Interacting specificity of a histidine kinase and its cognate response regulator: the PrrBA system of *Rhodobacter sphaeroides*

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Using a yeast two-hybrid assay system, it was demonstrated that the four-helix bundle of the *Rhodobacter sphaeroides* PrrB histidine kinase both serves as the interaction site for the regulatory domain of its cognate response regulator PrrA and is the primary determinant of the interaction specificity. The α -helix 1 and its flanking turn region within the dimerization domain (DD) of the PrrB histidine kinase appear to play an important role in conferring the recognition specificity for the PrrA response regulator on the DD. The catalytic ATP-binding domain of the histidine kinase, which functions as the catalytic unit for the phosphotransfer reaction from ATP to the conserved histidine residue in the DD, also appears to contribute to the enhancement of the recognition specificity conferred by the DD. It was also revealed that replacement of Asp-63 and Lys-113 of the PrrA response regulator by alanine abolished protein–protein interactions between PrrA and its cognate histidine kinase PrrB, whereas mutations of Asp-19, Asp-20 and Thr-87 to alanine did not affect protein–protein interactions, indicating that among the active site residues of PrrA, Asp-63 and Lys-113 are important not only in the function of PrrA but also for protein–protein interactions between PrrA and PrrB.

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INTRODUCTION

Two-component signal-transduction systems play major roles in cellular adaptation to various environmental conditions in prokaryotes (Grebe & Stock, 1999; West & Stock, 2001). The typical two-component system consists of a histidine kinase and a cognate response regulator. The orthologous form of the histidine kinase comprises the N-terminal sensing domain responsible for signal sensing and the C-terminal transmitter domain that is involved in ATP-dependent phosphorylation of the cognate response regulator. The transmitter domain is further divided into the dimerization domain (DD) and the catalytic ATP-binding (CA) domain (Dutta *et al.*, 1999). The DD consists of ~70 amino acid residues that form a secondary structure of α -helix–turn– α -helix (Tomomori *et al.*, 1999). Histidine kinases exist as homodimers, in which the DDs of two identical subunits form a four-helix bundle. In the middle of the first (N-terminal) α -helix of the DD a histidine residue is conserved among all histidine kinases and the histidine residue serves as the autophosphorylation site. The CA domain exhibits a structural homology with ATPases such as Hsp90, DNA gyrase B and MutL, and contains conserved

signatures of histidine kinases (N, G1, F and G2 boxes), which form the unique ATP-binding pocket (Dutta *et al.*, 1999). This domain is responsible for the phosphotransfer reaction from ATP to the histidine residue conserved in the DD. The CA domain of one subunit is juxtaposed to the DD of the other subunit. Due to the highly flexible interdomain hinges between CA and DDs, the 3D structure of the intact transmitter domain of a histidine kinase is yet to be solved. However, the structures of each independent domain of the histidine kinase have been resolved (Tanaka *et al.*, 1998; Tomomori *et al.*, 1999).

The response regulator usually has a two-domain structure, with a conserved N-terminal regulatory domain and a variable C-terminal effector domain (West & Stock, 2001). The regulatory domain is also called the receiver domain and contains a conserved aspartate residue that receives the phosphoryl group from a phosphorylated histidine kinase. The phosphorylation of the regulatory domain brings about the conformational change, which leads to the activation of the effector domain that usually functions as the DNA-binding domain. Many histidine kinases are bifunctional enzymes that have both kinase and phosphatase activities acting on their cognate response regulators (Stock *et al.*, 2000; Oh & Kaplan, 2001; Oh *et al.*, 2004). Depending on the presence or absence of an environmental signal, the equilibrium of the kinase/phosphatase activities of a histidine

Abbreviations: 3AT, 3-amino-1,2,4-triazole; CA domain, catalytic ATP-binding domain; DD, dimerization domain; GAL4AD, GAL4 activation domain; GAL4BD, GAL4 DNA-binding domain.

kinase is regulated, and this in turn controls the cellular abundance of the phosphorylated response regulator that forms the phosphorelay couple with the histidine kinase.

The photosynthetic bacterium *Rhodobacter sphaeroides* can grow in various environments and has been shown to possess extensive metabolic capability (Kiley & Kaplan, 1988; Zeilstra-Ryalls *et al.*, 1998; Oh & Kaplan, 2001). In order to adapt to various environmental conditions, *R. sphaeroides* possesses approximately 30 different pairs of two-component systems, excluding the CheA-CheY chemotaxis systems (www.rhodobacter.org). In organisms with multiple two-component systems, signal transduction through these two-component systems requires precise interactions between histidine kinases and their cognate response regulators to elicit the correct response in adaptation to a given environmental signal. Currently little is known about the mechanisms underlying the specific interaction between a histidine kinase and a cognate response regulator. Using the PrrBA and DevSR two-component systems that are involved in redox sensing in *R. sphaeroides* and *Mycobacterium smegmatis* (Eraso & Kaplan, 1994, 1995; Mayuri *et al.*, 2002), respectively, we performed *in vivo* mapping of the interaction domains of the histidine kinases and their cognate response regulators by means of yeast two-hybrid assays, and revealed regions (domains) on the histidine kinases that confer recognition specificity for their cognate response regulators.

METHODS

Strains, plasmids, and growth conditions. The *Escherichia coli* strains, *Saccharomyces cerevisiae* strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown at 37 °C in Luria-Bertani (LB) medium as described elsewhere (Sambrook *et al.*, 1989). Ampicillin (100 µg µl⁻¹) and kanamycin (50 µg µl⁻¹) were added when appropriate. *S. cerevisiae* strains were grown at 30 °C in YPD (Clontech) or synthetic defined dropout (SD) medium (Q-Biogene) lacking adenine, histidine, leucine and tryptophan (SD/-Ade/-His/-Leu/-Trp) in the presence of different concentrations of 3-amino-1,2,4-triazole (3AT; Sigma) as described in the manufacturer's manual (www.clontech.com/clontech/techinfo/manuals/PDF/PT3024-1.pdf).

Molecular genetic techniques. Standard protocols and manufacturer's instructions were followed for general recombinant DNA manipulations (Sambrook *et al.*, 1989). *S. cerevisiae* strains were cotransformed with different pairs of two-hybrid plasmids according to the lithium acetate (LiAc)-mediated method as described elsewhere (Guthrie & Fink, 1991).

Construction of plasmids

i) pPLA and pBBC. To construct pPLA, a 0.64 kb *EcoRI*-*Bam*HI fragment containing *prfA* was amplified by PCR using the primers PrrA-(*EcoRI*) + (5'-ccttgaattccgaatgacagccatgtgtc-3') and PrrA(*Bam*HI) - (5'-cttcggatcctcagcgcgggctgcgttc-3') using the plasmid pUI1643 as the template and *pfu* DNA polymerase. The PCR product was restricted with *EcoRI* and *Bam*HI and cloned into pGADT7 encoding the GAL4 activation domain (GAL4AD) digested with the same restriction enzymes, yielding pPLA. Most *prfA* derivatives were cloned into pGADT7 by the same procedure as described above to produce the fusion proteins (GAL4AD-PrrA derivatives). To construct

pBBC, a 0.91 kb *Bam*HI-*Pst*I fragment containing a portion of *prfB* was amplified by PCR using the primers PrrB(*Bam*HI) + (5'-tata-ggatccgcatgttcgaattcggc-3') and PrrB(*Pst*I) - (5'-atatctgcagatcaggtct-ggatcagg-3'), digested with *Bam*HI and *Pst*I, and cloned into pGBKT7, giving pBBC. Most PrrB derivatives were fused to the C terminus of the GAL4 DNA-binding domain (GAL4BD) employing the vector pGBKT7.

ii) pPLR and pBSC. A 0.74 kb *EcoRI*-*Bam*HI fragment containing *devR* was amplified by PCR using the primers DevR(*EcoRI*) + (5'-atgcgaattcaccgccggtctggtgcgc-3') and DevR(*Bam*HI) - (5'-atgc-ggatccatagttgcgcgggtccagttt-3') from *M. smegmatis* MC2 chromosomal DNA as the template. A 0.69 kb *EcoRI*-*Bam*HI fragment containing a portion of *devS* was amplified with the primers DevSc-(*EcoRI*) + (5'-atgcgaattcccttcaccgcagaacagttg-3') and DevS(*Bam*HI) - (5'-atgcggatcctcagtcggggagcggcggtg-3') by the same procedure. The DNA fragments containing *devR* and a portion of *devS* were digested with *EcoRI* and *Bam*HI and cloned into pGADT7 and pGBKT7 restricted with the same restriction enzyme, yielding pPLR and pBSC, respectively.

iii) pBBSDI and pBBSDI. A recombinant PCR method was performed to generate PrrB-DevS chimeric proteins. Two rounds of PCR were carried out using *pfu* polymerase. The plasmids pUI1643 and pBSC were used as the templates for amplification of portions of *prfB* and *devS*, respectively. Two primary PCR reactions were performed with the primers PrrB(*Bam*HI) + and BSI - (5'-ctgctgcacctcggccgaacgaagctcctcggaagctcg-3') and with BSI + (5'-cggagcttcgagagagcttcgttcggcgaggtgcagcag-3') and DevS(*Bam*HI) - (5'-atgcggatcctcagtcggggagcggcggtg-3') to generate two DNA fragments each containing a 42 bp overlapping region. Two primary PCR products were used as the templates for the secondary PCR, which was performed using the primers PrrB(*Bam*HI) + and DevSD(*Bam*HI) - (5'-atatggatccatcaagaatggcgtgcg-3'). The secondary PCR product was restricted with *EcoRI* and *Bam*HI and cloned into pGBKT7 digested with the same restriction enzymes to yield pBBSDI. The plasmid pBBSDI was constructed in the same way as pBBSDI, except for using the primers BSI + (5'-cgaacgtgcgcggg-gatcgtgcagctcaccagcttgatcgt-3') and BSI - (5'-acgatcaagctgtgagctc-gacgatccgcgcgcagcttcg-3') in place of BSI + and BSI -.

iv) pPR and pBZD. To construct pPR, a 0.72 kb *EcoRI*-*Bam*HI fragment containing *ompR* was amplified by PCR using the primers OmpR(*EcoRI*) + (5'-atatgaattcatgcaagagaactacaag-3') and OmpR(*Bam*HI) - (5'-atatggatcctcagctttagagccgtc-3') from *E. coli* chromosomal DNA as the template. The PCR product was restricted with *EcoRI* and *Bam*HI and cloned into pGADT7 digested with the same restriction enzymes, yielding pPR. A 0.21 kb *EcoRI*-*Bam*HI fragment containing a portion of *envZ* was amplified with the primers EnvZDD (*EcoRI*) + (5'-atatgaattcatgctgcgctgtgttaag-3') and EnvZDD (*Bam*HI) - (5'-atatggatccctcctgcccgtgcgcag-3') by PCR, digested with *EcoRI* and *Bam*HI, and cloned into pGBKT7, giving pBZD.

Site-directed mutagenesis. To mutate codons corresponding to Asp-19, Asp-20, Asp-63, Thr-91 and Lys-113 of PrrA to alanine, mutagenesis was performed using the Quick Change Site-directed Mutagenesis kit (Stratagene) using pULAR as a template plasmid. Synthetic oligonucleotides 33 bases long containing an alanine codon (GCC, GCT, GCG) in place of the codon of the corresponding amino acid in the middle of their sequences were used to mutagenize the *prfA* gene. Following the verification of mutations by DNA sequencing, a 0.48 kb *EcoRI*-*Bam*HI fragment containing the mutated sequence was cloned into pGADT7, giving pPLAR19, pPLAR20, pPLAR63, pPLAR91 and pPLAR113.

Analysis of *in vivo* protein-protein interactions. The *S. cerevisiae* strains co-transformed with both pGADT7 and pGBKT7

Table 1. Strains and plasmids used in this study

Abbreviations: AD, activation domain; DNA-BD, DNA-binding domain; ATP BD, ATP binding domain.

Strain/plasmid	Relevant phenotype/genotype	Source/reference
Strains		
<i>E. coli</i> DH5 α	(Φ 80dlacZ Δ M15) Δ lacU169 <i>recA1 endA1 hsdR17 supE44 thil gyrA96 relA1</i>	Jessee (1986)
<i>S. cerevisiae</i> AH109	<i>MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1_{UAS}-GAL1_{TATA}-HIS3, GAL2_{UAS}-GAL2_{TATA}-ADE2, URA3::MEL1_{UAS}-MEL1_{TATA}-lacZ</i>	James <i>et al.</i> (1996)
Plasmid		
pUC19	Ap ^r ; <i>lacPOZ'</i>	Yanisch-Perron <i>et al.</i> (1985)
pUI1643	pBSIIKS+::4.0 kb <i>Bam</i> HI– <i>Hind</i> III fragment containing <i>prpB</i> and <i>prpA</i>	Eraso & Kaplan (1995)
pGADT7	Ap ^r , <i>LEU2</i> , GAL4(768–881:1561–1899) AD	Chien <i>et al.</i> (1991)
pGBKT7	Km ^r , <i>TRP1</i> , GAL4(1–147:762–1202) DNA-BD	Louret <i>et al.</i> (1997)
pULAR	pUC19::0.48 kb <i>Eco</i> RI– <i>Bam</i> HI fragment containing the 3' part of <i>prpA</i>	This study
pPLA	GAL4AD:PrrA	This study
pBBC	GAL4BD:PrrBc	This study
pPLAR	GAL4AD:PrrAr	This study
pPABD	GAL4AD:PrrAe	This study
pPLAR10	GAL4AD:PrrAr-10	This study
pPLAR20	GAL4AD:PrrAr-20	This study
pP10LA	GAL4AD:-10PrrA	This study
pP20LA	GAL4AD:-20PrrA	This study
pBBCA	GAL4BD:PrrBc320	This study
pBBCA50	GAL4BD:PrrBc270	This study
pBBCA53	GAL4BD:PrrBc267	This study
pBBCA56	GAL4BD:PrrBc264	This study
pBBCA60	GAL4BD:PrrBc260	This study
pBBD	GAL4BD:PrrB DD	This study
pPLAR19	GAL4AD:PrrArD19A	This study
pPLAR20	GAL4AD:PrrArD20A	This study
pPLAR63	GAL4AD:PrrArD63A	This study
pPLAR91	GAL4AD:PrrArT91A	This study
pPLAR113	GAL4AD:PrrArK113A	This study
pPLR	GAL4AD:DevR	This study
pBSC	GAL4BD:DevSc	This study
pBSD	GAL4BD:DevS DD	This study
pBSDATP	GAL4BD:DevS DD + ATP BD	This study
pBBSDI	GAL4BD: α -helix 1 and turn of PrrB fused with α -helix 2 of DevS	This study
pBBSDII	GAL4BD: α -helix 1 of PrrB fused with turn and α -helix 2 of DevS	This study
pPR	GAL4AD:OmpR	This study
pBZD	GAL4BD:EnvZ DD	This study

derivatives were grown in SD/–Leu/–Trp medium. The cultures were diluted to OD₆₀₀ 0.4–0.45 and spotted onto both solid SD/–Leu/–Trp medium and SD/–Leu/–Trp/–His medium containing different concentrations of 3AT. These plates were incubated for 3–7 days. The crude cell extracts were prepared by three freeze/thaw cycles using liquid nitrogen. Determination of β -galactosidase activity was performed using ONPG as substrate, as described elsewhere (Schneider *et al.*, 1996).

Immunoblotting. SDS-PAGE and Western blotting were performed as described elsewhere (Laemmli, 1970; Mouncey & Kaplan, 1998). The protein extracts were resolved by 12.5% (w/v) SDS-PAGE and transferred to PVDF membranes. The mouse mAbs against GAL4AD (SantaCruz) and GAL4BD (BD Biosciences) were used at dilutions of 1:200 and 1:10 000, respectively. The alkaline phosphatase-conjugated anti-mouse IgG (Sigma) was used at a 1:10 000 dilution

for the detection of the primary antibodies. Detection was carried out by staining the membrane with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT).

RESULTS

Detection of specific protein–protein interactions between histidine kinases and their cognate response regulators in a yeast two-hybrid system

This study was designed to reveal which regions (domains) of the PrrA response regulator and its cognate PrrB histidine kinase confer recognition specificity for protein–protein

interaction. We first determined whether the yeast two-hybrid system could be applied to detect specific interactions between the histidine kinase and its cognate response regulator. To employ the yeast two-hybrid assay, the genes encoding the PrrA response regulator and the PrrB histidine kinase were cloned into pGADT7 (prey vector) and pGBKT7 (bait vector), respectively, to generate GAL4AD–PrrA and GAL4BD–PrrB fusion proteins in the yeast strain AH109. In the case of PrrB, the 3' portion of the *prrB* gene encoding the transmitter domain (PrrBc) lacking the N-terminal membrane-spanning domain (amino acids 1–161), was cloned into the bait vector, since the PrrB transmitter domain was predicted to interact with PrrA on the basis of the *in vitro* phosphorylation assay (Bird *et al.*, 1999; Comolli *et al.*, 2002; Oh *et al.*, 2004). The DNA fragment which was cloned into the prey vector to produce a GAL4AD–PrrA fusion protein contained the 84 bp DNA sequence upstream of the *prrA* start codon in addition to the entire *prrA* gene. Since this additional DNA sequence does not contain a stop codon that lies in the same reading frame as the *GAL4AD* and *prrA* genes, the PrrA protein expressed from pPLAC (pGADT7:: *prrA*) has an additional 28 amino acid residues at its N terminus. In order to assess the specificity of the interaction between the histidine kinase and its cognate response regulator using the yeast two-hybrid assay, another two-component system, the DevSR two-component system from *M. smegmatis*, was included. The genes for the DevS histidine kinase and DevR response regulator were cloned into the bait and prey vectors, respectively, in the same way as for the PrrBA two-component system (DevR of the GAL4AD–DevR fusion protein also has an additional 28 amino acid residues at its N terminus). When protein–protein interactions between GAL4AD and GAL4BD fusion proteins occur in the yeast strain AH109 carrying both bait and prey plasmids, the yeast strain is enabled to grow on histidine-deficient medium and synthesize β -galactosidase due to the induction of the *HIS3* and *lacZ* reporter genes on its chromosomal DNA. It was further possible to comparatively determine the strength of the protein–protein interactions by using various concentrations of 3AT, which serves as an inhibitor of the *HIS3* product.

As shown in Fig. 1, the yeast strain (PrrA/PrrBc) expressing both the GAL4AD–PrrA and the GAL4BD–PrrBc fusion proteins grew on histidine-deficient medium (SD medium lacking histidine), indicating that PrrA interacts with PrrBc. Likewise, the yeast strain with the DevR/DevSc pair grew on SD medium without histidine, and its growth in the presence of 5 mM 3AT was more robust than that of the yeast strain (PrrA/PrrBc) under the same conditions. This result indicated that the protein–protein interactions between DevR and DevSc are stronger than those between PrrA and PrrBc. The yeast strains (PrrA/DevSc and DevR/PrrBc) which expressed heterologous pairs of fusion proteins did not grow on SD medium lacking histidine. Taken together, the data presented in Fig. 1 demonstrate that the yeast two-hybrid system used in this study is

Fig. 1. Detection of specific protein–protein interactions between histidine kinases and their cognate response regulators by the yeast two-hybrid assay. Growth of the yeast strains transformed with both the bait and prey plasmids in the yeast two-hybrid assay is shown. The yeast strains were grown at 30 °C in SD/–Leu/–Trp medium. All yeast cultures were diluted to OD₆₀₀ 0.4–0.45 and spotted onto a SD/–Leu/–Trp plate or histidine-deficient SD plates (SD/–Leu/–Trp/–His) containing different concentrations of 3AT (the numbers to the left indicate the concentration of 3AT). Pairs of fusion constructs are indicated in the order X/Y, where X and Y are the proteins fused to GAL4AD and GAL4BD, respectively. The level of growth is indicated with + or –. The SD/–Leu/–Trp plate and SD/–Leu/–Trp/–His/+3AT plates were incubated at 30 °C for 3 and 5 days, respectively.

applicable for the study of the specific protein–protein interactions between a histidine kinase and its cognate response regulator.

The regulatory domain of PrrA is the region that interacts with PrrB

We next examined which domain of PrrA is responsible for its specific interaction with PrrB. The PrrA response regulator is composed of the N-terminal regulatory domain (amino acids 1–131) and the C-terminal effector domain (amino acids 136–184), which are connected by a linker consisting of four consecutive proline residues (Eraso & Kaplan, 1994). The regulatory domain of PrrA contains an aspartate residue (Asp-63) that receives the phosphoryl group from autophosphorylated PrrB (Comolli *et al.*, 2002), while the effector domain functions as the DNA-binding domain (Laguri *et al.*, 2003).

As shown in Fig. 2(A), the yeast strain (PrrAr/PrrBc) expressing both GAL4AD–PrrAr (regulatory domain of PrrA) and GAL4BD–PrrBc fusion proteins grew on SD medium lacking histidine. The growth of the yeast strain (PrrAr/PrrBc) on histidine-deficient medium supplemented with 3AT was more robust than that of the yeast strain (PrrA/PrrBc) under the same growth conditions. In keeping with this observation, the β -galactosidase assay revealed that a 1.9-fold higher activity of β -galactosidase was detected in

Fig. 2. Mapping of the PrrA domain interacting with PrrB. (A) Schematic representation of PrrA derivatives is on the right of the figure. The numbers indicate the amino acid boundaries of the PrrA domains. The regulatory and effector domains of PrrA are connected by the linker consisting of four consecutive proline residues. The panel on the left shows the results of the yeast two-hybrid assay. Cultures were spotted in the same way as described in Fig. 1 and fusion pairs are indicated in the same order as described in Fig. 1. P (prey) refers to the absence of a protein fused to GAL4AD. The strains grown on the SD/-Leu/-Trp plate were incubated for 3 days and the other plates for 6 days. The numbers to the left of the growth panel indicate the specific β -galactosidase (β -gal) activities that are normalized by the value for the control strain (P/PrrBc); the numbers at the top of the growth panel show 3AT concentration. (B) Western blotting analysis for the detection of GAL4AD fusion proteins using a GAL4AD mAb. The numbers to the left indicate the molecular mass (kDa).

cell lysates of the yeast strain (PrrAr/PrrBc) compared to the yeast strain (PrrA/PrrBc). The PrrA derivative (PrrAe), in which the regulatory domain was removed from PrrA, did not interact with PrrBc in the yeast strain (PrrAe/PrrBc). The yeast strain (P/PrrBc) harbouring the empty prey vector (P) as a negative control did not grow on histidine-deficient medium. Taken together, these results indicate that the regulatory domain of PrrA is the region in which protein–protein interactions between PrrA and PrrB occur, and that the PrrA derivative (PrrAr) consisting of the regulatory domain alone appears to interact with PrrBc more strongly than does intact PrrA, suggesting a negative effect of the effector domain on this interaction.

In order to delineate more precisely the region within the PrrA regulatory domain that is involved in protein–protein interactions with PrrB, C-terminal deletion derivatives of PrrAr were constructed and their interactions with PrrBc were examined. The PrrAr derivatives in which 10 and 20 amino acids were removed from the C terminus of PrrAr lost their ability to interact with PrrBc, as judged by the two-hybrid assay. Interestingly, the removal of 10 and 20 amino acids from the N terminus of PrrA with the 28 extra amino acid extension at its N terminus rendered the PrrA derivatives (10PrrA and 20PrrA) unable to interact with PrrBc, as observed for the yeast strains (10PrrA/PrrBc and 20PrrA/PrrBc), although the PrrA protein itself remained intact. The PrrA derivative from which all 28 extra amino acids had been removed did not interact with PrrBc either (data not shown).

To determine whether the expression and/or stability of the GAL4AD–PrrA fusion proteins in yeast cells were affected by these deletions, we performed Western blotting analysis with a mAb against GAL4AD. As shown in Fig. 2(B), all GAL4AD fusion proteins were detected in the Western blotting assay, ruling out the possibility that the inability of some PrrA derivatives to interact with PrrBc might have resulted from instability or degradation of their fusion proteins. From the results it can be inferred that the intact form or conformation of the PrrA regulatory domain is required for the correct protein–protein interactions between PrrA and PrrB, and that it is necessary to insert a linker of the proper length between GAL4AD and PrrA when the GAL4AD–PrrA fusion proteins for the yeast two-hybrid assay are constructed.

D63A and K113A substitutions in the regulatory domain of PrrA abolish protein–protein interactions between PrrA and PrrB

The regulatory domains of response regulators have a $(\beta/\alpha)_5$ topology with five parallel β -strands surrounded by five α -helices (Volz & Matsumura, 1991; Stock *et al.*, 1993). The active site located in the regulatory domain of the response regulator is believed to be composed of three acidic residues, a lysine residue and a hydroxyl amino acid (threonine or serine), which are all conserved in all known response regulators (Fig. 3A). The corresponding conserved active site residues in PrrA are Asp-19, Asp-20, Asp-63, Thr-91 and Lys-113. Asp-63 has been demonstrated to be the site of phosphorylation (Comolli *et al.*, 2002). Asp-19 and Asp-20 are predicted to be involved in coordination of a Mg^{2+} ion that is essential for phosphorylation and dephosphorylation of the response regulator (Stock *et al.*, 1993). It is assumed that Thr-91 and Lys-113 are involved in the phosphorylation-induced conformational change of the response regulator (Lukat *et al.*, 1991; Appleby & Bourret, 1998).

In order to investigate the possible roles of the conserved active site residues in protein–protein interactions between PrrA and PrrB, the conserved amino acid residues in the PrrA regulatory domain were individually changed to an

Fig. 3. Effect of replacement of conserved amino acids in the regulatory domain of PrrA on protein-protein interactions between PrrA and PrrB. (A) Multiple alignment of the receiver domain of PrrA with those of other response regulators. The amino acid residues which are conserved in the regulatory domains of all response regulators and play a crucial role in the phosphorylation reaction are boxed. These amino acids are substituted with alanine by site-directed mutagenesis. Abbreviations: RsPrrAr, PrrA of *R. sphaeroides*; RcRegAr, RegA of *R. capsulatus*; EcOmpR, OmpR of *E. coli*; RmFixJ, FixJ of *Sinorhizobium meliloti*; MsDevR, DevR of *M. smegmatis*. The final 'r' signifies the regulatory domain. Identical amino acids are indicated by asterisks. The similar and less similar amino acid residues are marked with : and ., respectively. (B) Yeast two-hybrid assay. All yeast strains tested carried pBBC expressing the GAL4BD-PrrBc fusion protein. Pairs of fusions are indicated in the order *X/Y*, where *X* and *Y* are the proteins fused to GAL4AD and GAL4BD, respectively. The level of growth is indicated with + or -. P refers to the absence of a protein fused to GAL4AD. The SD/-Leu/-Trp plate and the SD/-Leu/-Trp/-His plates supplemented with 3AT were incubated at 30 °C for 3 and 5 days, respectively. (C) Western blotting analysis for the detection of GAL4AD fusion proteins using a GAL4AD mAb. The number to the left indicates the molecular mass (kDa).

alanine residue by site-directed mutagenesis, and interactions of the mutant forms of the PrrA regulatory domain with PrrBc were assessed by the yeast two-hybrid assay. As shown in Fig. 3(B), the positive-control yeast strain (PrrAr/PrrBc) grew on histidine-deficient medium, while the yeast strain (P/PrrBc) carrying the empty prey vector did not grow on histidine-deficient medium. The mutant forms (PrrArD19A, PrrArD20A and PrrArT91A) of the PrrA regulatory domain in which Asp-19, Asp-20 and Thr-91, respectively, were replaced with alanine, were shown to interact with PrrBc to the same extent as the wild-type form of the PrrA regulatory domain (PrrAr), indicating that D19A, D20A and T91A mutations of PrrAr do not affect the protein-protein interactions between PrrAr and PrrBc to any significant degree. In contrast, replacement of Asp-63 or Lys-113 with alanine abolished protein-protein interactions between PrrAr and PrrBc, as judged by the inability of the corresponding yeast strains (PrrArD63A/PrrBc, PrrArK113A/PrrBc) to grow on histidine-deficient medium.

To determine whether the inability of the yeast strains expressing the mutant forms (D63A and K113A) of PrrAr to grow on histidine-deficient medium was the result of instability of the corresponding GAL4AD-PrrAr fusion

proteins in the yeast cell, we performed Western blotting analysis with a mAb against GAL4AD. As shown in Fig. 3(C), all GAL4AD fusion proteins with molecular masses of ~43 kDa were detected by Western blotting analysis, indicating that inability of the yeast strains (PrrArD63A/PrrBc, PrrArK113A/PrrBc) to grow on histidine-deficient medium was not the result of instability or degradation of the fusion proteins in the yeast cell.

The DD of PrrB is the region that interacts with PrrA and confers recognition specificity for interactions between PrrB and PrrA

The transmitter domain (PrrBc) of the PrrB histidine kinase, which catalyses the phosphotransfer reaction from ATP to the PrrA response regulator, is composed of two distinct domains, the DD and the CA domain (Bird *et al.*, 1999; Comolli *et al.*, 2002; Oh *et al.*, 2004).

To examine the contributions of these individual domains to the PrrB interaction between the PrrB transmitter domain and PrrA, protein-protein interactions between the different truncated PrrBc derivatives and PrrA were analysed in the yeast two-hybrid assay. As shown in Fig. 4(A), the yeast

strain (PrrA/PrrBc) expressing both GAL4AD–PrrA and GAL4BD–PrrBc fusion proteins grew on histidine-deficient medium, whereas the negative-control strain (PrrA/B) harbouring the empty bait vector (B) did not grow on the same growth medium. The PrrBc derivatives (PrrBc320, PrrBc270 and PrrBc267) in which 142, 192 and 195 amino acids were removed from the C terminus of PrrBc,

respectively, retained the intact DD, but a part or the whole of the CA domain was deleted in these PrrBc derivatives. The PrrBc derivatives containing the intact DD interacted with PrrA. On the other hand, the PrrBc derivatives (PrrBc264 and PrrBc260) whose DDs were impaired by C-terminal deletion did not interact with PrrA. PrrB DD is a PrrB derivative that consists of 70 amino acids

Fig. 4. Mapping of the PrrB domain interacting with PrrA. (A) Yeast two-hybrid assay. All PrrB derivatives are schematized to the right. All strains contain the pPLA plasmid encoding PrrA fused with GAL4AD in common. A 'B' indicates the absence of a protein fused to GAL4BD. The numbers to the left side of the growth panel indicate the specific β -galactosidase (β -gal) activities that are normalized by the value for the control strain (PrrA/B). The strains were grown on the SD/–Leu/–Trp plate and the SD/–Leu/–Trp/–His plates containing different concentrations of 3AT at 30 °C for 3 and 5 days, respectively. The numbers above the schematic diagrams indicate the amino acid boundaries of the PrrB derivatives. (B) Western blotting analysis for the detection of GAL4BD fusion proteins using a GAL4BD mAb. The numbers to the left indicate the molecular mass (kDa). (C) The amino acid sequence of PrrB DD. The DD of the histidine kinase is composed of the two helices α -helix 1 and α -helix 2, linked by a turn.

(Fig. 5A, C). The yeast strain (PrrA/PrrB DD) expressing both GAL4AD–PrrA and GAL4BD–PrrB DD grew on histidine-deficient medium, while the yeast strain (DevR/PrrB DD) expressing the heterologous pair of the fusion proteins was unable to grow on the same medium (Fig. 5B). This result indicated that the DD of PrrB is sufficient *in vivo* to provide recognition specificity for its cognate response regulator, PrrA. In the case of the DevS histidine kinase, the DD of DevS (DevS DD) interacted with both DevR and PrrA, although interactions of DevS DD with its partner DevR appeared to be significantly stronger than those with PrrA. The control yeast strain (P/DevS DD) harbouring the empty prey vector did not grow on histidine-deficient medium (data not shown). The DevS derivative DevS DD + ATP, consisting of both the DD and CA domain, did not interact with PrrA, but gave interaction signals with its cognate response regulator, DevR. Taken together, the results shown in Fig. 5 suggest that the DD of the histidine kinase serves as the interaction region with its cognate response regulator and is sufficient to make both specific and non-specific interactions with its cognate response regulator and other related proteins. However, the presence of the CA domain masks the non-specific interactions (either directly or indirectly) and allows only for the specific interaction between a kinase and its cognate response regulator.

The first α -helix and turn within the PrrB DD are important for specific interactions between PrrB and PrrA

As demonstrated above, the DD of the histidine kinase to some extent confers recognition specificity for its cognate response regulator. The DD of the histidine kinase has a secondary structure of α -helix–turn– α -helix (Tomomori *et al.*, 1999). Our next question to be addressed was which portion of the DD plays a significant role in providing recognition specificity for its interaction with its cognate response regulator? To address this question, DNA fragments encoding the chimeric DDs (BS DD I and BS DD II) consisting of the α -helices with two different origins (PrrB and DevS) were generated by recombinational PCR and cloned into the bait vector. The BS DD I polypeptide is composed of the first α -helix (α -helix 1) and turn derived from PrrB and the second α -helix (α -helix 2) from DevS (Fig. 6A). Protein–protein interactions between BS DD I and PrrA were observed in the yeast two-hybrid assay. In contrast, BS DD I did not interact with DevR (Fig. 6B). Another chimeric polypeptide BS DD II is made up of the α -helix 1 from PrrB and the α -helix 2 and turn from DevS. BS DD II did not interact with either PrrA or DevR. These results suggest that α -helix 1 and the flanking turn structure of the PrrB DD determine the recognition specificity for the PrrA response regulator.

As shown in Fig. 6(C), Western blotting analysis with a GAL4BD-specific antibody revealed that all the chimeric proteins fused to GAL4BD were normally expressed, indicating that the lack of interactions in the yeast

Fig. 6. Protein–protein interactions between PrrB–DevS chimeric proteins and the response regulators PrrA and DevR. (A) The chimeric proteins of PrrB and DevS are schematized. Each chimeric protein has the DD composed of the two helices from the different origins. The numbers indicate the amino acid boundaries of each histidine kinase. (B) Yeast two-hybrid assay. Pairs of fusions are indicated in the same order as described for Fig. 5. The strains were grown on the SD/–Leu/–Trp plate and the SD/–Leu/–Trp/–His plates containing different concentrations of 3AT at 30 °C for 3 and 6 days, respectively. (C) Western blotting analysis for the detection of GAL4BD fusion proteins using a GAL4BD mAb.

two-hybrid assay observed for BS DD II is not due to instability of the chimeric proteins in the yeast two-hybrid system.

The EnvZ protein of *E. coli* is a histidine kinase that is involved in osmosensing and controls the phosphorylation state of the OmpR response regulator. The amino acid sequence of the EnvZ DD shows a high level of similarity with that of the PrrB DD, especially in the region encompassing α -helix 1 and the turn (Fig. 7A). If the α -helix 1 and turn of the DD are important for specific interactions between the DD and its cognate response regulator, the DD of EnvZ might be expected to interact not only with OmpR but also with PrrA. As shown in Fig. 7(B), the DD of EnvZ indeed interacted with PrrA as well as with its cognate response regulator, OmpR. The control yeast strain (P/EnvZ DD) harbouring the empty prey vector did not grow on histidine-deficient medium (data not shown). β -Galactosidase assays were performed on cell-free lysates of

Fig. 7. Protein–protein interactions between the DD of EnvZ and PrrA. (A) Comparison of the amino acid sequences of the PrrB and EnvZ DDs. Identical or conservatively substituted amino acids are indicated by asterisks or colons, respectively. RsPrrB DD, DD of *R. sphaeroides* PrrB; EcEnvZ DD, DD of *E. coli* EnvZ. (B) Interactions between the DDs of EnvZ and PrrA or its cognate response regulator, OmpR, in the yeast two-hybrid assay. The SD/–Leu/–Trp plate and SD/–Leu/–Trp/–His plates containing 3AT were incubated at 30 °C for 2 and 4 days, respectively. β -Galactosidase (β -gal) activities detected in the strains are shown below the panel.

the yeast strains as a measure of the strength of the protein–protein interactions. The interaction of the EnvZ DD with OmpR was shown to be much stronger than that with PrrA.

DISCUSSION

In vivo yeast two-hybrid assay is an efficient method for detecting a weak and transient protein–protein interaction that is difficult to investigate by other *in vitro* methods. Yeast two-hybrid systems have been successfully employed to study the specific interaction between components of the NtrBC two-component system of *Klebsiella pneumoniae* (Martinez-Argudo *et al.*, 2001, 2002) and to identify the histidine kinases which specifically interact with the DivK response regulator of *Caulobacter crescentus* (Ohta & Newton, 2003).

While studying protein–protein interactions between the PrrB histidine kinase and the PrrA response regulator, we found that insertion of a linker of approximately 60 amino acids (including the linker of 32 amino acids provided by the prey vector itself) between GAL4AD and PrrA was required for the successful yeast two-hybrid assay. However, the GAL4AD–DevR fusion protein without the additional linker interacted with GAL4BD–DevS (data not shown), implying that the necessity of the additional linker between GAL4AD and response regulator depends on the type of response regulator.

Protein-protein interactions between the GAL4AD-PrrA and GAL4BD-PrrBc fusion proteins were clearly detected in the spotting assay, while those between GAL4AD-PrrBc and GAL4BD-PrrA were not (data not shown). This phenomenon has been observed elsewhere for the NtrBC two-component system and explained by instability of the GAL4AD-NtrB fusion protein in the yeast cell (Martinez-Argudo *et al.*, 2002).

It was demonstrated in this study that the interaction surfaces of PrrA and PrrB reside in the regulatory domain and the DD, respectively, which is consistent with the fact that the autophosphorylated histidine in the DD must be closely juxtaposed to the conserved aspartate in the regulatory domain for the phosphotransfer reaction to ensue. Yeast two-hybrid studies on the NtrBC system have revealed that the 110 amino acid long NtrB fragment containing the DD specifically interacts with the regulatory domain of NtrC (Martinez-Argudo *et al.*, 2002). In this study we further delineated the PrrB region with which PrrA interacts, and clearly showed that the PrrB DD consisting of only 70 amino acids interacted with PrrA and that impairment of the DD by further deletions abolished the interaction between PrrB and PrrA.

It has been suggested by structural (crystallographic and NMR titration) studies on the Spo0B-Spo0F and EnvZ-OmpR systems that the DDs of histidine kinases other than the Hpt (histidine-containing phosphotransfer)-containing

CheA proteins are the sites with which their cognate response regulators interact (Tomomori *et al.*, 1999; Zapf *et al.*, 2000).

D63A and K113A mutations in the regulatory domain of PrrA abolished protein–protein interactions between PrrA and PrrB, suggesting that Asp-63 and Lys-113 among the active site residues of the PrrA regulatory domain are important not only in the function of PrrA but also for protein–protein interactions between PrrA and PrrB. Asp-63 of PrrA has been demonstrated elsewhere to be the amino acid that receives the phosphoryl group from PrrB (Comolli *et al.*, 2002). Although there has been no report regarding the role of Lys-113 in PrrA function, it can be assumed from the earlier study on CheY that Lys-113 might be involved in a phosphorylation-induced conformational change in PrrA (Lukat *et al.*, 1991). X-ray crystallographic studies on the D57A mutant form of CheY, which corresponds to the D63A form of PrrA, have revealed that the D57A mutation leads to a repositioning of the Lys-109 that is the counterpart of Lys-113 of PrrA and the ϵ -amino group of which is in close proximity to the Asp-57 β -carboxylate group (Sola *et al.*, 2000). From this observation, together with our result, we assume that the precise positioning of Asp-63 and Lys-113 within the active site of PrrA is important in maintaining the correct conformation of PrrA that is required for its interaction with PrrB. The T91A mutation of PrrA did not affect protein–protein interactions between PrrA and PrrB, which is in good agreement with the earlier finding that the T87A mutant form of CheY is phosphorylated normally by phosphorylated CheA (Appleby & Bourret, 1998), although this mutation renders CheY non-functional. Asp-19 and Asp-20 of PrrA are predicted to be involved in coordination of the catalytically essential Mg^{2+} ion together with Asp-63 (Lukat *et al.*, 1990). Mutations of the corresponding amino acids to alanine or asparagine have been reported to convert the PhoB and OmpR response regulators into non-phosphorylatable forms (Brissette *et al.*, 1991; Zundel *et al.*, 1998). The D19A and D20A mutations of PrrA did not affect the protein–protein interactions between PrrA and PrrB, indicating that the mutations did not alter PrrA conformation to an extent sufficient to abolish its interaction with PrrB.

We showed here that the recognition specificity of a histidine kinase for its cognate response regulator is conferred primarily by its DD. Using the chimeric DDs it can be inferred that α -helix 1 and its neighbouring turn structure are more important in conferring the recognition specificity on the DD. Although α -helix 1 of the DD shows some sequence conservation around the conserved histidine, the amino acid sequence of α -helix 1 and its neighbouring turn region appears to be sufficiently variable to provide the recognition specificity when the primary structures of different histidine kinases are compared (Grebe & Stock, 1999). An NMR titration experiment has suggested that OmpR interacts with the four-helix bundle of EnvZ most strongly at the lower part that corresponds to the C-terminal

region of α -helix 1, turn, and N-terminal region of α -helix 2 (Tomomori *et al.*, 1999). On the basis of the results presented here as well as those reported elsewhere (Tomomori *et al.*, 1999; Martinez-Argudo *et al.*, 2002; Ohta & Newton, 2003), we assume that the lower part of the four-helix bundle within the histidine kinase serves as a docking site for its cognate response regulator and that α -helix 1 and its neighbouring turn of the DD provide to some extent the recognition specificity for its response regulator.

The CA domain of the histidine kinase is known to catalyse the phosphotransfer reaction from ATP to the conserved histidine residue within the DD (Park *et al.*, 1998). As shown in Fig. 5(B), the DevS derivative containing both the DD and CA domain exhibited a higher recognition specificity for DevR than did the DevS derivative consisting of the DD alone, which interacted also with the non-cognate response regulator PrrA. This result clearly reveals that the CA domain of the histidine kinase provides additional recognition specificity to the histidine kinase in case the DD of the histidine kinase alone does not provide sufficient recognition specificity for its interaction with the response regulator. The specificity for protein–protein interactions between a histidine kinase and its response regulator appears to be provided not only by the DD that serves as an interaction surface, but also by the CA domain that catalyses the phosphotransfer reaction.

Deletion of the CA domain from PrrBc led to an increase in the interaction affinity of PrrBc for PrrA. This observation can be explained by the following: (i) the CA domain provides recognition specificity additional to that of the DD, which reduces the strength of protein–protein interactions between PrrBc and PrrA, or (ii) the GAL4BD–PrrBc fusion protein might phosphorylate the GAL4AD–PrrA fusion protein in the yeast cell. Phosphorylation might reduce the affinity of PrrA for PrrB. Earlier, it has been reported that the affinity of the phosphorylated OmpR for EnvZ is weaker than that of the non-phosphorylated OmpR (Mattison & Kenney, 2002).

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REFERENCES

- Appleby, J. L. & Bourret, R. B. (1998). Proposed signal transduction role for conserved CheY residue Thr87, a member of the response regulator active-site quintet. *J Bacteriol* **180**, 3563–3569.
- Bird, T. H., Du, S. & Bauer, C. E. (1999). Autophosphorylation, phosphotransfer, and DNA-binding properties of the RegB/RegA two-component regulatory system in *Rhodobacter capsulatus*. *J Biol Chem* **274**, 16343–16348.

- Brissette, R. E., Tsung, K. L. & Inouye, M. (1991). Suppression of a mutation in OmpR at the putative phosphorylation center by a mutant EnvZ protein in *Escherichia coli*. *J Bacteriol* **173**, 601–608.
- Chien, C. T., Bartel, P. L., Sternglanz, R. & Fields, S. (1991). The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. *Proc Natl Acad Sci U S A* **88**, 9578–9582.
- Comolli, J. C., Carl, A. J., Hall, C. & Donohue, T. (2002). Transcriptional activation of the *Rhodobacter sphaeroides* cytochrome c_2 gene P2 promoter by the response regulator PrrA. *J Bacteriol* **184**, 390–399.
- Dutta, R., Qin, L. & Inouye, M. (1999). Histidine kinases: diversity of domain organization. *Mol Microbiol* **34**, 633–640.
- Eraso, J. M. & Kaplan, S. (1994). *prpA*, a putative response regulator involved in oxygen regulation of photosynthesis gene expression in *Rhodobacter sphaeroides*. *J Bacteriol* **176**, 32–43.
- Eraso, J. M. & Kaplan, S. (1995). Oxygen-insensitive synthesis of the photosynthetic membranes of *Rhodobacter sphaeroides*: a mutant histidine kinase. *J Bacteriol* **177**, 2695–2706.
- Grebe, T. W. & Stock, J. B. (1999). The histidine protein kinase superfamily. *Adv Microb Physiol* **41**, 139–227.
- Guthrie, C. & Fink, G. R. (1991). Guide to yeast genetics and molecular biology. *Methods Enzymol* **194**, 1–932.
- James, P., Halladay, J. & Craig, E. A. (1996). Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. *Genetics* **144**, 1425–1436.
- Jessee, J. (1986). New subcloning efficiency competent cells: $>1 \times 10^6$ transformants/ μ g. *Focus* **8**, 9.
- Kiley, P. J. & Kaplan, S. (1988). Molecular genetics of photosynthetic membrane biosynthesis in *Rhodobacter sphaeroides*. *Microbiol Rev* **52**, 50–69.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Laguri, C., Phillips-Jones, M. K. & Williamson, M. P. (2003). Solution structure and DNA binding of the effector domain from the global regulator PrrA (RegA) from *Rhodobacter sphaeroides*: insights into DNA binding specificity. *Nucleic Acids Res* **31**, 6778–6787.
- Louret, O. F., Doignon, F. & Crouzet, M. (1997). Stable DNA binding yeast vector allowing high bait expression for use in the two-hybrid system. *Bio Techniques* **23**, 816–819.
- Lukat, G. S., Stock, A. M. & Stock, J. B. (1990). Divalent metal ion binding to the CheY protein and its significance to phosphotransfer in bacterial chemotaxis. *Biochemistry* **29**, 5436–5442.
- Lukat, G. S., Lee, B. H., Mottonen, J. M., Stock, A. M. & Stock, J. B. (1991). Roles of the highly conserved aspartate and lysine residues in the response regulator of bacterial chemotaxis. *J Biol Chem* **266**, 8348–8354.
- Martinez-Argudo, I., Martin-Nieto, J., Salinas, P., Maldonado, R., Drummond, M. & Contreras, A. (2001). Two-hybrid analysis of domain interactions involving NtrB and NtrC two-component regulators. *Mol Microbiol* **40**, 169–178.
- Martinez-Argudo, I., Salinas, P., Maldonado, R. & Contreras, A. (2002). Domain interactions on the *ntr* signal transduction pathway: two-hybrid analysis of mutant and truncated derivatives of histidine kinase NtrB. *J Bacteriol* **184**, 200–206.
- Mattison, K. & Kenney, L. J. (2002). Phosphorylation alters the interaction of the response regulator OmpR with its sensor kinase EnvZ. *J Biol Chem* **277**, 11143–11148.
- Mayuri Bagchi, G., Das, T. K. & Tyagi, J. S. (2002). Molecular analysis of the dormancy response in *Mycobacterium smegmatis*: expression analysis of genes encoding the DevR-DevS two-component system, Rv3134c and chaperone α -crystallin homologues. *FEMS Microbiol Lett* **211**, 231–237.
- Mouncey, N. J. & Kaplan, S. (1998). Redox-dependent gene regulation in *Rhodobacter sphaeroides* 2.4.1^T: effects on dimethyl sulfoxide reductase (*dor*) gene expression. *J Bacteriol* **180**, 5612–5618.
- Oh, J. I. & Kaplan, S. (2001). Generalized approach to the regulation and integration of gene expression. *Mol Microbiol* **39**, 1116–1123.
- Oh, J. I., Ko, I. J. & Kaplan, S. (2004). Reconstitution of the *Rhodobacter sphaeroides* *ccb3*-PrrBA signal transduction pathway *in vitro*. *Biochemistry* **43**, 7915–7923.
- Ohta, N. & Newton, A. (2003). The core dimerization domains of histidine kinases contain recognition specificity for the cognate response regulator. *J Bacteriol* **185**, 4424–4431.
- Park, H., Saha, S. K. & Inouye, M. (1998). Two-domain reconstitution of a functional protein histidine kinase. *Proc Natl Acad Sci U S A* **95**, 6728–6732.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schneider, S., Buchert, M. & Hovens, C. M. (1996). An *in vitro* assay of beta-galactosidase from yeast. *Biotechniques* **20**, 960–962.
- Sola, M., Lopez-Hernandez, E., Cronet, P., Lacroix, E., Serrano, L., Coll, M. & Parraga, A. (2000). Towards understanding a molecular switch mechanism: thermodynamic and crystallographic studies of the signal transduction protein CheY. *J Mol Biol* **303**, 213–225.
- Stock, A. M., Martinez-Hackert, E., Rasmussen, B. F., West, A. H., Stock, J. B., Ringe, D. & Petsko, G. A. (1993). Structure of the Mg^{2+} -bound form of CheY and mechanism of phosphoryl transfer in bacterial chemotaxis. *Biochemistry* **32**, 13375–13380.
- Stock, A. M., Robinson, V. L. & Goudreau, P. N. (2000). Two-component signal transduction. *Annu Rev Biochem* **69**, 183–215.
- Tanaka, T., Saha, S. K., Tomomori, C. & 12 other authors (1998). NMR structure of the histidine kinase domain of the *E. coli* osmosensor EnvZ. *Nature* **396**, 88–92.
- Tomomori, C., Tanaka, T., Dutta, R. & 11 other authors (1999). Solution structure of the homodimeric core domain of *Escherichia coli* histidine kinase EnvZ. *Nat Struct Biol* **6**, 729–734.
- Volz, K. & Matsumura, P. (1991). Crystal structure of *Escherichia coli* CheY refined at 1.7-Å resolution. *J Biol Chem* **266**, 15511–15519.
- West, A. H. & Stock, A. M. (2001). Histidine kinases and response regulator proteins in two-component signaling systems. *Trends Biochem Sci* **26**, 369–376.
- Yanisch-Perron, C., Vieira, J. & Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.
- Zapf, J., Sen, U., Madhusudan Hoch, J. A. & Varughese, K. I. (2000). A transient interaction between two phosphorelay proteins trapped in a crystal lattice reveals the mechanism of molecular recognition and phosphotransfer in signal transduction. *Structure* **8**, 851–862.
- Zeilstra-Ryalls, J. H., Gomelsky, M., Yeliseev, A. A., Eraso, J. M. & Kaplan, S. (1998). Transcriptional regulation of photosynthesis operons in *Rhodobacter sphaeroides* 2.4.1. *Methods Enzymol* **297**, 151–166.
- Zundel, C. J., Capener, D. C. & McCleary, W. R. (1998). Analysis of the conserved acidic residues in the regulatory domain of PhoB. *FEBS Lett* **441**, 242–246.