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Protein-Protein Interactions between Histidine Kinases and Response Regulators of *Mycobacterium tuberculosis* H37Rv§

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Using yeast two-hybrid assay, we investigated protein-protein interactions between all orthologous histidine kinase (HK)/response regulator (RR) pairs of *M. tuberculosis* H37Rv and identified potential protein-protein interactions between a noncognate HK/RR pair, DosT/NarL. The protein interaction between DosT and NarL was verified by phosphotransfer reaction from DosT to NarL. Furthermore, we found that the DosT and DosS HKs, which share considerable sequence similarities to each other and form a two-component system with the DosR RR, have different cross-interaction capabilities with NarL: DosT interacted with NarL, while DosS did not. The dimerization domains of DosT and DosS were shown to be sufficient to confer specificity for DosR, and the different cross-interaction abilities of DosS and DosT with NarL were demonstrated to be attributable to variations in the amino acid sequences of the α2-helices of their dimerization domains.

Keywords: histidine kinase, *Mycobacterium tuberculosis*, protein interaction, response regulator, two-component system

Introduction

Two-component systems (TCSs) comprise simple signal transduction pathways which play a major role in bacterial adaptation to environmental changes (Stock *et al.*, 2000; West and Stock, 2001). The TCS consists of a histidine kinase (HK) and its cognate response regulator (RR). The typical class I HK is composed of the N-terminal sensory domain and the C-terminal kinase domain which can be further divided into the DHp (dimerization and histidine phosphotransfer) and the CA (catalytic and ATP-binding) domains (Dutta *et al.*, 1999). The DHp domain consists of ~70 amino acid residues that form a secondary structure of α-helix-loop-α-helix (Tomomori *et al.*, 1999; Marina *et al.*, 2005; Casino *et al.*, 2009; Yamada *et al.*, 2009). The DHp domains of two identical subunits of the HK form a four-helix bundle and serve as an interacting domain of HK with RR and play a major role in dictating the specificity on HK-RR interactions (Tomomori *et al.*, 1999; Seok *et al.*, 2006; Skerker *et al.*, 2008; Casino *et al.*, 2009). A portion of the CA domain was also suggested to be involved in HK-RR interactions and thereby is assumed to contribute higher levels of specificity on HK-RR interactions (Seok *et al.*, 2006; Casino *et al.*, 2009).

BLAST search using the kinase domain of HK and the receiver domain of RR revealed that the genome of *Mycobacterium tuberculosis* H37Rv contains 14 and 16 genes encoding the HK and RR homologues, respectively. The *rv0600c* and *rv0601c* gene products among the 14 HK homologues are not the orthologous HKs, i.e., Rv0601c contains only the Hpt (histidine-containing phosphotransfer) domain found in the CheA family of HKs, whereas Rv0600c has only the CA domain (Shrivastava *et al.*, 2007). It was demonstrated that Rv0600c and Rv0601c function together to phosphorylate the Rv0602c RR. The other 12 HKs belong to the class I HKs composed of both the DHp and CA domains. Some of the functionally crucial amino acids involved in phosphorylation and dephosphorylation of the RR (two consecutive acidic amino acids coordinating a Mg^{2+} ion, phosphorylatable aspartate, serine/threonine and lysine involved in the phosphorylation-induced conformational change) are not conserved in the Rv0195, Rv0260c, and Rv2884 RR homologues. Rv3143 consists of only the N-terminal receiver domain without the effector domain. Therefore, *M. tuberculosis* appears to possess 12 functional RRs.

Although phosphotransfer from a HK to its cognate RR is very specific, some cross-talk (cross-phosphorylation) between HKs and non-cognate RRs has been observed (Yamamoto *et al.*, 2005; Drepper *et al.*, 2006; Noriega *et al.*, 2010). This cross-talk might contribute to either the integration of multiple signals into a single response or the multiple responses from a single signal (Laub and Goulian, 2007).

We, as well as other research groups, previously reported that yeast two-hybrid (Y2H) assay could be used to detect specific protein-protein interactions between HKs and RRs (Martinez-Argudo *et al.*, 2001; Ohta and Newton, 2003; Seok *et al.*, 2006). In this study, protein-protein interactions between all the orthologous HKs and RRs in *M. tuberculosis* were investigated using Y2H assay to identify possible cross-interactions between TCSs in this bacterium.

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Materials and Methods

Strains, plasmids, and growth conditions

The *Escherichia coli* strains were grown at 37°C in Luria-Bertani (LB) medium as described elsewhere (Sambrook, 2001). Ampicillin and kanamycin were added to the final concentration of 100 μg/ml and 50 μg/ml when appropriate. *Saccharomyces cerevisiae* AH109 strains (James *et al.*, 1996) were grown at 30°C in YPD (Clontech, USA) or synthetic defined dropout (SD) medium (Q-Biogene, Canada) as described in the manufacturer's manual (Clontech yeast protocols handbook #PT3024-1). The plasmids used in this study are listed in Supplementary Materials.

DNA manipulation and transformation of yeast

Standard protocols and manufacturer's instructions were followed for general recombinant DNA manipulations (Sambrook, 2001). *S. cerevisiae* strains were cotransformed with different pairs of two-hybrid plasmids according to the lithium acetate (LiAc)-mediated method as described previously (Guthrie and Fink, 1991). All PCR reactions in this study were performed using *pfu* DNA polymerase.

Construction of plasmids

pGADT7linker: In order to insert an additional linker of 28 amino acids between GAL4AD (GAL4 activation domain) and RR, a 84-bp DNA fragment was generated by means of PCR using the primers (5′-CCCCCATATGATGACAGCC ATGTGTCAAC-3′ and 5′-CCCTGAATTCGATTGTCGC CCATCTGC-3′) and the plasmid pPLA as the template and cloned into the prey vector pGADT7 restricted with *Nde*I-*Eco*RI, resulting in pGADT7linker.

GAL4AD-RR fusion plasmids: All the RR genes except *rv0602c* were amplified by PCR using appropriate primers from *M. tuberculosis* H37Rv genomic DNA as the template. The amplified PCR products were cloned into pGADT7 linker, yielding the plasmids carrying the genes for the GAL4AD-RR fusions.

GAL4BD-HK fusion plasmids: The gene portions of the cytoplasmic kinase domains of 12 HKs (SenX3: amino acid numbering 127–410, PhoR: 229–485, Rv0845: 201–425, PrrB: 200–446, MprB: 209–504, KdpD: 602–860, TrcS: 247–509, DosT: 352–573, DosS: 355–578, PdtaS: 263–501, MtrB: 265–567, TcrY: 216–475) were amplified by PCR and cloned into the bait vector pGBKT7, resulting in the plasmids carrying the genes for the GAL4BD (GAL4 DNA-binding domain)-HK fusions.

GAL4AD-HK fusion plasmids: The GAL4AD-HK fusion plasmids were constructed by directly cloning the HK gene-containing DNA fragments restricted from their corresponding GAL4BD-HK fusion plasmids into pGADT7, when it was possible to clone the HK genes in the same reading frame of the GAL4AD gene on pGADT7. Otherwise, the HK genes were generated by PCR using their corresponding GAL4BD-HK fusion plasmids as the templates and cloned into pGADT7.

GAL4BD-RR fusion plasmids: The GAL4BD-RR fusion plasmids were constructed by cloning the RR gene-containing DNA fragments restricted from their corresponding GAL4AD-RR fusion plasmids into pGBKT7, when it was possible to clone the RR genes in the same reading frame of the GAL4BD gene on pGBKT7. Otherwise, the RR genes were amplified by PCR using their corresponding GAL4AD-RR fusion plasmids as the templates and cloned into pGBKT7.

GAL4BD-DHp domain fusion plasmids: To construct GAL4BD-DHp domain fusion plasmids, 216-bp DNA fragments encoding the DHp domains of DosT and DosS were amplified by PCR using pBDosT and pBDosS plasmids as the templates, respectively. The PCR products were digested with *Eco*RI and *Bam*HI and cloned into pGBKT7, resulting in pBSDosTDHp and pBDosSDHp. The chimeric DosST DHp domain consists of the N-terminal portion of the DosS DHp domain (S374 to V421) and the C-terminal portion of the DosT DHp domain (R419 to D442). To construct the chimeric DNA fragment encoding the DosST DHp domain, a 162–bp DNA fragment encoding the N-terminal portion of DosS DHp was obtained by PCR using pBDosS as the template and the primer pair (5′-AATTGAATTCTCGCA ACGTCGGATGCGC-3′ and 5′-GCTGTAGATGGATTCC CGCACTTCAGGATTACGTTC-3′). A 150-bp DNA fragment encoding the C-terminal portion of DosT DHp was obtained by PCR using pBDosT as the template and the primer pair (5′-GAACGTAATCCTGAAGTGCGGGAAT CCATCTACAGC-3′ and 5′-GTCGATGACCTTGTCCAG-3′). A 216-bp DNA fragment encoding the chimeric DosST DHp domain was generated by using both the primary PCR products as the templates and the primer pair (5′-AATT GAATTCTCGCAACGTCGGATGCGC-3′ and 5′-AATTG GATCCGTCGAAGATCGCAGATCG-3′) in the secondary PCR. This product was digested with *Eco*RI and *Bam*HI and cloned into the pGBKT7 vector, yielding pBDosSTDHp.

Overexpression plasmids: A 699-bp DNA fragement including *regX3* and the six histidine codons immediately before its stop codon was amplified by PCR using the *M. tuberculosis* H37Rv genomic DNA as the template. The PCR product was restricted with *Nde*I and *Pst*I and cloned into pT7-7 digested with the same restriction enzymes, yielding pT7RegX3His. pT7NarLHis and pT7DosRHis were constructed for overexpression of *narL* and *dosR*, respectively, in the same way as pT7RegX3His except for the primers used for PCR.

Analysis of *in vivo* protein-protein interactions

The *S. cerevisiae* AH109 strains cotransformed with both pGADT7linker (or pGADT7) and pGBKT7 derivatives were grown in SD medium lacking leucine and tryptophan (SD/-Leu/-Trp). The cultures were diluted to an OD_{600} of 0.5–0.6 and spotted onto both solid SD/-Leu/-Trp medium and SD/-Leu/-Trp/-His medium containing appropriate concentrations of 3AT (3-1,2,3-triazole) (Sigma, USA). These plates were incubated for three to five days. The crude cell extracts of yeast strains were prepared by three freeze/thaw cycles using liquid nitrogen. Determination of β-galactosidase activity was performed using *o*-NPG as a substrate as described elsewhere (Schneider *et al.*, 1996).

Protein purification

DosS and DosT: The *E. coli* BL21 (DE3) strains (Promega,

WI) carrying pHis-DosS and pHis-DosT were used for overexpression of *dosS* and *dosT*, respectively. The overexpression of the genes and the purification procedure by means of Ni-Sepharose affinity chromatography were performed as previously described for the intact DevS protein (Lee *et al.*, 2008).

RegX3, NarL, and DosR: The genes encoding His_6-tagged RegX3, NarL, and DosR were overexpressed in the *E. coli* BL21 (DE3) carrying pT7RegX3His, pT7NarLHis, and pT7DosRHis, respectively. All purification steps of the RRs were same as those for DosS and DosT.

In vitro kinase assay

Autophosphorylation of DosT and DosS was performed at 30°C in an assay buffer (Tris-HCl; pH 8.0, 30 mM KCl, 5 mM $MgCl_2$) in the presence of a mixture of [γ-^{32}P]ATP and unlabeled ATP to a final concentration of 200 μM (500 Ci/mol) for 2 h. After autophosphorylation reaction, 158 pmol of either DosT or DosS was added to each of the RRs (5 μg) for phosphotransfer reactions (the total reaction volume is 50 μl). At various time intervals, 10 μl of aliquots were removed and the reactions were stopped by the addition of 3 μl of 5× loading buffer [50 mM Tris-HCl (pH 6.8), 4% (w/v) sodium dodecyl sulfate (SDS), 20% (w/v) glycerol, 20 mM dithiothreitol, 1% (v/v) β-mercaptoethanol, 0.1% (w/v) bromophenol blue, and 100 mM EDTA]. The phosphorylation level of the proteins was determined by SDS-PAGE and subsequent autoradiography.

Results

Identification of cross-interactions between noncognate HK-RR pairs of *M. tuberculosis* using Y2H assay

To identify cross-interactions between noncognate HK/RR pairs of *M. tuberculosis*, we used a Y2H system which is suitable for the detection of weak and transient protein-protein interactions. To generate GAL4AD-HK and GAL4BD-RR fusion proteins in the yeast strain AH109, 12 HK genes and 11 RR genes were cloned into the pGADT7 prey vector and the pGBKT7 bait vector for Y2H assay, respectively. The Rv0602c RR was excluded from the experiment, since it forms three-protein TCS together with Rv0600c and Rv0601c (Shrivastava *et al.*, 2007, 2009). 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 can grow on histidine-deficient medium and synthesize β-galactosidase due to the induction of the *HIS3* and *lacZ* reporter genes on its chromosomal DNA. As shown in Table 1 and Fig. 1A, the yeast strains expressing HKs and their cognate RRs showed robust growth on SD medium lacking histidine (SenX3/RegX3, PhoR/PhoP, Rv0845/NarL, PrrB/PrrA, KdpD/KdpE, DosT/DosR, DosS/DosR, PdtaS/PdtaR, MtrB/MtrA, TcrY/TcrX), validating that the Y2H assay is applicable to detect specific HK-RR interactions. The yeast strains expressing GAL4AD-MprB and GAL4AD-TrcS did not grow on histidine-deficient medium in any combination with GAL4BD-RRs,

Table 1. Protein-protein interactions between HKs and RRs of *M. tuberculosis* H37Rv

HK / RR	SenX3 (Rv0490)	PhoR (Rv0758)	Rv0845	PrrB (Rv0902c)	MprB (Rv0982)	KdpD (Rv1028c)	TrcS (Rv1032c)	DosT (Rv2027c)	DosS (Rv3132c)	PdtaS (Rv3220c)	MtrB (Rv3245c)	TcrY (Rv3764c)
RegX3 (Rv0491)	+++											
	+++			ns			ns				ns	
PhoP (Rv0757)		+++										
		+++		ns		+	ns				ns	
NarL (Rv0844c)		+++	+++					+				
			+++	ns			ns	+++				
PrrA (Rv0903c)		+		+++				+++				
				ns			ns					
MprA (Rv0981)												
			+	ns		+	ns				ns	
KdpE (Rv1027c)						+++						
			+++	ns		+++	ns				ns	
TrcR (Rv1033c)												
			+	ns		+	ns				ns	
PdtaR (Rv1626)										+++		
				ns			ns			+++	ns	
DosR (Rv3133c)								+++	+			
				ns			ns	+++	+++		ns	
MtrA (Rv3246c)											+++	
				ns			ns				ns	
TcrX (Rv3765c)		+								+++		+++
				ns			ns				ns	+++

The upper and lower cells for each HK/RR pair indicate growth of the yeast strains expressing the corresponding GAL4AD-HK/GAL4BD-RR (Fig. 1A) and GAL4BD-HK /GAL4AD-RR (Fig. 1B), respectively. The level of growth is indicated with +++ (strong) and + (weak). The "ns" denotes nonspecific protein-protein interactions. The cognate and noncognate HK/RR pairs showing strong cross-interactions are indicated with grey and dark-grey shading, respectively.

which might be due to instability or poor expression of the GAL4AD-MprB and GAL4AD-TrcS fusion proteins in the yeast cell. The similar phenomenon has been observed for the GAL4AD-NtrB HK (Martinez-Argudo *et al.*, 2002). Intriguingly, the Y2H assay revealed protein-protein interactions between some noncognate HK/RR pairs (Table 1 and Fig. 1A). The PhoR HK interacted with NarL and PrrA in addition to its cognate RR, PhoP. DosT also showed protein-protein interactions with PrrA and NarL, although its interaction with NarL was weak. Cross-interaction was also observed between PdtaS and TcrX.

To confirm the result obtained from the Y2H assay with GAL4AD-HK/GAL4BD-RR pairs, another Y2H assay was performed using the yeast strains expressing GAL4BD-HK/GAL4AD-RR pairs. As shown in Table 1 and Fig. 1B, the yeast strains expressing PrrB, TrcS, and MtrB fused to GAL4BD grew well on histidine-deficient medium, even when cotransformed with the empty pGADT7linker, indicating that expression of GAL4AD-(PrrB, TrcS, or MtrB) alone gave rise to false positive results. As in the Y2H assay with GAL4AD-HK/GAL4BD-RR pairs, strong protein-protein interactions between the cognate HK/RR pairs were also detected in the Y2H assay with GAL4BD-HK/GAL4AD-RR pairs (Table 1 and Fig. 1B). Strong cross-interactions between noncognate HK/RR pairs were observed for the DosT/NarL and Rv0845/KdpE pairs. The yeast strains with Rv0845/MprA, Rv0845/TrcR, KdpD/PhoP, KdpD/MprA, KdpD/TrcR, and TrcY/NarL exhibited weak growth on SD medium lacking histidine. Taken together, the results shown in Table 1 and Fig. 1 indicate the followings: i) the cognate HK/RR pairs, which were proven by phosphotransfer reactions (Via *et al.*, 1996; Himpens *et al.*, 2000; Haydel *et al.*,

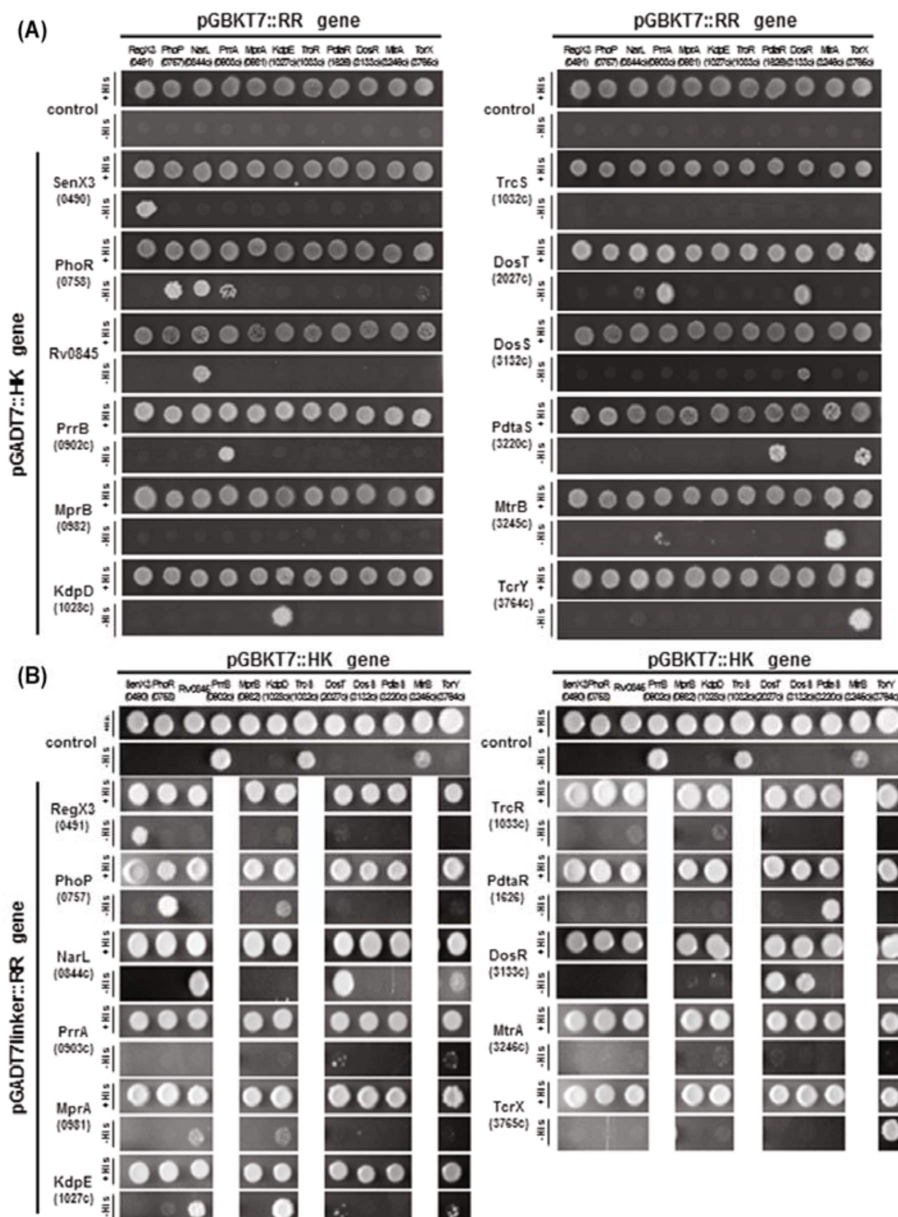

Fig. 1. Determination of protein-protein interactions between HKs and RRs of *M. tuberculosis* in Y2H assay. Growth of yeast strains transformed with both pGBKT7 and pGADT7 (linker) derivatives is shown. The yeast strains were grown at 30°C in SD/-Leu/-Trp medium. All yeast cultures were diluted to an OD_{600} of 0.5–0.6 and spotted onto SD/-Leu/-Trp plates (+His) and histidine-deficient SD/-Leu/-Trp plates containing 0.1 mM 3AT (-His). The SD/-Leu/-Trp plates and SD/-Leu/-Trp/-His/+3AT plates were incubated at 30°C for 3 to 5 days. (A) The HK genes on the left of the panels and the RR genes on the top of the panels were cloned pGADT7 and pGBKT7, respectively. The yeast strain containing empty prey vector pGADT7 was included as the control (control). (B) The RR genes on the left of the panels and the HK genes on the top of the panels were cloned pGADT7linker and pGBKT7, respectively. The empty prey vector pGADT7 linker was included as the control (control).

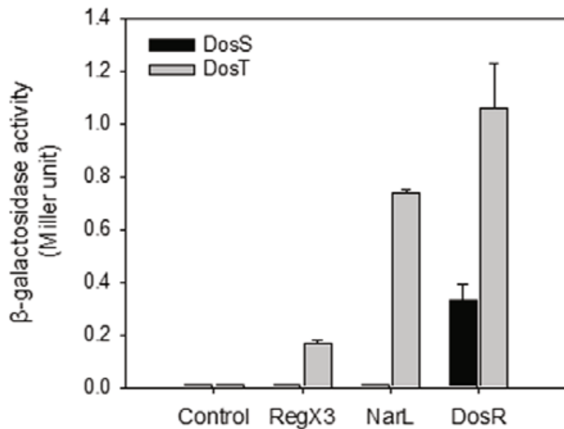

Fig. 2. Determination of the relative strength of protein-protein interactions between the DosS homologues (DosS and DosT) and their interacting RRs by means of β-galactosidase assay. The yeast strains carried either pBDosT expressing the GAL4BD-DosT fusion protein (grey bar) or pBDosS expressing the GAL4BD-DosS fusion protein (black bar).The yeast strains were cotransformed with either empty pGADT7linker (control) or pGADT7linker::RR gene expressing the corresponding GAL4AD-RR (RegX3, NarL, and DosR). The yeast strains were grown aerobically in liquid SD/-Leu/-Trp medium at 30°C to an OD_{600} of 0.65 to 0.75 and β-galactosidase activity was determined and normalized to that detected in the control strain expressing DosT alone (DosT control). The β-galactosidase activities are expressed as the Miller unit.

2002; Zahrt *et al.*, 2003; Ewann *et al.*, 2004; Saini *et al.*, 2004a; Morth *et al.*, 2005; Gupta *et al.*, 2006; Bhattacharya *et al.*, 2010) or predicted by the adjacent location of HK and RR genes, displayed strong protein-protein interactions in Y2H assay with both sets of GAL4-HK/GAL4-RR (GAL4AD-HK/GAL4BD-RR and GAL4BD-HK/GAL4AD-RR) except for the MprB/MprA and TrcS/TrcR pairs. ii) the PhoR/NarL, Rv0845/KdpE, DosT/PrrA, and PdtaS/TcrX pairs showed relatively strong cross-interactions which were detected in Y2H assay with only a set of GAL4-HK/GAL4-RR (either GAL4AD-HK/GAL4BD-RR or GAL4BD-HK/GAL4AD-RR). iii) DosT and NarL showed protein-protein interactions in Y2H assay with both combinations of GAL4-HK/GAL4-RR like the cognate HK/RR pairs, indicating the plausible cross-interaction between the two proteins, iv) it appears to be more proper for the study of protein-protein interactions between HK/RR pairs to use the GAL4AD-HK/GAL4BD-RR pair set than the GAL4BD-HK/GAL4AD-RR set, since the former resulted in less false positive results.

Different cross-interaction capabilities of DosT and DosS

The DosS and DosT HKs share 89% homology to each other at the level of their primary structures and control the phosphorylation state of the DosR RR in response to hypoxia and nitric oxide (NO) exposure (Roberts *et al.*, 2004; Saini *et al.*, 2004b; Kumar *et al.*, 2007; Sousa *et al.*, 2007). In this study, we found that DosS and DosT showed different cross-interactions with the noncognate NarL RR. DosT interacted with NarL, while DosS did not (Table 1 and Fig. 1). To determine the relative strength of cross-interactions between DosT and its interacting RRs (DosR and NarL), we measured β-galactosidase activities in the yeast strains expressing GAL4BD-DosT and GAL4AD-RRs (DosT/RRs) (Fig. 2). As the negative controls, the yeast strains expressing DosT/RegX3 and DosT alone (control) were included in the experiment. The strongest activity was detected in the yeast strain with DosT/DosR. The yeast strain expressing DosT/NarL showed 70% of the activity detected in the yeast strain with DosT/DosR. In contrast, the Y2H assay using GAL4BD-DosS showed that DosS interacted only with its cognate DosR RR and that protein-protein interactions between DosS and DosR were weaker than those between DosT and DosR (Fig. 2). DosS interacted with neither NarL nor RegX3. This result is in good agreement with the result shown in Table 1.

To investigate whether DosT can transfer the phosphoryl group to NarL which was shown to cross-interact with DosT in Y2H assay, we performed phosphotransfer reactions using the purified DosT and RRs (Fig. 3). The kinase activity of a HK consists of the autokinase and phosphotransferase activities. Autophosphorylation of the HK is the rate-limiting step of the phosphorylation of the RR by the HK, i.e., the autophosphorylation rate of the HK is much slower than the phosphotransfer rate from the phosphorylated HK to the RR. Our assumption was that the level of the phosphorylated DosT would be decreased following the addition of the purified NarL to the fully autophosphorylated DosT, if DosT could interact with NarL and catalyze its phosphorylation. As shown in Fig. 3, the addition of the purified NarL to the phosphorylated DosT led to a decrease in the level of phosphorylated DosT. When RegX3 was used in the phosphotransfer assay as a negative control, its addition did not change the level of the phosphorylated DosT. When the phosphotransfer reaction was carried out in the presence of DosR, most of the phosphorylated DosT was dephosphorylated within 5 min and the phosphorylated DosR was detected only at the time point of 5 min and disappeared at the later time points, indicating much faster phosphotransfer rate from DosT to DosR than that from DosT to NarL as well as the faster dephosphorylation

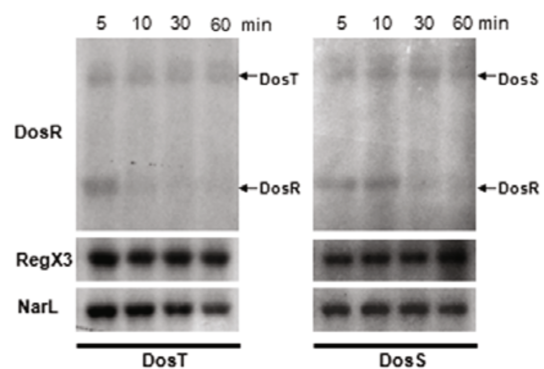

Fig. 3. The phosphotransferase activity of the DosS homologues to transfer the phosphoryl group to their interacting RRs. Following autophosphorylation reaction, 158 pmol of either DosT or DosS was added to 5 μg each of the RRs (DosR, RegX3, and NarL) for phosphotransfer reactions (the total reaction volume is 50 μl). 10 μl of aliquots were removed at the indicated time points, and the reaction was stopped by the addition of 5× loading buffer.

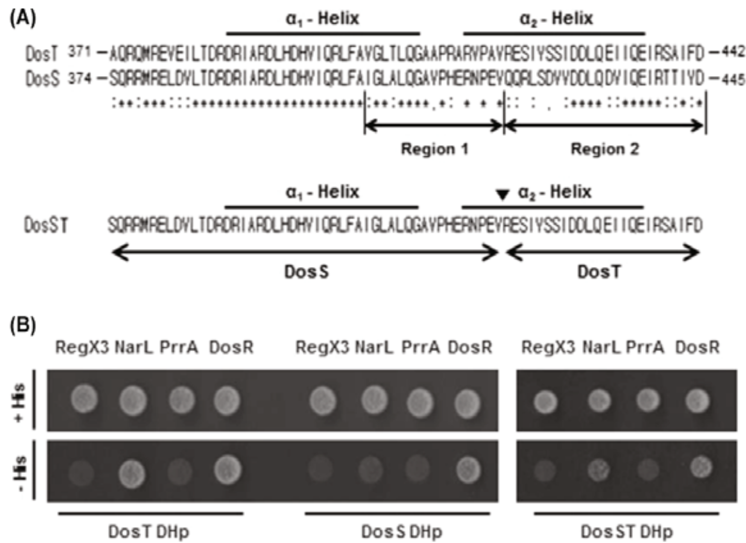

Fig. 4. Protein-protein interactions between the DHp domains of the DosS homologues and several RRs. (A) Comparison of the amino acid sequences of the DHp domains of DosS and DosT. The DHp domains of the DosS homologues and the position of the α1- and α2-helices were inferred from the known 3D structure of EnvZ by multiple alignment analysis. Identical or conservatively substituted amino acids are indicated by asterisks or colons, respectively. The DosST chimeric DHp domain consists of the N-terminal portion of DosS DHp and the C-terminal portion of DosT DHp. The fusion point is indicated by the inverted triangle. (B) Y2H assay. The gene portions encoding the DHp domains of DosT, DosS, and DosST were cloned into pGBKT7. The RR genes (*regX3*, *narL*, *prrA*, and *dosR*) were cloned into pGADT7linker. The yeast strains cotransformed with both pGBKT7 and pGADT7linker derivatives were grown at 30°C in SD/-Leu/-Trp medium. All yeast cultures were diluted to an OD_{600} of 0.5–0.6 and spotted onto SD/-Leu/-Trp plates (+His) and histidine-deficient SD/-Leu/-Trp plates containing 0.3 mM 3AT (-His).

rate of DosR than its phosphorylation rate under the given experimental conditions (in the absence of the reductants such as β-mercaptoethanol and DTT). When the same phosphotransfer reactions were performed with the purified DosS, the level of the phosphorylated DosS was not affected by the addition of the purified RegX3 and NarL. As with DosT, the phosphoryl group was rapidly transferred to DosR. The result obtained by the phosphotransfer assay is consistent with the different cross-interaction abilities of DosT and DosS found in Y2H assay.

The DHp domains of DosT and DosS confer recognition specificity for RRs

The DHp domain of a HK is composed of approximately 70–80 amino acid residues and has the secondary structure of α1-helix-loop-α2-helix. This domain has been proven to be the major interaction site with which the receiver domain of a RR comes in contact (Tomomori *et al.*, 1999; Zapf *et al.*, 2000; Skerker *et al.*, 2008; Casino *et al.*, 2009; Yamada *et al.*, 2009). The α1-helix of the DHp domain directly interacts with the α1-helix and the β5-α5 loop of the RR receiver domain (Casino *et al.*, 2009).

We investigated whether the different cross-interaction capabilities of DosT and DosS are due to the different amino acid sequences of their DHp domains or not. For this experiment, the DHp domains of DosT and DosS, which are composed of 72 amino acids, were fused to GAL4BD by cloning the corresponding gene portions into pGBKT7 (Fig. 4A). The yeast strain expressing either GAL4BD-DosT DHp or GAL4BD-DosS DHp was cotransformed with the plasmids expressing GAL4AD-RRs (RegX3, NarL, PrrA, and DosR), and Y2H assay was performed. As shown in Fig. 4B, the DHp domains of both DosT and DosS interacted with their cognate RR, DosR. Neither DosT nor DosS DHp domain interacted with RegX3 and PrrA used as the negative controls. As with the Y2H result with the whole kinase domains of DosT and DosS (Table 1 and Fig. 1B), the DHp domain of DosT cross-interacted with NarL, whereas that of DosS did not, indicating that the DHp domains alone can confer the cross-interaction specificity on DosT and DosS. The different cross-interaction abilities between DosT and DosS appear to be attributable to variations at the loop region connecting α1- and α2-helices of the DHp domain as well as at the α2-helix (Fig. 4A). To examine this assumption, we constructed the chimeric DosST DHp domain in which the N-terminal portion of the DosS DHp is fused to the C-terminal portion of the DosT DHp as depicted in Fig. 4A, and conducted Y2H assay to examine protein-protein interactions between DosST DHp and NarL (Fig. 4B). RegX3, PrrA, and DosR were included in the experiment as the controls. The Y2H assay revealed that the chimeric DosST DHp interacted with DosR and NarL, but not with RegX3 and PrrA in the same pattern as the DosT DHp, indicating that the different cross-interaction abilities of DosS and DosT are due to the variations in the region 2 of the DHp domain.

Discussion

Using Y2H assay, we comprehensively investigated protein-protein interactions between components of all orthologous TCSs in *M. tuberculosis* H37Rv. Relatively strong protein-protein interactions were observed between five noncognate HK/RR pairs (DosT/NarL, DosT/PrrA, PhoR/NarL, Rv0845/KdpE, and PdtaS/TcrX). Since only the DosT/NarL pair among them showed protein-protein interactions in both sets of GAL4BD-HK/GAL4AD-RR and GAL4AD-HK/GAL4BD-RR like the cognate HK/RR pairs, we further examined protein-protein interactions between DosT and NarL. DosT was shown to catalyze phosphorylation of NarL, although the phosphotransfer rate was slow. Phylogenetic analysis on the receiver domains of all the RRs in *M. tuberculosis* H37Rv revealed that DosR and NarL form the same subclade (data not shown), being in good agreement with the fact that DosR and NarL are members of the NarL family of RRs (Dasgupta *et al.*, 2000). These results strongly suggest the possibility that DosT indeed interacts with the noncognate NarL.

DosT and DosS are paralogues with 89% homology to each other and contain hemes as the ligand-binding cofactor in their N-terminal sensory domains (Sardiwal *et al.*, 2005; Ioanoviciu *et al.*, 2007; Podust *et al.*, 2008; Cho *et al.*, 2009). Nevertheless, their different roles in the hypoxic adaptation of mycobacteria have been suggested: DosT responds to a decrease in oxygen tension more sensitively and strongly than DosS, allowing DosT to play a more important role in the early phase of hypoxic conditions than DosS when the growth of *M. tuberculosis* gradually transits from aerobic to hypoxic conditions (Honaker *et al.*, 2009; Kim *et al.*, 2010). Although controversial, the different autooxidation property of the heme irons of DosT and DosS was also proposed (Kumar *et al.*, 2007; Cho *et al.*, 2009). In this study, we found that DosT interacted with DosR more strongly than DosS and that the cross-interaction with NarL was observed only for DosT, which is another different property of DosT and DosS. Previously it was demonstrated by X-ray crystallography and NMR titration studies that the DHp domain of a HK is the key determinant site which confers the specificity for HK-RR interactions (Tomomori *et al.*, 1999; Zapf *et al.*, 2000; Skerker *et al.*, 2008; Casino *et al.*, 2009; Yamada *et al.*, 2009). The Y2H assay as well as the domain swapping and mutagenesis studies have also revealed that the C-terminal portion of the α1-helix below the phosphoacceptor histidine and the loop between the α1- and α2-helices of the DHp domain dictate the specificity for HK-RR interactions (Seok *et al.*, 2006; Skerker *et al.*, 2008). We here demonstrated that the different cross-interaction abilities of DosT and DosS resulted from the variations in the amino acid sequences of the DHp α2-helix, indicating the α2-helix of the DHp domain also affects the determination of the specificity for HK-RR interactions.

Since nothing is known about the genes under the control of NarL and the nature of a signal activating the Rv0845 HK, it is difficult to envisage the physiological role of cross-interactions between the nocognate DosT/NarL pair. NarL of *M. tuberculosis* H37Rv shares 73% homology with NarL of *E. coli* at the level of the amino acid sequence. NarL of *E. coli* is known to regulate genes involved in nitrate and nitrite metabolism and its phosphorylation state is controlled by the NarX and NarQ HKs which recognize nitrate and nitrite (Noriega *et al.*, 2010). The N-terminal sensory domain of Rv0845 does not show any homology to those of NarX and NarQ, implying the possibility that Rv0845 is implemented in neither nitrate nor nitrate sensing. The DosT and DosS activities were demonstrated to be in the kinase-dominant state in the absence of O_2 or in the presence of diatomic gases such as NO and CO (Kumar *et al.*, 2007; Sousa *et al.*, 2007), while the signal activating the Rv0845 HK remains elusive. Although the cognate DosT/DosR pair shows the strong kinetic preference for phosphotransfer over DosT/NarL (Fig. 3), it is possible for DosT to enhance the expression of the NarL regulon by activating NarL together with Rv0845 under hypoxic conditions or in the presence of NO. On the other hand, DosT might mitigate the level of NarL phosphorylation under aerobic conditions, even though the signal, which Rv0845 recognizes, is present.

In conclusion, we for the first time conducted a comprehensive screening for protein-protein interactions between all orthologous TCSs of *M. tuberculosis*, which would provide a valuable basis for future studies on cross-talks between the TCSs in mycobacteria. It was demonstrated that the Rv0845 HK and NarL (Rv0844c) RR, whose partnership was predicted only from the adjacent location of their genes, indeed interact with each other at the protein level. Our study also revealed the novel aspect that, although DosT and DosS share the common DosR RR, their cross-interaction abilities with NarL are different.

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