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Purification of soluble and active RaxH, a transmembrane histidine protein kinase from *Xanthomonas oryzae* pv. *oryzae* required for AvrXa21 activity

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SUMMARY

The RaxHR two-component regulatory system (TCS) of the rice pathogen *Xanthomonas oryzae* pv. *oryzae* is required for AvrXa21 activity. RaxH is a typical transmembrane histidine protein kinase (HK), whereas RaxR is its concomitant response regulator (RR). Here, we report the isolation of soluble, active amounts of recombinant His-tagged full-length RaxH and RaxR following growth of *Escherichia coli* over-expressing strains in the presence of sorbitol and glycine betaine. Full-length His-RaxH showed similar autophosphorylation activities to that of a truncated version of the protein (His-t-RaxH), lacking the N-terminal transmembrane region. Transphosphorylation assays revealed that only full-length RaxH was able to induce phosphorylation of His-RaxR, indicating that the N-terminal region of RaxH may be required for transphosphorylation of RaxR. Using site-directed mutagenesis we also demonstrated that residues histidine 222 in RaxH and aspartate 51 in RaxR are essential for phosphorylation activities of these proteins. Utilization of compatible solutes may be widely applied for purification of soluble, active recombinant transmembrane proteins, and in particular for purification of transmembrane HKs.

INTRODUCTION

Xanthomonas oryzae pv. *oryzae* is the causal agent of bacterial leaf blight (BLB) of rice (*Oryza sativa* L.), which is responsible for significant reductions of rice yields worldwide. *Xa21* is a rice disease resistance (*R*) gene encoding a presumed receptor kinase (RK), which confers resistance to multiple races of this pathogen (Dardick and Ronald, 2006; Wang *et al.*, 1996). Xa21 belongs to the leucine-rich repeats (LRR) subclass of plant RKs and contains an extracellular domain carrying imperfect units of 24-amino acid LRR (Song *et al.*, 1995). The extracellular LRR domain of Xa21 is responsible for recognition of several *X. oryzae* pv. *oryzae* strains (Wang *et al.*, 1998).

AvrXa21, the presumed bacterial molecule that interacts with Xa21 to elicit the plant defence response, is yet to be identified. However, in the search for AvrXa21, several *X. oryzae* pv. *oryzae* genes that are required for AvrXa21 avirulence activity (*rax* genes) were identified. In contrast to wild-type strain PXO99, which is avirulent on rice lines expressing *Xa21*, PXO99 knock-out strains impaired in *rax* genes lose AvrXa21 activity and become totally or partially virulent on Xa21-rice lines (Burdman *et al.*, 2004; da Silva *et al.*, 2004; Shen *et al.*, 2002). Three of these genes, *raxA*, *raxB* and *raxC*, encode proteins that share similarity with components of type I secretion systems of Gram-negative bacteria (da Silva *et al.*, 2004). Three additional genes, *raxP*, *raxQ* and *raxST*, encode proteins involved in sulphur metabolism (da Silva *et al.*, 2004; Shen *et al.*, 2002). *raxST* is linked to *raxA* and *raxB*, and sequence analyses suggest these genes are part of one operon, named *raxSTAB* (da Silva *et al.*, 2004). Sequencing downstream of *raxSTAB* led to the identification of two genes, *raxR* and *raxH*, encoding a response regulator (RR) and a transmembrane histidine protein kinase (HK) of bacterial two-component regulatory systems (TCSs), respectively. The RaxHR system belong to the OmpR/EnvZ family of TCSs (Burdman *et al.*, 2004).

The role of the RaxHR system in the AvrXa21/Xa21 interaction was investigated and it was found that this TCS is required for expression of wild-type levels of AvrXa21 activity (Burdman *et al.*, 2004). PXO99 mutant strains impaired in *raxR* and/or *raxH* genes caused lesions significantly longer and grew to significantly higher levels than the wild-type strain in Xa21-rice leaves. Promoter-reporter studies revealed that the *raxSTAB* operon is a target for RaxHR regulation (Burdman *et al.*, 2004). Moreover, evidence from real-time PCR studies has indicated that not only the *raxSTAB* operon but also other *rax* genes (*raxPQ* and *raxC*) are positively

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regulated by the RaxHR system, and that regulation occurs in a cell density-dependent manner, (S. W. Lee, unpublished data).

In prokaryotes, TCSs regulate a wide variety of biological processes, including expression of toxins and other proteins related to virulence and pathogenicity. Upon activation by determined environmental stimuli, the HK, which usually is a transmembrane protein, autophosphorylates at a conserved histidine residue, creating a high-energy phosphoryl group that is subsequently transferred to an aspartate residue in the RR. This induces a conformational change in the regulatory domain of the latter, which results in activation of its effector domain triggering activation or inhibition of a gene or a set of genes (Grebe and Stock, 1999; Stock *et al.*, 2000; West and Stock, 2001).

The involvement of the RaxHR system in the AvrXa21/Xa21 interaction suggests a model in which the bacterium senses a component in the extracellular environment, perhaps a rice or a bacterial peptide, that activates this TCS to control AvrXa21 activity, directly or via additional regulatory elements. One important approach to assess this model and characterize the RaxHR-mediated regulatory mechanism is the generation of recombinant, active proteins of this system. Despite many studies aimed at characterization of TCSs, attempts to obtain soluble and functional full-length HKs have consistently failed. Instead, most studies were performed with truncated versions of HKs lacking their transmembrane (TM) domains.

By cultivating *Escherichia coli* cells under osmotic stress in the presence of the protein-stabilizing compatible solute glycine betaine, Blackwell and Horgan (1991) obtained large amounts of soluble, active dimethylallyl pyrophosphate:5′-AMP transferase (DMAPP:AMP transferase). Curiously, despite the well-known protein-stabilizing features of compatible solutes such as glycine betaine (Caldas *et al.*, 1999; da Costa *et al.*, 1998; Diamant *et al.*, 2001; Galinski, 1995) and the great potential of this method for production of soluble recombinant proteins, this technique has not been widely exploited. Moreover, no mention of this technique is found in manufacturers' handbooks dealing with expression and purification of recombinant proteins. Here we report on the isolation of soluble, active amounts of recombinant histidine (His 6X)-tagged full-length RaxH and RaxR following growth of *E. coli* over-expressing strains in the presence of sorbitol and glycine betaine. In addition, we show that in the case of this HK, the TM domain is essential for transphosphorylation of the corresponding RR. We also demonstrate that residues histidine 222 in RaxH and aspartate 51 in RaxR are essential for phosphorylation activities of these proteins.

RESULTS

Expression of recombinant RaxH and RaxR in *E. coli*

raxH and *raxR* coding sequences (CDSs) were cloned downstream of a histidine tag (His 6X) into vector pET15b to produce plasmids pETraxH and pETraxR, respectively. The calculated molecular weights (MW) of the recombinant proteins (including the His 6X tag) are 48.9 kDa for His-RaxH and 28.8 kDa for His-RaxR. Because difficulties were expected to purify full-length RaxH in soluble form, we also generated plasmid pETtraxH encoding a His-tagged version of RaxH lacking its TM domain. The calculated MW of this protein, named His-t-RaxH, is 31.5 kDa. The plasmids were transformed into *E. coli* BL21 cells for over-expression of the recombinant proteins upon addition of isopropyl-β-D-thiogalactopyranoside (IPTG). All recombinant proteins were over-expressed in *E. coli* under standard conditions (LB medium; 37 °C; 1 mM IPTG; shown in Fig. 1 for His-RaxH).

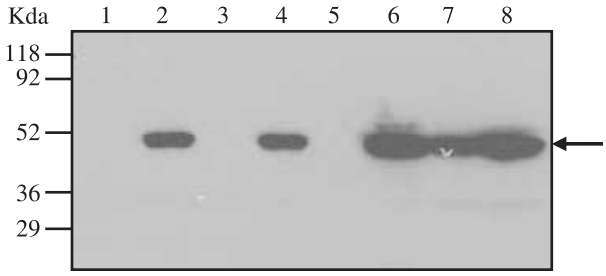

Fig. 1 Production of soluble His-RaxH following incubation of *E. coli* BL21/pETraxH cells in modified LB medium containing 1 M sorbitol and 2.5 mM glycine betaine (LB*). Bacteria were incubated in LB or in LB*, with or without 1 mM IPTG for 4 h at 37 °C. Cells were then lysed and soluble/insoluble fractions were obtained, according to standard procedures (The QIA*expressionist*, Qiagen). Samples were run in SDS-PAGE and transferred to nitrocellulose membranes for detection by ECL after incubation of the membrane with anti-His 6X monoclonal antibodies (1 : 3000) and anti-mouse IgG coupled to horseradish peroxidase (1 : 10 000), as primary and secondary antibodies, respectively. Lanes: (1) LB; (2), LB w/IPTG; (3) LB w/IPTG, soluble fraction; (4) LB w/IPTG, insoluble fraction; (5) LB*; (6) LB* w/IPTG; (7) LB* w/IPTG, soluble fraction; (8) LB* w/IPTG, insoluble fraction. The arrow indicates the position of His-RaxH.

In all tested conditions, over-expression of His-RaxR and His-t-RaxH was clearly observed by Coomassie staining of SDS-PAGE gels of bacterial crude extracts (Fig. 2A and 2C, respectively). By contrast, His-RaxH expressed to significantly lower levels (Fig. 2B), and its over-expression had to be confirmed by Western blotting using anti-His 6X monoclonal antibodies. In addition, BL21 cells over-expressing His-RaxH grew more slowly and to a lesser extent than cells over-expressing the other proteins, suggesting a negative, toxic effect of His-RaxH expression in *E. coli*.

Attempts to obtain soluble His-RaxH following induction of LB-grown BL21/pETraxH cells with IPTG failed (Fig. 1). Unexpectedly, we were also not able to obtain soluble His-RaxR following induction of BL21/pETraxR cells in LB medium (data not shown). For both proteins, several modifications were assessed, including utilization of different combinations of IPTG concentrations, growth temperatures and incubation times (see Experimental procedures), all without success (data not shown). By contrast, BL21/pETtraxH

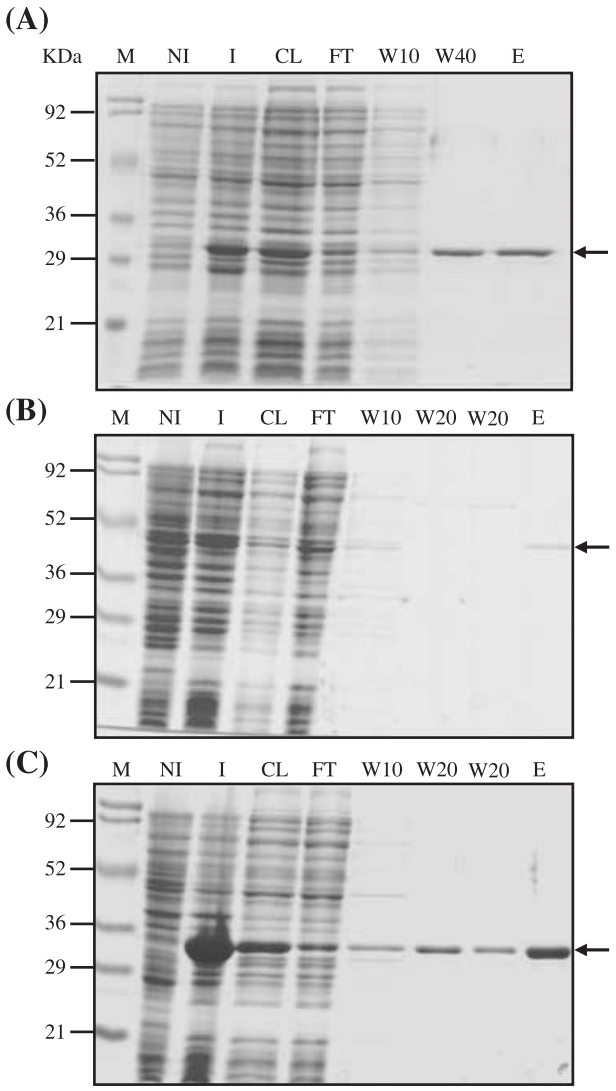

Fig. 2 Native purification of recombinant proteins. (A) His-RaxR; (B) His-RaxH; (C) His-t-RaxH. BL21 cells were incubated in LB* (His-RaxR, His-RaxH) or in LB (His-t-RaxH), with or without IPTG (1 mM) for 4 h at 37 °C (His-RaxH, His-t-RaxH) or 30 °C (His-RaxR). Lyses and purifications were performed according to standard procedures (The QIA*expressionist*, Qiagen). SDS-PAGE of selected fractions are shown. Lanes: (M) standard markers; (NI) non-induced cells; (I) cells induced with IPTG; (CL) cleared lysate (soluble fraction); (FT) flow-through; (Wn′) washings with n′ mM imidazole; (E) eluates with 250 mM imidazole. Arrows indicate the position of the eluted recombinant proteins.

cells expressed soluble His-t-RaxH under standard conditions (incubation in LB for 3–4 h at 37 °C in the presence of 1 mM IPTG; data not shown).

To obtain soluble His-RaxH and His-RaxR, BL21 cells were incubated under osmotic stress in a modified LB medium (named LB*), containing 1 M sorbitol and 2.5 mM glycine betaine (Blackwell and Horgan, 1991). Under osmotic stress, halophilic and halotolerant bacteria control cytoplasmic osmolarity by accumulating a range of organic osmolytes, referred to as compatible solutes or osmoprotectants, with glycine betaine being one of the best studied osmoprotectants (Galinski, 1995). Indeed, incubation of BL21/pETraxH and BL21/pETraxR in LB* medium led to the production of soluble amounts of both His-RaxH (approximately one-third of total protein; Fig. 1) and His-RaxR (about one-half of total protein; data not shown).

Purification of soluble forms of His-RaxH, His-t-RaxH and His-RaxR

Small-scale experiments were carried out to optimize expression of the recombinant proteins and to increase their solubilization. In all cases, the concentration of IPTG that led to the isolation of larger amounts of soluble protein was 1 mM (as compared with 0.1 and 0.5 mM). Optimal incubation temperatures were 37 °C for His-RaxH and His-t-RaxH (the latter in regular LB), and 30 °C for His-RaxR, although differences between temperatures (25, 30, 37 °C) were not significantly pronounced. Figure 2 summarizes the native purification flows of soluble His-RaxR, His-RaxH and His-t-RaxH, using the optimized procedures. The level of purity of the recombinant proteins varied between approximately 95 and 98% among the different trials. As expected from over-expression studies, His-RaxH was obtained at lower concentrations than the other proteins (Fig. 2), probably because of its toxic effect on BL21 cells.

Following optimization of purification procedures, the recombinant proteins were purified on a large scale for activity assays. Under optimized conditions the yields for the recombinant proteins from different purification batches ranged from 0.02 to 0.04 mg/L of culture for His-RaxH, 1.0–1.5 mg/L for His-t-RaxH and 0.4–0.7 mg/L for His-RaxR. Fractions containing purified recombinant proteins (Fig. 2) were dialysed to remove imidazole and to equilibrate the proteins with storage buffer.

To overcome the relatively low yield of His-RaxH, this protein was further concentrated using Amicon Ultra-4 (10 000 MWCO) centrifugal filter tubes (Millipore Corp., Billerica, MA). Glycerol was added to a final concentration of 25–30% and proteins were stored at –20 °C.

Phosphorylation activities of recombinant proteins

Several autophosphorylation assays were carried out with both His-RaxH and His-t-RaxH to determine optimal activity conditions. Both recombinant proteins autophospohorylated under all tested conditions. However, among the tested activity buffers, the one described by Potter *et al.* (2002) seemed to give better results than the others (Fig. 3), and thus this buffer was selected for further assays. Both His-RaxH and His-t-RaxH autophosphorylation initiated a few seconds after addition of [γ-^{32}P]ATP and autophosphorylation increased with time up to 40 min of incubation

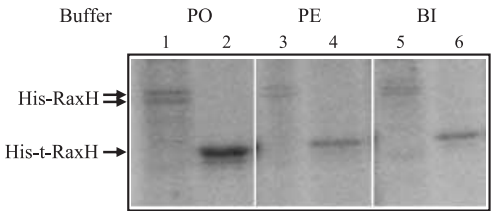

Fig. 3 Autophosphorylation of RaxH in different buffers. Approximately 25 pmol of His-RaxH (lanes 1, 3 and 5) or His-t-RaxH (lanes 2, 4 and 6) were incubated in 30 μL of Potter *et al.* (2002; PO), Pernestig *et al.* (2001; PE) or Bird *et al.* (1999; BI) reaction buffers (detailed in Experimental procedures). Reactions were initiated by addition of 10 μCi of [γ-^{32}P]ATP, and terminated after 1 h by addition of 5 μL 6× SDS-PAGE loading buffer. Samples (15 μL) were run in SDS-PAGE. Gels were dried under vacuum and labelled proteins were visualized by phosphor imaging.

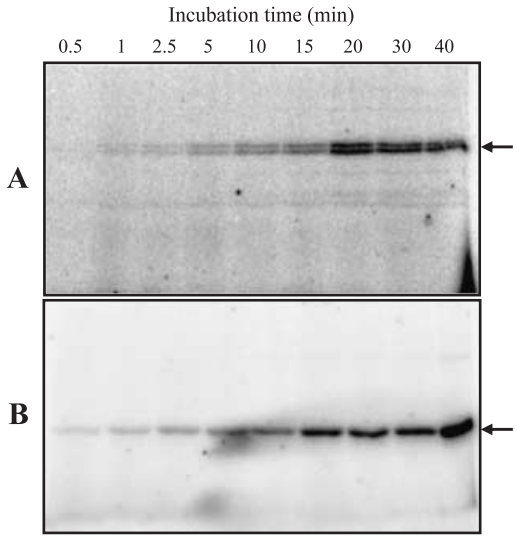

Fig. 4 Time-course of RaxH autophosphorylation. (A) His-RaxH; (B) His-t-RaxH. Approximately 120 pmol of recombinant proteins were incubated in 180 μL of Potter *et al.* (2002) autophosphorylation buffer. Reactions were initiated by addition of 50 μCi of [γ-^{32}P]ATP. Aliquots of 20 μL were collected at different times, and reactions were terminated by addition of 4 μL 6× SDS-PAGE loading buffer. Samples (15 μL) were run in SDS-PAGE. Gels were dried under vacuum and labelled proteins were visualized by phosphor imaging. Arrows indicate the position of the recombinant proteins.

(Fig. 4). Further incubation (1 h) did not significantly increase the phosphorylation signal of both proteins (data not shown). A double-band pattern was often obtained, especially for His-RaxH (also in transphosphorylation studies). Although the presence of contaminants cannot be completely discarded, double-band patterns are commonly found in studies involving recombinant proteins and are often attributed to partial truncated translation or protein degradation (The QIA*expressionist*, Qiagen, Valencia, CA).

Both His-RaxH and His-t-RaxH were assessed in transphosphorylation assays with His-RaxR (Fig. 5). His-RaxR phosphorylated

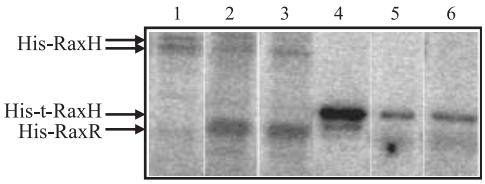

Fig. 5 Transphosphorylation of RaxR in the presence of RaxH. Reactions were carried out in 30 μL of Potter *et al.* (2002) phosphor transfer buffer using approximately 25 pmol of His-RaxH or His-t-RaxH and 40 pmol of His-RaxR. All reactions were initiated by addition of 10 μCi of [γ-^{32}P]ATP, and terminated by addition of 4 μL 6× SDS-PAGE loading buffer. Samples (15 μL) were run in SDS-PAGE; gels were dried under vacuum and labelled proteins were visualized by phosphor imaging. Treatments: His-RaxH or His-t-RaxH were incubated for 1 h in the presence of [γ-^{32}P]ATP (lanes 1 and 4, respectively); His-RaxH or His-t-RaxH were incubated for 1 h in the presence of [γ-^{32}P]ATP, then His-RaxR was added for an additional hour (lanes 2 and 5, respectively); His-RaxH or His-t-RaxH were incubated with His-RaxR for 2 h in the presence of [γ-^{32}P]ATP (lanes 3 and 6, respectively).

in the presence of His-RaxH~P, and no differences were found between treatments involving pre-incubation of His-RaxH for 1 h followed by addition of His-RaxR and incubation for one additional hour, or incubation of both proteins together for 2 h (Fig. 5). By contrast, phosphotransfer from His-t-RaxH~P to His-RaxR did not occur (or occurred only to a very minor, undetectable extent) in any of the above treatments, thus suggesting the importance of the TM domain of the HK for *in vitro* phosphorylation of the RR in this system.

Time-course transphosphorylation assays confirmed the above results, showing that phosphorylation of His-RaxR is accompanied by dephosphorylation of His-RaxH, which does not occur in combination of the first with His-t-RaxH (Fig. 6). Figure 6 also shows that His-RaxR is not able to autophosphorylate (lane R), thus confirming that His-RaxH is essential for transphosphorylation of this RR.

Sequence analysis of RaxH and RaxR revealed that the putative phosphorylation residues of this proteins are histidine 222 (H222) and aspartate 51 (D51), respectively (Burdman *et al.*, 2004). To verify this, we generated pET15b plasmids carrying mutated versions of recombinant RaxH and RaxR, in which these residues were substituted by alanine by site-directed mutagenesis, and the phosphorylation activities of His-RaxHH222A and His-RaxRD51A were assessed in comparison with the wild-type proteins. Figure 7 clearly shows that both His-RaxHH222A and His-RaxRD51A lack phosphorylation activities, demonstrating that H222 and D51 are indeed the phosphorylation sites of RaxH and RaxR, respectively.

DISCUSSION

In *Xanthomonas oryzae* pv. *oryzae*, the RaxHR TCS is required for expression of wild-type levels of AvrXa21 activity (Burdman *et al.*, 2004). TCSs have been shown to regulate a wide variety

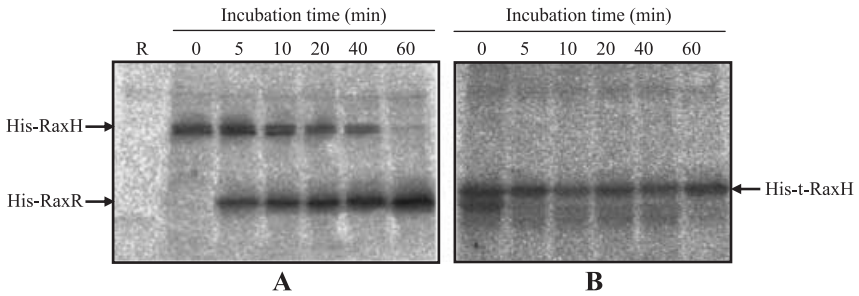

Fig. 6 Time-course of transphosphorylation of RaxR in the presence of RaxH. Reactions were carried out in 180 μL of Potter *et al.* (2002) phosphor transfer buffer. Approximately 120 pmol of His-RaxH (A) or His-t-RaxH (B) were incubated for 1 h with 50 μCi of [γ-^{32}P]ATP. Then, about 200 pmol of His-RaxR was added, and aliquots of 20 μL were collected at different times (time 0, before addition of His-RaxR). Lane R (in A) indicates incubation of approximately 40 pmol of His-RaxR for 1 h (35 μL buffer, 50 μCi of [γ-^{32}P]ATP). Reactions were terminated by addition of 4 μL 6× SDS-PAGE loading buffer and samples (15 μL) were run in SDS-PAGE. Gels were dried under vacuum and labelled proteins were visualized by phosphor imaging. Arrows indicate the position of the recombinant proteins.

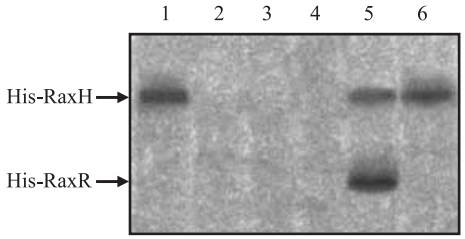

Fig. 7 Autophosphorylation (lanes 1 and 2) and transphosphorylation (lanes 3–6) of mutated RaxH and RaxR in comparison with the wild-type proteins. Reactions were carried out as described in Fig. 5. Treatments: His-RaxH (lane 1) or His-RaxHH222A (lane 2) were incubated for 1 h in the presence of [γ-^{32}P]ATP; His-RaxHH222A was incubated for 1 h in the presence of [γ-^{32}P]ATP, then His-RaxR (lane 3) or His-RaxRD51A (lane 4) were added for an additional hour; His-RaxH was incubated for 1 h in the presence of [γ-^{32}P]ATP, then His-RaxR (lane 5) or His-RaxRD51A (lane 6) were added for an additional hour.

of biological processes in bacteria, including expression of genes related to virulence and pathogenicity (Grebe and Stock, 1999; Stock *et al.*, 2000; West and Stock, 2001). The abundance of TCSs in prokaryotes can be assessed from surveys of different bacterial genomes. For instance, the *E. coli* genome encodes 62 two-component proteins (Mizuno, 1997). The recently sequenced genomes of *X. oryzae* pv. *oryzae* and related xanthomonads encode around 30 predicted TCSs (da Silva *et al.*, 2002; Lee *et al.*, 2005; Thieme *et al.*, 2005).

One limitation in characterizing bacterial TCSs has been the difficulty in obtaining soluble, folded and active full-length histidine protein kinases (HKs), which are usually present as transmembrane proteins. Therefore, most studies have been performed with truncated versions of HKs lacking the TM region. Exceptions of these are the *E. coli* NarX and the *Rhodobacter sphaeroides* RegB. Walker and DeMoss (1993) were able to purify soluble, active NarX from membrane fractions of NarX over-expressing *E. coli* strains. For purification of soluble, active RegB, Potter *et al.* (2002) utilized pTTQ18His, a membrane protein expression plasmid. In addition, the *E. coli* KdpD has been expressed successfully as an enriched protein in *E. coli*, but in contrast to the others, this protein did not retain functional activity after purification from membranes (Stallkamp *et al.*, 1999). Similarly, Sanowar and Le Moual (2005) were able to solubilize full-length His-tagged PhoQ of *Salmonella typhimurium* using different detergents; however, in all cases no activity of the purified protein was detected. In this case, typical HK catalytic activities were reconstituted following insertion of recombinant PhoQ into liposomes (Sanowar and Le Moual, 2005). Similar results to those obtained with PhoQ were observed with recombinant EnvZ of *E. coli* (Jung *et al.*, 2001).

Under osmotic stress, halophilic and halotolerant bacteria control cytoplasmic osmolarity by accumulation of organic osmolytes, referred to as compatible solutes or osmoprotectants (da Costa *et al.*, 1998; Galinski, 1995). One of the best studied osmoprotectants is glycine betaine, which accumulates in *E. coli* cells to molar concentrations at high osmolarity, usually by uptake from the extracellular environment, although betaine can be synthesized from choline by several strains (Caldas *et al.*, 1999).

The underlying principle of hyperosmotic-stress-governed and solute-assisted expression is poorly understood. In addition to the predominant role that internalized osmoprotectants play in cellular osmotic adjustment, these solutes should directly participate in other intracellular processes. Protective effects of betaine and other solutes on protein stability under increased salinity or temperature have been reported (Arakawa and Timasheff, 1985; Murata *et al.*, 1992). Bourot *et al.* (2000) were able to restore the growth of a conditional lysine auxotrophic mutant by addition of lysine or osmoprotectants such as glycine betaine in minimal media. They demonstrated that the growth rate increased proportionally with the augmentation of the intracellular levels of glycine betaine. This and other evidence from *in vitro* assays led to the conclusion that glycine betaine may actively assist *in vivo* protein folding in a chaperone-like manner (Bourot *et al.*, 2000).

Blackwell and Horgan (1991) reported expression of soluble, active DMAPP:AMP transferase under osmotic stress in the presence of glycine betaine and sorbitol: under these conditions, an increase of up to 427-fold in the active yield was achieved as compared with standard growth conditions. The use of glycine betaine as a means to counteract the insolubility of recombinant proteins has also been described for ferritin (Van Wuytswinkel *et al.*, 1995) and immunotoxins (Barth *et al.*, 2000). However, in spite of the above, the use of compatible solutes has not been widely exploited for purification of recombinant proteins.

Here we generated His-tagged recombinant RaxH and RaxR proteins for expression in *E. coli* cells, purification and further characterization. Not surprisingly, all attempts to obtain soluble His-RaxH under standard growth and induction conditions failed. We also failed to obtain His-RaxR in soluble form, probably because of the large amounts obtained for this protein. This problem was solved by growing and inducing cells under osmotic pressure, in the presence of glycine betaine and sorbitol. Although a lower over-expression of recombinant proteins was obtained with this procedure, especially for His-RaxH, sufficient amounts of soluble protein were obtained for functional assays. To the best of our knowledge, this is the first report on glycine betaine-assisted purification of a soluble, active transmembrane HK.

Functional activity of the recombinant proteins was demonstrated in HK autophosphorylation and RR transphosphorylation assays. In these assays, full-length His-RaxH showed similar autophosphorylation activity as a truncated version of this protein lacking the TM domain. Moreover, transphosphorylation of His-RaxR was obtained only in the presence of the full-length recombinant RaxH protein, suggesting that in the case of this HK, the TM domain is required for RR transphosphorylation. The importance of the TM domain for functionality of an HK was previously demonstrated for the *R. sphaeroides* RegB, for which *in vivo* mutagenesis studies revealed the importance of the central part of this domain for sensing and signal transduction (Oh *et al.*, 2001). In support of the important regulatory activity of the TM domain, Potter *et al.* (2002) revealed important differences in functional assays between full-length and truncated RegB.

Typical prokaryotic HKs and RRs possess conserved phosphorylation domains. HKs catalyse ATP-dependent autophosphorylation of a specific histidine residue within the dimerization domain, while RRs catalyse transfer of the phosphoryl group from the HK phospho-histidine to one of its own aspartate residues in the conserved regulatory (or receiver) domain (Stock *et al.*, 2000; West and Stock, 2001). Sequence analysis of RaxH and RaxR revealed that the putative phosphorylation residues of these proteins are H222 and D51, respectively (Burdman *et al.*, 2004). Using site-directed mutagenesis combined with phosphorylation assays we demonstrate that H222 and D51 are indeed the phosphorylation sites of RaxH and RaxR, respectively, and that these proteins have no alternative phosphorylation sites, at least under tested conditions.

Findings from this study confirm the functionality of the RaxHR system and open new possibilities for further characterization of this TCS. As stated above, most *in vitro* activities of TCSs have been characterized using truncated HKs, which in most studies show constitutive autophosphorylation activities. As with truncated HKs, *in vitro* autophosphorylation of full-length NarK and RegB appears to be independent of the ligand/signal required for autophosphorylation *in vivo*. For example, NarK is part of the *E. coli* nitrate sensing system; however, autophosphorylation *in vitro* of purified full-length NarK is independent of nitrate concentration (Walker and DeMoss, 1993). RegB is involved in regulation of the global redox switch between aerobic and anaerobic growth in *R. sphaeroides*; however, Potter *et al.* (2002) showed that purified full-length RegB has identical *in vitro* autophosphorylation kinetics under both aerobic and anaerobic conditions. As for these proteins, we showed that full-length RaxH is able to autophosphorylate *in vitro* in standard activity buffer. One possibility exists that the presumable ligand required for RaxH autophosphorylation *in vivo* is not required in *in vitro* assays. Alternatively, a ligand or other bacterial regulator protein may be required for repression (instead of stimulation) of RaxH activity. Based on the autophosphorylation assays described here, we are now generating an assay to test if RaxH activity is modulated by bacterial or plant-derived molecules.

Lastly, we support the suggestion that glycine betaine-assisted protein folding may be widely exploited for purification of soluble, active transmembrane proteins, and in particular, of transmembrane HKs. In light of the importance of full-length HKs for functionality, as demonstrated using the RaxHR and the RegAB system, utilization of full-length versions of HKs instead of their truncated versions may be needed in future studies.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids and DNA manipulations

Escherichia coli strain DH10B (Gibco BRL, Gaithersburg, MD) was used for routine cloning and maintenance of plasmids. *E. coli* strain BL21(DE3) pLysS (BL21; Novagen, Merck KGaA, Darmstadt, Germany) was used for over-expression and purification of recombinant proteins. Cultures were grown in Luria-Bertani medium (LB; Difco Laboratories, Detroit, MI) at 37 °C, or as otherwise indicated. For solid media, agar was added at 16 g/L. Constructs for over-expression of recombinant proteins were generated with pET15b plasmid (Novagen), and carbenicillin (50 μg/mL) was added for growth of *E. coli* cells carrying these vectors. Plasmid DNA preparations, restriction enzyme digestions and other routine DNA manipulations were performed using standard procedures (Sambrook *et al.*, 1989). Genomic DNA from *Xanthomonas oryzae* pv. *oryzae* PXO99 (Hopkins *et al.*, 1992) was prepared according to Ausubel *et al.* (1994). DNA sequencing of cloned fragments

was performed by the dideoxy chain termination method (Sanger *et al.*, 1992) using an automated sequencer (Model 400 I, Li-Cor Inc., Lincoln, NE). Primers were purchased from Operon Biotechnologies Inc. (Huntsville, AL). PCR was performed with Vent DNA polymerase (New England Biolabs Inc., Beverly, MA) using a Mastercycler® gradient (Eppendorf, Hamburg, Germany), according to standard conditions. DNA fragments were purified from agarose gels using the Zymoclean Gel DNA Recovery kit (Zymo Research Corp., Orange, CA). Restriction enzymes and T4 DNA ligase were from New England Biolabs Inc. Bacterial transformations were carried out with a Electroporator 2510 (Eppendorf).

Construction of plasmids for over-expression of recombinant proteins

pETraxR and pETraxH, for over-expression of His-RaxR and His-RaxH, respectively, were as previously described (Burdman *et al.*, 2004). Basically, these are pET15b-based vectors containing the *raxR* (717 bp) and *raxH* (1293 bp) coding sequences from *X. oryzae* pv. *oryzae* PXO99, respectively, cloned into the *Nde*I-*Bam*HI sites, and fused to a tag of six sequential copies of histidine (His 6X tag) in their N-terminus. For generation of pET15b over-expressing truncated RaxH (t-RaxH; lacking the TM domain), a 828-bp fragment starting at position 465 bp of the *raxH* coding sequence was PCR-amplified using pUCraxH (Burdman *et al.*, 2004) as template and primers HK_FP (forward), 5′-GCTAGGTACCCG**CATATG**GCTCGGCTGGTGCGCCCGCTGGTT-3′ (*Kpn*I and *Nde*I sites shown in bold type), and HK_R2 (reverse), 5′-CGTA**GGATCC**TTAGCGAGCCGTGGCAGTAG-3′ (*Bam*HI, bold type). The PCR reaction was run in a 1% agarose gel, and the t-*raxH* product was excised, purified, treated with *Kpn*I and *Bam*HI and cloned into pUC18 cut with the same enzymes to create pUCtraxH. Following verification by sequencing, the t-*raxH* fragment was isolated using *Nde*I and *Bam*HI, purified, and ligated into *Nde*I-*Bam*HI cut pET15b, to generate pETtraxH over-expressing His-t-RaxH. His-RaxRD51A and His-RaxHH222A were generated by site-directed mutagenesis using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions, and employing pETraxR and pETraxH plasmids as templates, respectively. Complementary mutagenesis oligonucleotide pairs RaxRDA-F, 5′-GCCCTGATCCTTGCCTGGATGATGCCGCGC-3′, and RaxRDA-R, 5′-GCGCGGCATCATCCAGGCAAGGATCAGGGC-3′, were used to generate pETraxRD51A. Oligonucleotide pairs RaxHHA-F, 5′-GACAGCGCCAGCGCCGAATTGCGCACGCCC-3′, and RaxHHA-R, 5′-GGGCGTGCGCAATTCGGCGCTGGCGCTGTC-3′, were used to generate pETraxHH222A. Underlined nucleotides are those differing from wild-type sequences. The temperature cycles used to generate the mutated plasmids were as follows: one cycle of 95 °C for 30 s, and 16 cycles of 95 °C for 30 s, 55 °C for 1 min and 68 °C for 8 min. Correct incorporation of each mutation was confirmed by DNA sequencing.

Assessment of over-expression and solubility of recombinant proteins

The pET15b-based vectors were transformed into *E. coli* BL21 and various small-scale optimization experiments were performed. Essentially, cells were cultured at 37 °C in 5 mL LB or LB* (10 g/L tryptone, 5 g/L yeast extract, 1 g/L NaCl, 2.5 mM betaine and 1 M sorbitol; Blackwell and Horgan, 1991) with carbenicillin, to an absorbance at 600 nm of 0.5 or 0.8 (for LB and LB*, respectively). Protein expression was induced through addition of 0.1–1 mM IPTG, and after 3–4 h incubation at different temperatures (25, 30 or 37 °C) cells were harvested to assess expression and solubility according to standard procedures (The QIA*expressionist*, Qiagen).

Purification of recombinant proteins

Cultures (500 mL) of BL21 cells carrying expression vectors were used for purification of soluble recombinant proteins. Cells carrying pETtraxH were grown in LB at 37 °C to an absorbance at 600 nm of 0.5. Expression was induced by addition of 1 mM IPTG and cells were harvested after a further 4 h incubation at 37 °C. Cells carrying pETraxH or pETraxR were grown in LB* at 37 °C to an absorbance at 600 nm of 0.8. Expression was induced by addition of 1 mM IPTG and cells were harvested after 4 h incubation at 37 °C for BL21/pETraxH, and 30 °C for BL21/pETraxR. Procedures for lysis of cells and purification of His-tagged proteins were according to the manufacturer's instructions (The QIA*expressionist*) for purification under native conditions. Essentially, cells were collected by centrifugation (5000 ***g***, 20 min, 4 °C) and lysed in a buffer containing 50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole; pH 8.0 (lysis buffer). Residual cells and debris were discarded by centrifugation (6000 ***g***, 20 min, 4 °C; twice) and recombinant proteins from the supernatant lysate were allowed to bind to 1 mL of Ni^{2+}-NTA resin (Qiagen) at 4 °C for 1 h. Following centrifugation (1000 ***g***, 1 min), the supernatant was removed and the resin was washed with 10 volumes of lysis buffer (once) and 10 volumes of the same buffer but containing 20 mM imidazole (twice). For His-RaxR, a further washing was performed with 10 volumes of buffer containing 40 mM imidazole. Recombinant proteins were then eluted with elution buffer (as lysis buffer but containing 250 mM imidazole; twice, 1–2 mL each time), and dialysed overnight at 15 °C against storage buffer (50 mM Tris-HCl, 50 mM KCl, 10 mM $MgCl_2$, 0.1 mM DTT; pH 7.6) using Slide-A-Lyser dialysis cassettes of 10 000 MWCO (Pierce Biotechnology Inc., Rockford, IL). His-RaxH protein was further concentrated using Amicon Ultra-4 (10 000 MWCO) centrifugal filter tubes (Millipore Corp., Billerica, MA) following the manufacturer's instructions. After dialysis and/or concentration, glycerol was added to a final concentration of 25–30%, and samples were kept at –20 °C. Protein concentration was assayed using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) and protein purification was verified by

SDS-PAGE and confirmed by Western blot using anti-His 6X monoclonal antibodies (Sigma, St Louis, MO). Bacterial growth and purification flows for His-RaxRD51A and His-RaxHH222A were identical to those for His-RaxR and His-RaxH, respectively.

Protein electrophoresis and Western blotting

Proteins were resolved by SDS-PAGE (with 12% polyacrylamide in the separating gel) and stained with R250-Coommassie blue or transferred to Hybond ECL nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ) by standard procedures (Sambrook *et al.*, 1989). Membranes were incubated overnight in blocking solution consisting of 5% (w/v) skimmed milk powder in T-TBS [10 mM Tris-HCl, pH 8.0, containing 150 mM NaCl and 0.1% (v/v) Tween 20]. The membranes were then incubated in the presence of anti-His 6X monoclonal antibodies (Sigma Co.; 1 : 3000) in T-TBS for 1 h at room temperature. Following three 10-min washes in T-TBS, the membranes were incubated for 1 h at room temperature in T-TBS containing 1 : 10 000 dilutions of anti-mouse IgG coupled to horseradish peroxidase (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). After three washes as described above, the membranes were incubated for 5 min with the Super Signal West Pico chemiluminescent substrate (Pierce Biotechnology Inc.) and developed by autoradiography using X-ray films (Fujifilm Medical Systems Inc., Stamford, CT).

In vitro phosphorylation assays

Autopohosphorylation assays were performed at 25 °C in 180 μL of activity buffer (unless stated different). Three different buffers were assessed: (1) 50 mM Tris-HCl (pH 7.6), 50 mM KCl, 10 mM $MgCl_2$ and 0.1 mM DTT (Potter *et al.*, 2002); (2) 33 mM HEPES (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA and 10% glycerol (Pernestig *et al.*, 2001); and (3) 25 mM HEPES (pH 8.0), 150 mM KCl, 5 mM $MgCl_2$, 3 mM $CaCl_2$, 10 mM DTT, 25 μg/mL bovine serum albumin and 15% glycerol (Bird *et al.*, 1999). Unless stated otherwise, transphosphorylation of His-RaxR in the presence of phosphorylated His-RaxH or His-t-RaxH was assayed at 25 °C in 180 μL of activity buffer as described by Potter *et al.* (2002), containing 50 mM Tris-HCl (pH 7.6), 50 mM KCl, 10 mM $MgCl_2$ and 1 mM DTT. Phosphorylation reactions were initiated by addition of 50 μCi of [γ-^{32}P]ATP (PerkinElmer Life and Analytical Sciences, Boston, MA). Aliquots of 20 μL were collected at different times, and reactions were terminated by addition of 4 μL 6× SDS-PAGE loading buffer (Sambrook *et al.*, 1989). Samples (15 μL) were resolved by SDS-PAGE as described above. Gels were then dried under vacuum and labelled proteins were visualized by phosphor imaging using a FujiFilm FLA-500 phosphoimager (FujiFilm, Tokyo, Japan). Phosphorylation assays were repeated twice for each of two different batches of purified proteins (wild-type and mutated), giving similar results.

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