5 and PhoB3 and subcloned into the *Eco*RI and *Hin*dIII sites of the arabinose-inducible expression vector pMPM-A6 (42) to create plasmids pARA-RBB and pARA-RRB, respectively. *E. coli* strain DH5α was used for cloning. Standard cloning techniques were described previously (50). Restriction enzymes were obtained from Life Technologies, T4 DNA ligase was obtained from Roche, and *Pfu* polymerase was purchased from Stratagene. The correct DNA sequence of the final construct was confirmed by automatic sequencing done by the Molecular Microbiology & Immunology Core Facility at Oregon Health & Science University.

Expression and purification of the response regulators. The *ompR-phoB* chimeras and wild-type *phoB* were subcloned into pET22b (Novagen) by using the *Xba*I and *Hin*dIII restriction sites. *E. coli* strain BL21(DE3) (Novagen) was used for protein expression. One liter of cells in Luria-Bertani medium was grown at 37°C to an optical density at 600 nm (OD_{600}) of approximately 0.6 before induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). The cells were harvested 3 h after induction. The cell pellet was resuspended in TGED (25 mM Tris [pH 7.6], 5% glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol) contain-

ing 1 mM AEBSF [4-(2-aminoethyl)benzene-sulfonyl fluoride; ICN Biomedicals]. After lysis with a French press (two passages at 10,000 lb/in^2) and centrifugation for 1 h at 30,000 × *g*, the supernatant was dialyzed overnight in TGED before loading onto a Hi-Trap heparin column (Pharmacia). Proteins were eluted with a linear gradient of 0 to 500 mM NaCl. The desired fractions were combined. NaCl (2 M) was added to the pooled fractions before loading onto a phenyl-Sepharose column (Pharmacia) and eluted with a linear gradient of 2 to 0 M NaCl. Pooled fractions were judged to be >90% pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). OmpR and $OmpR_N$ proteins were purified as described by Kenney et al. (27) with the modifications detailed in reference 16.

DNA binding. Fluorescence anisotropy was used to measure equilibrium binding, and the experiments were performed as previously described (16). The sequences of the oligonucleotides used were the same as those used in the previous study. The results from the binding curves were fit by nonlinear least-squares regression as described (16). The apparent dissociation constants from the individual binding curves are reported in Table 2. The values shown in Table 2 represent the mean ± the standard deviation.

EnvZ115 and PhoR purification and phosphorylation. EnvZ115 was expressed and purified as previously described (20), and the kinase assay was conducted as described previously (28, 59). Thioredoxin-tagged PhoR was a gift from William McCleary, and the kinase assays were performed as for EnvZ. Modifications to the kinase assay are listed in the figure legends.

β-Galactosidase and alkaline phosphatase assays. Wild-type *ompR*, *BRR*, and *BBR* were subcloned into pFR29*, an *envZ* null derivative of plasmid pFR29 (48). The plasmid was used to transform the *ompR* deletion strains MH513.101 and MH225.101, which contain chromosomal copies of the *ompF-lacZ* and *ompC-lacZ* fusions, respectively (15). β-Galactosidase assays were carried out according to the method of Tran et al. (58). β-Galactosidase activity was expressed in Miller units and calculated by using the formula $1,000 \times OD_{420}/(\text{time [min]} \times \text{culture volume [ml]} \times OD_{600})$.

Plasmids pARA-BBR and pARA-BRR were transformed into strain BW25113 or BW28669 (Barry Wanner, Purdue University) for the *phoA* assays. BW28669 is an *envZ* mutant derivative of BW25113 constructed by following a method described previously (9). Alkaline phosphatase assays were performed as follows. Transformants were grown to mid to late log phase, and OD readings were obtained by using a Molecular Devices UVmax microtiter plate reader. A 1-ml volume of cells was resuspended in 1 ml of 1 M Tris (pH 8.2) and lysed with SDS and chloroform, and debris was removed by brief centrifugation. Assays were carried out on microtiter plates with 200 μl of cell extract and 20 μl of 4 mM *o*-nitrophenyl phosphate (Sigma). After 15 min at 37°C, 80 μl of 1 M KH_2PO_4 was added to each reaction mixture before obtaining OD_{405} readings. Alkaline phosphatase activity (arbitrary units) was expressed as $100 \times OD_{405}/OD_{570}$.

RESULTS

PhoB$_N$-OmpR$_C$ chimeras (BRR and BBR) are unable to activate transcription of OmpR-dependent genes. We were interested in whether or not chimeras containing the OmpR DNA binding domain were able to activate the transcription of *ompF* or *ompC* (BBR and BRR [Fig. 1]). The chimeras were expressed in multicopy in *ompR* null strains containing chromosomal copies of *ompF-lacZ* or *ompC-lacZ* operon fusions (15). The results of the β-galactosidase assays are depicted in Fig. 2. In rich media, the strains containing a chromosomal

TABLE 2. Summary of DNA binding measurements

Oligonucleotide[a]	K_d (nM) ± SD	
	BRR	BBR
F1	108 ± 32	NSB[b]
F123	133 ± 13	NSB
C1	73 ± 39	295 ± 52
C123	NSB	NSB

[a] Neither chimera bound to the low-affinity sites F2, F3, C2, and C3 (data not shown).
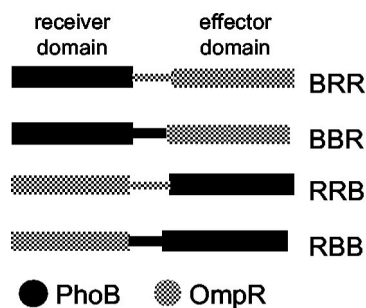[b] NSB, nonsaturable binding.

FIG. 1. Structures of the OmpR-PhoB chimeras. The PhoB N terminus, linker, and C terminus are depicted in black. The OmpR domains are shown in grey. The three-letter chimera designations, from left to right, refer to the sources of the N terminus, linker, and C terminus, respectively.

copy of *ompR* expressed both *ompF* and *ompC* while transcription of either porin gene was not observed in the *ompR* mutant strains (Fig. 2). When the chimeras (BRR and BBR) were expressed in the *ompR* null reporter strains, neither was able to activate transcription of *ompF* or *ompC* (Fig. 2). These results indicate that both chimeras are defective in activating the transcription of OmpR-dependent genes. We also tested whether or not OmpR$_C$ activated the transcription of single-copy reporter fusions. Overexpression of OmpR$_C$ did not result in the activation of either *ompF* or *ompC*, indicating that OmpR requires its N terminus for transcriptional activation (Fig. 2).

PhoB$_N$-OmpR$_C$ chimeras (BRR and BBR) are phosphorylated by PhoR. One explanation for the inability of the PhoB$_N$-OmpR$_C$ chimeras to activate the transcription of OmpR-dependent genes is that they may not be phosphorylated by PhoR, the cognate kinase for the PhoB N terminus.
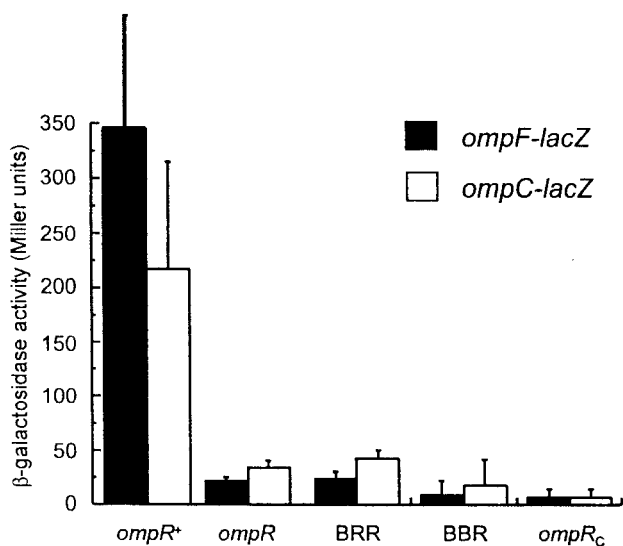

FIG. 2. Transcriptional activation by the PhoB$_N$-OmpR$_C$ chimeras. β-Galactosidase assays were performed as described in Materials and Methods. Activities of the *ompF-lacZ* strain MH513.101 and the *ompC-lacZ* strain MH225.101 are shown. The *ompR$^+$* strains are derivatives of MH225.101 and MH513.101 that contain a chromosomal copy of *ompR*. At least two independent assays were performed on each strain; the error bars indicate +1 standard deviation. *ompR*, *ompR* null.
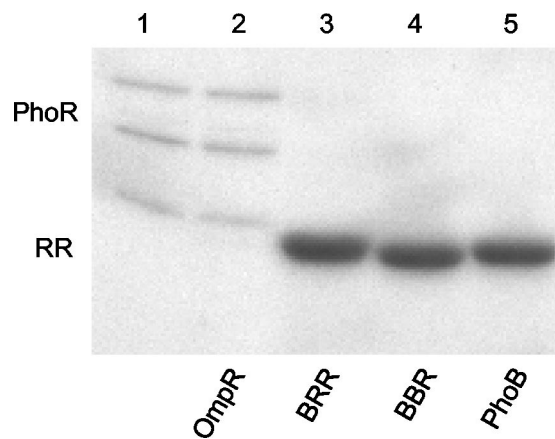

FIG. 3. Phosphorylation of the PhoB$_N$-OmpR$_C$ chimeras by PhoR. The kinase assay is described in Materials and Methods. Lane 1 contains 2 μg of thioredoxin-tagged PhoR that has been autophosphorylated by [γ-^{32}P]ATP; the three bands correspond to the proteolytic cleavage products of PhoR. The response regulator (RR; 5.5 μg) in each reaction is indicated by the labels under the gel. The reactions were carried out for 30 min at 37°C and stopped by the addition of SDS-PAGE loading buffer.

We used a kinase assay to examine the phosphotransfer between PhoR and the chimeras (Fig. 3). Preincubation of PhoR with [γ-^{32}P]ATP resulted in autophosphorylation (PhoR-P), as shown in lane 1 of Fig. 3. During expression and/or purification of this preparation, PhoR underwent proteolysis, as evident from the three bands of varying size corresponding to phosphoprotein (William McCleary, personal communication). To examine kinase specificity, PhoR-P was mixed with the noncognate response regulator OmpR. No evidence of phosphotransfer between PhoR and OmpR was indicated (Fig. 3, lane 2). We also tested a higher ratio of PhoR to OmpR (1:1) and did not observe phosphotransfer (data not shown). In contrast, when PhoB or either chimera containing PhoB$_N$ was incubated with PhoR-P, phosphotransfer occurred (Fig. 3, lanes 3 through 5). The results shown in Fig. 3 also demonstrate that phosphotransfer between PhoR and the chimeras occurs with an efficiency similar to that for the phosphotransfer between PhoR and PhoB, suggesting that the presence of a heterologous C terminus does not affect the phosphorylation of PhoB$_N$ when PhoR-P is the phosphodonor. Thus, the lack of transcriptional activation observed in Fig. 2 is not due to an inability of the chimeras to be phosphorylated.

PhoB and PhoB$_N$-OmpR$_C$ chimeras (BRR and BBR) are phosphorylated by the noncognate kinase EnvZ. Although it would not be expected that OmpR$_C$ would bind to PhoB-specific DNA, it has been suggested that cross talk occurs at the kinase level (59, 61) (see Discussion). As a result, we examined whether or not wild-type PhoB could be phosphorylated by EnvZ. Lane 1 of Fig. 4 demonstrates that EnvZ underwent autophosphorylation in the presence of [γ-^{32}P]ATP (EnvZ-P). When EnvZ-P was mixed with its cognate response regulator, OmpR, phosphotransfer was observed (Fig. 4, lane 2). When PhoB was incubated with EnvZ-P, phosphotransfer was also seen, but to a lesser extent than that with OmpR (Fig. 4, compare lanes 2 and 5). However, the reverse reaction, i.e., phosphorylation of OmpR by the noncognate kinase PhoR,
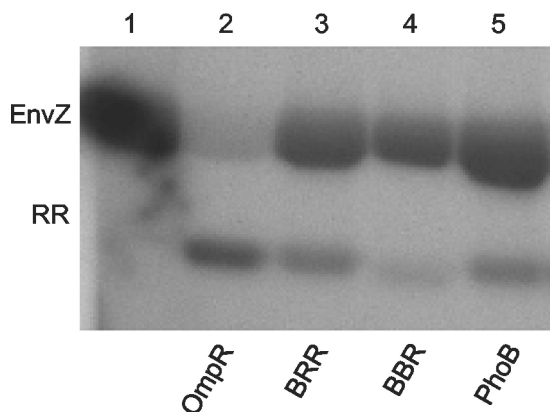
FIG. 4. Phosphorylation of the PhoB$_N$-OmpR$_C$ chimeras by EnvZ. The kinase assay is described in Materials and Methods. Lane 1 contains 35 µg of EnvZ that has been autophosphorylated by [γ-^{32}P]ATP. The response regulator in each reaction (RR; 5.5 µg) is indicated by the labels under the gel. The reactions were carried out for 30 min at 37°C and stopped by the addition of SDS-PAGE loading buffer.

does not appear to have occurred in vitro (Fig. 3, lane 2). Thus, it appears that cross talk is not bidirectional, since EnvZ phosphorylates PhoB (Fig. 4, lane 5), but PhoR does not appear to phosphorylate OmpR in vitro (Fig. 3, lane 2) or requires higher concentrations of PhoR.

We also tested the PhoB$_N$-OmpR$_C$ chimeras to determine if they were phosphorylated by the noncognate kinase EnvZ. When BRR was included in a kinase assay with EnvZ-P, phosphotransfer occurred to a level similar to that observed with wild-type PhoB (Fig. 4, compare lanes 3 and 5). This result further demonstrates that the transcriptional activation defect of BBR and BRR is not due to a defect in phosphorylation. Not surprisingly, phosphorylation of the PhoB$_N$-OmpR$_C$ chimeras by EnvZ is less efficient than is phosphorylation by PhoR. Interestingly, BBR, containing the PhoB linker, is phosphorylated less efficiently by the noncognate kinase EnvZ than is wild-type PhoB or BRR (Fig. 4, lane 4). Perhaps the shorter interdomain linker in BBR positions PhoB$_N$ in a manner that alters the phosphorylation site, making it less accessible.

The linker affects the DNA binding properties of OmpR$_C$. We next examined the DNA binding properties of the PhoB$_N$-OmpR$_C$ chimeras to determine the basis for their defects in transcriptional activation (Fig. 2). Protein-DNA interactions were quantified by fluorescence anisotropy as previously described (16). A representative DNA binding curve is shown in Fig. 5. BRR, containing the OmpR linker, exhibits saturable binding to the high-affinity site F1 (average $K_d = 108$ nM). In contrast, in the presence of BBR, containing the PhoB linker, no saturable binding was observed. The results of our binding assays are summarized in Table 2. The chimera containing the OmpR linker (BRR) binds to *ompF* DNA with high affinity. In addition, when it binds to the isolated site, C1, the K_d is 73 nM. Interestingly, BRR does not bind to the composite *ompC* site C123. Binding at C1, but not C123, has been observed previously (40) (see Discussion). BBR is unable to bind to *ompF* DNA and only weakly binds to the highest affinity site C1 ($K_d = 295$ nM). The results from our binding assays indicate that the source of the interdomain linker governs the DNA binding properties of OmpR$_C$. When the PhoB N terminus is linked to

OmpR$_C$ via the PhoB linker (BBR), DNA binding is prevented. In contrast, the PhoB N terminus is unable to inhibit the DNA binding of OmpR$_C$ at *ompF* when the OmpR linker (BRR) is present. Thus, PhoB$_N$ requires its cognate interdomain linker to inhibit the heterologous OmpR C terminus.

OmpR$_N$-PhoB$_C$ (RRB and RBB) chimeras activate transcription of *phoA*. The inverse chimeras containing the OmpR N terminus and the PhoB C terminus were constructed to determine how PhoB$_C$ behaves when linked to OmpR$_N$ via either interdomain linker (RBB and RRB [Fig. 1]). We examined their ability to activate the transcription of a chromosomal copy of the PhoB-specific reporter gene *phoA*. The chimeric genes were expressed in multicopy in isogenic *envZ*$^+$ and *envZ* null backgrounds. The strain containing RRB exhibited a low level of basal *phoA* activity in the absence of induction (Fig. 6, control). In the absence of the *envZ* kinase gene, the chimeras activated the transcription of *phoA* to similar levels (Fig. 6, black bars). Thus, neither OmpR$_N$-PhoB$_C$ chimera (RRB nor RBB) requires EnvZ for activity. Because the chimeras were constitutively active in an *envZ* null background, the results also indicate that OmpR$_N$, unlike PhoB$_N$, cannot inhibit DNA binding by PhoB$_C$. In an *envZ*$^+$ background, RBB activated transcription to similar levels as in an *envZ* null background (RBB) (Fig. 6, white bar). Interestingly, the activity of RRB was increased almost threefold in an *envZ*$^+$ background compared to that in an *envZ* mutant background (Fig. 6, compare white and black bars of RRB columns). Presumably, OmpR$_N$ is phosphorylated by EnvZ, resulting in the activation of PhoB$_C$, whose activation requires the OmpR linker.

OmpR$_N$-PhoB$_C$ chimeras (RRB and RBB) are phosphorylated by EnvZ. Since RRB was activated by EnvZ in vivo whereas RBB was not (Fig. 6), it was of interest to determine whether EnvZ could phosphorylate the chimeras in vitro. EnvZ was preincubated with [γ-^{32}P]ATP (Fig. 7, lane 1) and
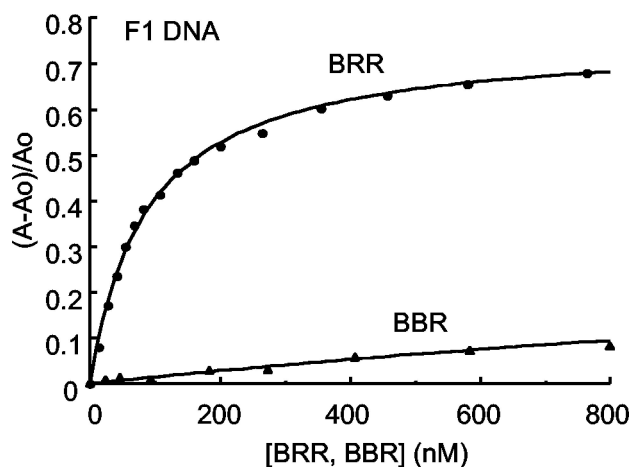

FIG. 5. DNA binding of BRR and BBR to the F1 binding site. BRR (circles) or BBR (triangles) was titrated into the binding reaction in the presence of 3 nM fluorescein-labeled F1 oligonucleotide. The means from five separate measurements performed at each titration point were plotted. The curve illustrates the change in anisotropy (A), where $(A - A_o)/A_o$ represents the difference in anisotropy in the presence of protein minus the anisotropy in the absence of protein divided by the anisotropy in the absence of protein. This value is plotted as a function of the total protein concentration.
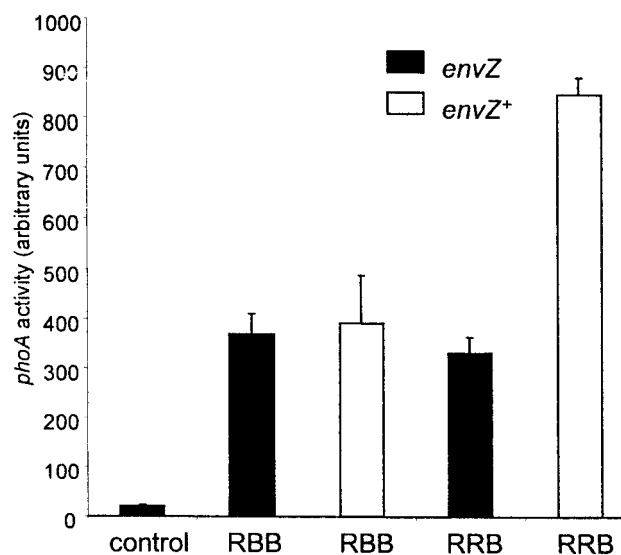
FIG. 6. Transcriptional activation by the OmpR$_N$-PhoB$_C$ chimeras. Alkaline phosphatase assays were performed as described in Materials and Methods. The activities of strains BW25115 (envZ$^+$) and BW28669 (envZ null [envZ]) are indicated by the solid and open bars, respectively; the error bars indicate +1 standard deviation. Assays were performed in triplicate on three independent colonies. The control strain was grown with plasmid pARA-RRB without arabinose induction. RBB and RRB refer to strains containing plasmids pARA-RBB and pARA-RRB, respectively.

then mixed with the indicated response regulator. OmpR$_N$ was used as a positive control for phosphotransfer (Fig. 7, lane 2). As shown in Fig. 7, both chimeras were phosphorylated by EnvZ to similar levels in vitro (lanes 3 and 4). The observation that EnvZ can phosphorylate RRB accounts for the enhancement of transcription observed in the presence of the envZ gene (Fig. 6). However, it was surprising that the RBB chimera was also phosphorylated by EnvZ (Fig. 7, lane 4) yet the phosphorylation of RBB did not result in the activation of its PhoB C terminus. In isogenic envZ$^+$ and envZ null strains, expression of RBB resulted in similar levels of phoA activity (Fig. 6). Because the chimeras differ only in the interdomain linker, the result shown in Fig. 7 further demonstrates that the presence of the OmpR linker activates PhoB$_C$. An interesting observation was that a difference of approximately 600 Da between RBB and RRB resulted in a visible difference in mobility on SDS-PAGE (Fig. 7).

DISCUSSION

OmpR requires its N terminus for transcriptional activation. The PhoB$_N$-OmpR$_C$ chimeras with either linker are unable to activate the transcription of ompF or ompC (Fig. 2). Apparently, PhoB$_N$ cannot function as a substitute for OmpR$_N$ in order to activate the transcription of OmpR-dependent genes. This requirement for OmpR$_N$ is further demonstrated by the inability of an isolated OmpR$_C$ fragment to activate transcription (reference 60 and this work) (Fig. 2). These results are consistent with the observation that PhoB$_N$ regulates its C terminus by an inhibitory mechanism, whereas OmpR$_N$ acts positively on its C terminus. OmpR$_N$ may be required to

stabilize the OmpR$_C$ conformation competent for DNA binding and transcriptional activation. It is noteworthy that OmpR and PhoB interact with different subunits of RNAP holoenzyme in order to activate transcription. PhoB uses the loop between α helices 2 and 3 of its C terminus (i.e., the transactivation loop) to make contacts with σ^{70} of RNAP holoenzyme to activate transcription (19, 29, 35, 36). In contrast, OmpR interacts with the α subunit of RNAP to activate transcription (19, 49, 52). However, the evidence is less clear whether the transactivation loop of OmpR makes contacts with α to activate transcription or whether contacts outside of this region are required. For example, a screen for positive control mutants of OmpR identified residue R42 of the N terminus as being important for transcriptional activation, which is suggestive that the N terminus is important for interaction with RNAP (46). Evidence exists for an interaction with RNAP via the N terminus of another response regulator, Spo0A. A screen for suppressors of a Spo0F-null phenotype identified a mutation in the N terminus of Spo0A (P60S) that results in enhanced interaction with RNAP (8). The possibility that OmpR$_N$ is required for RNAP interactions would account for the inability of the PhoB$_N$-OmpR$_C$ chimeras to activate transcription at OmpR-dependent promoters.

OmpR requires its linker for activation of its C terminus. The inverse chimeras containing the PhoB DNA binding domain (RBB and RRB) demonstrate a requirement for the OmpR interdomain linker to stimulate transcriptional activation by PhoB$_C$. In an envZ$^+$ background, RRB displays an enhanced ability to activate transcription, compared to that in an envZ null background (Fig. 6). Clearly, OmpR$_N$ can activate a heterologous effector domain. In contrast, in the presence of RBB, alkaline phosphatase activity was independent of EnvZ (Fig. 6). The results shown in Fig. 6 indicate that OmpR$_N$ activation of PhoB$_C$ is dependent on the OmpR linker. Because OmpR$_C$ and the PhoB$_N$-OmpR$_C$ chimeras are unable to activate transcription (Fig. 2), our findings indicate that OmpR$_N$ and the OmpR linker are both required for the acti-

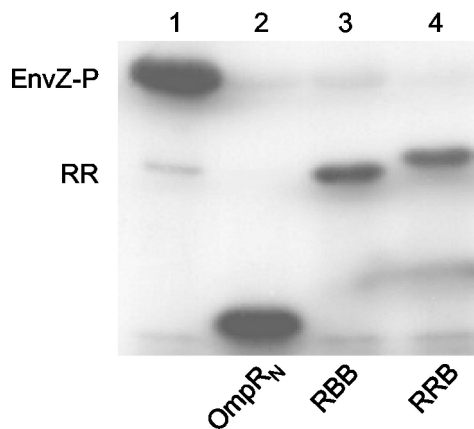FIG. 7. Phosphorylation of the OmpR$_N$-PhoB$_C$ chimeras by EnvZ. The kinase assays are described in Materials and Methods. The phosphotransfer reaction was performed for 1 h at room temperature and stopped by the addition of SDS-PAGE loading buffer. Lane 1 contains 2 μg of EnvZ autophosphorylated by [γ-^{32}P]ATP. The response regulator (RR; 0.3 μg) in each reaction is indicated by the labels under the gel.

vation of OmpR$_C$. In addition, OmpR$_N$ does not contain an inhibitory function, a finding that further distinguishes the activation mechanism of OmpR from PhoB.

One explanation for the lack of phosphorylation-induced activation of RBB is that it is not phosphorylated in vivo. However, direct measurements of phosphorylation by the kinase EnvZ (Fig. 7) demonstrate that RBB is phosphorylatable in vitro. Another, somewhat unlikely possibility is that the phosphorylated form of RBB could be unstable in vivo. Both RBB and RRB display similar phosphorylation properties, yet only RRB is activated by EnvZ in vivo. This observation makes a third possibility likely, that phosphorylation of the RBB chimera in vivo is without consequence. In other words, phosphorylation of the N terminus does not result in enhanced DNA binding by the C terminus. The linkers of PhoB and OmpR differ in both sequence and length. The shorter PhoB linker (6 residues versus 15 residues for OmpR) could alter the interdomain interface of RBB to disrupt signaling. Alternatively, the linkers of OmpR and PhoB could have different roles in signaling phosphorylation-dependent changes in the N terminus to the C terminus.

The OmpR linker is required for DNA binding by OmpR$_C$. Although BBR and BRR did not activate transcription, DNA binding measurements revealed that their binding properties depend on the source of the interdomain linker. BRR was able to bind to DNA, whereas BBR containing the PhoB linker was not (Fig. 5; Table 2). BRR bound to *ompF* DNA with high affinity and was able to bind to an isolated *ompC* site (C1) but not to a composite site (C123 [Table 2]). This binding behavior resembles that of the OmpR mutant T83I (40). The observation that T83I binds to *ompF* but not to *ompC* composite sites led to the conclusion that the substitution locks OmpR in a low-osmolarity conformation that can bind to *ompF* sites (F123) and the high-affinity *ompC* site C1. The BRR binding data indicate that it is also locked in this low-osmolarity conformation, which prevents binding at *ompC* (i.e., PhoB$_N$ does not inhibit binding to *ompC*). BBR did not bind to *ompF* or *ompC* DNA (Table 2). One explanation for this result is that PhoB$_N$ inhibits DNA binding by OmpR$_C$, but only when the PhoB interdomain linker is present. In a previous report, an internal deletion of the PhoB linker resulted in constitutive *phoA* transcription, suggesting that the linker, in addition to the α5 helix, is required for PhoB$_C$ inhibition (3). Our DNA binding results with the chimera containing the PhoB interdomain (BBR) linker could be interpreted as a requirement for the PhoB linker in effector domain inhibition.

OmpR linker length is important for effector domain regulation. Our results with OmpR and PhoB chimeras demonstrate a role for the interdomain linker and raise the question of which feature of the linker is important for signaling, its length or specific amino acid sequence? This question was directly addressed in a separate study in which linker substitutions (in particular, a polyQ derivative) were used to examine the role of linker length on function. While specific amino acid substitutions resulted in an *ompF*(Con) *ompC* null phenotype, changing the linker length had more profound effects on signaling. Increasing the linker length to 20 amino acids (Q$_{20}$) decreased the level of *ompF* activation compared to that in the wild type, and its expression was constitutive. Conversely, with

a Q$_{10}$ linker, there was barely any activation of *ompF* and, at shorter linker lengths, *ompF* or *ompC* was not expressed (41).

Determinants of kinase specificity differ between OmpR and PhoB. Interestingly, we found that EnvZ phosphorylates PhoB yet PhoR does not appear to phosphorylate OmpR in vitro under the conditions tested (Fig. 3 and 4). The in vivo relevance of this unidirectional cross talk is not known, but our data suggest that the determinants of kinase specificity differ between OmpR and PhoB. Our results agree with previous reports suggesting a role for noncognate kinases in the regulation of PhoB-dependent genes under certain conditions. In *phoR creC ompR*-null mutants, *envZ* is required for the activation of PhoB (30). However, this process also requires the genes for acetyl phosphate synthesis (*ackA* and *pta*) and requires that OmpR, the cognate response regulator for EnvZ, be absent from the cell. OmpR is also subject to phosphorylation by noncognate histidine kinases. The gene for the hybrid kinase EvgA was isolated as a multicopy suppressor of an EnvZ-null phenotype with respect to *ompC* expression (61). Another report demonstrated that a C-terminal fragment of the hybrid kinase ArcB weakly phosphorylates OmpR in vitro (i.e., compared to phosphorylation of the cognate response regulator ArcA [59]). Thus, noncognate histidine kinases may phosphorylate OmpR and PhoB in vivo. However, between the EnvZ/OmpR and PhoR/PhoB two-component systems, cross talk appears to be unidirectional.

Is there a role for the α5 helix in OmpR signaling? Because the PhoB α5 helix is involved in signaling (3) and the OmpR α5 helix is highly homologous to that of PhoB (70% identical), it would seem likely that the α5 helix of OmpR has a role in signaling to the C terminus. This prediction is supported by OmpR α5 helix mutants E111K and R115S, which are both defective at the transcriptional activation of *ompF* and *ompC* (7, 44). The R115S mutant binds to DNA with similar affinity as unphosphorylated OmpR, but phosphorylation does not stimulate DNA binding. These results suggest that R115S is a true signaling mutant and not simply a DNA binding mutant, in agreement with a role for the α5 helix in postphosphorylation signaling.

Characterization of CheY-PhoB chimeras and N-terminal truncation mutants of PhoB indicate that the α5 helix of PhoB inhibits its effector domain and that phosphorylation relieves this inhibition (3). Thus, we constructed a third OmpR$_N$-PhoB$_C$ chimera containing the PhoB α5 helix and the PhoB linker to examine the behavior of the PhoB α5 helix when fused to OmpR$_N$. In the isogenic *envZ*$^+$ and *envZ* null strains, this chimera was able to activate the transcription of *phoA*, suggesting that it does not require phosphorylation by EnvZ for function (data not shown). It is apparent that the PhoB α5 helix behaves differently (i.e., it is unable to inhibit its C terminus) when fused to OmpR$_N$ than when present in PhoB$_N$. This result further highlights the mechanistic differences with which OmpR$_N$ and PhoB$_N$ regulate their C termini. Presumably, the OmpR N terminus is unable to maintain the PhoB α5 helix in an inhibitory conformation, even in the absence of phosphorylation.

Conclusions. OmpR and PhoB are among the most closely related response regulators of the OmpR subfamily of winged-helix-turn-helix proteins (23, 26), yet they use different mechanisms to regulate their DNA binding domains. Phosphoryla-

tion of OmpR$_N$ stimulates a basal activity of OmpR$_C$ that results in enhanced DNA binding and transcriptional activation. In contrast, phosphorylation of PhoB$_N$ relieves inhibition mediated by the α5 helix, which enables DNA binding and transcriptional activation by PhoB$_C$. In OmpR and PhoB, effector domain regulation requires both the N terminus and an interdomain linker of proper length. Clearly, the different properties exhibited by OmpR and PhoB domains indicate that structural homology does not always imply functional homology.

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