[26] Phosphotransfer Profiling: Systematic Mapping of Two-Component Signal Transduction Pathways and Phosphorelays

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Abstract

Two-component signal transduction systems, composed of histidine kinases and response regulators, enable bacteria to sense, respond, and adapt to changes in their internal and external conditions. The importance of these signaling systems is reflected in their widespread distribution and prevalence in the bacterial kingdom, with some organisms encoding as many as 250 two-component signaling proteins. In many cases, a histidine kinase and a response regulator are encoded in the same operon and, in such cases, the two molecules usually interact in an exclusive one-to-one fashion. However, in many organisms, the vast majority of two-component signaling genes are encoded as orphan genes, precluding the mapping of signaling pathways based on sequence information and genome position alone. There is also a growing number of examples of two-component signaling pathways with more complicated topologies, including one-to-many and many-to-one relationships, which cannot be inferred from sequence. To address these problems, we have developed an *in vitro* technique called phosphotransfer profiling, which enables the systematic identification of two-component signaling pathways. Purified histidine kinases are tested for their ability to transfer a phosphoryl group to each response regulator encoded in a genome of interest. As histidine kinases typically exhibit a strong kinetic preference *in vitro* for their *in vivo* cognate substrates, this technique allows the rapid mapping of cognate pairs and is applicable to any organism containing two-component signaling genes. The technique can be further extended to mapping phosphorelays and the cognate partners of histidine phosphotransferases. Here, we describe protocols and strategies for the successful implementation of this system-level technique.

Overview

Two-component signal transduction systems, comprising histidine kinases and response regulators, are the single most prevalent paralogous family of signaling proteins in the bacterial kingdom and are also found in archaea, plants, yeasts, and other lower eukaryotes. These signaling systems

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enable cells to sense and respond to a wide range of stimuli, both intracellular and extracellular, and thus help coordinate a wide range of adaptive responses. Both histidine kinases and response regulators are easily identified by sequence homology and can be systematically enumerated in any fully sequenced genome. Some organisms encode more than 200 two-component signaling proteins, with an average bacterial genome containing 50 to 100.

The canonical two-component signaling pathway (Fig. 1A,B) contains a histidine kinase, which, in response to a stimulus, autophosphorylates on a conserved histidine residue. The phosphorylated histidine kinase then binds and transfers its phosphoryl group to a conserved aspartate residue on the response regulator. The phosphorylation of a response regulator, which occurs on its receiver domain, typically activates an output domain that can trigger changes in gene expression, protein–protein interactions, or enzymatic activity (for a comprehensive review of two-component signaling pathways, see Stock *et al.*, 2000). In addition to this canonical case involving two proteins, one histidine kinase and one response regulator, these classes of signaling proteins can be arranged into more complicated pathways. In some cases, a histidine kinase can have multiple phosphotransfer targets or a regulator can receive input from multiple kinases. Another common arrangement is the so-called phosphorelay (Fig. 1C,D). In these pathways, the top-level histidine kinase is usually a hybrid histidine kinase such that the N-terminal portion of the molecule is similar to that of a canonical histidine kinase and the C-terminus contains a receiver domain, similar to that found on response regulators. In response to an input stimulus, these hybrid kinases catalyze a histidine autophosphorylation and then transfer the phosphoryl group intramolecularly to their receiver domain. The phosphoryl group can then be transferred to a different type of protein, called a histidine phosphotransferase, which subsequently donates the phosphoryl group to a soluble response regulator, leading to an output response.

Identifying the connectivity of two-component signal transduction proteins and phosphorelays remains a major challenge and is the focus of this chapter. In *E. coli*, nearly all histidine kinases and response regulators occur in operon pairs and, most probably, form exclusive one-to-one pairs. Operon pairs in other organisms also usually define exclusive phosphotransfer relationships. In many bacteria, however, histidine kinases are often encoded as orphans and the cognate response regulator(s) cannot be predicted based on sequence alone. Moreover, orphan two-component signaling proteins frequently have one-to-many or many-to-one connectivity or are involved in phosphorelays. In some cases, phenotypic analysis of mutants can help delineate these pathways. However, for some complicated pathways, biochemical connectivity is difficult to infer from mutant

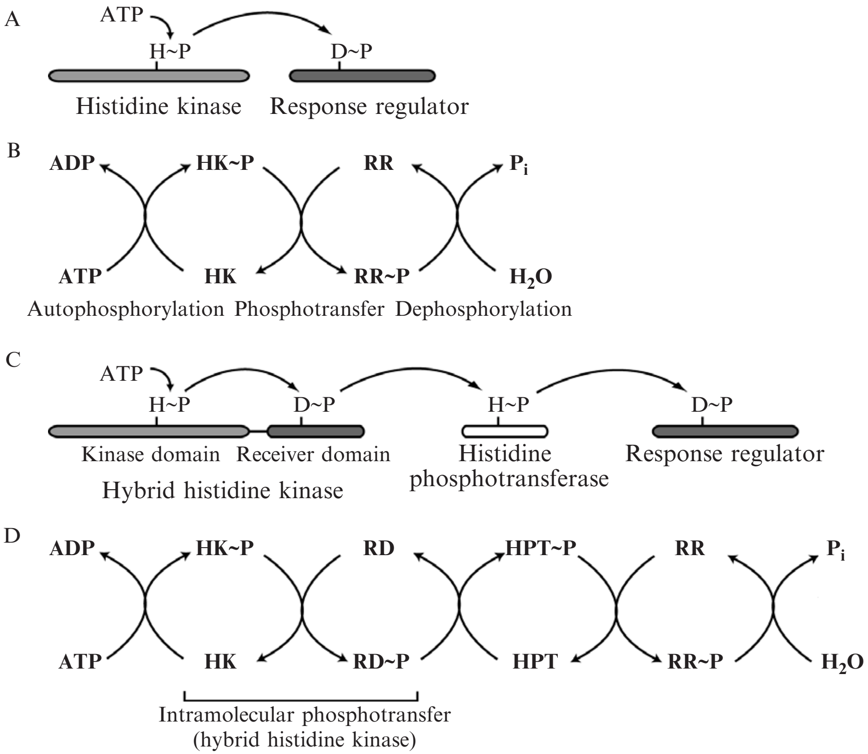

Fig. 1. Schematic overview of two-component signaling pathways. (A) In a canonical two-component signaling system, receipt of an input stimulates a histidine kinase to catalyze autophosphorylation on a conserved histidine residue. The phosphoryl group is subsequently passed to a cognate response regulator which, after phosphorylation, triggers changes in cellular physiology. (B) Schematic of the flow of phosphate through a two-component signal transduction system. Autophosphorylation involves the transfer of the gamma phosphoryl group from ATP to the histidine kinase (HK). Phosphotransfer shuttles the phosphoryl group to a cognate response regulator (RR). Dephosphorylation of the response regulator occurs by hydrolysis of the aspartyl-phosphate bond in a reaction stimulated by either an intrinsic autophosphatase activity in the regulator or by the phosphatase activity of a bifunctional histidine kinase. (C) In a canonical phosphorelay, receipt of an input stimulates autophosphorylation of a hybrid histidine kinase that harbors both a histidine kinase domain and a C-terminal receiver domain equivalent to those found in soluble response regulators. After autophosphorylation, the phosphoryl group is passed intramolecularly to the receiver domain, then to a histidine phosphotransferase, and finally to a soluble, terminal response regulator. (D) Schematic of the flow of phosphate through a canonical phosphorelay. After autophosphorylation of the kinase domain (HK) of a hybrid histidine kinase, the phosphoryl group is passed intramolecularly to the receiver domain (RD), then to a histidine phosphotransferase (HPT), and finally to a terminal response regulator (RR).

phenotypes and, for genetically intractable organisms, such analyses are simply not possible. To address these problems, we have developed an *in vitro* technique, called phosphotransfer profiling, which enables the systematic mapping of *in vivo* connectivity of two-component signal transduction proteins and phosphorelays (Biondi *et al.*, 2006; Skerker *et al.*, 2005). This technique leverages the fact that histidine kinases exhibit a kinetic preference *in vitro* for their *in vivo* cognate response regulator substrate(s). Kinetic preference was first demonstrated with subsets of two-component signaling proteins (Fisher *et al.*, 1996; Grimshaw *et al.*, 1998), and subsequently extended to a genome-wide level (Skerker *et al.*, 2005). The latter observation now enables the efficient mapping of two-component pathways in any organism with a sequenced genome.

In a typical phosphotransfer profiling experiment (Fig. 2A), the purified cytoplasmic, soluble kinase domain of a histidine kinase is first autophosphorylated *in vitro* with [γ-^{32}P]ATP. The radiolabeled kinase is then tested, in parallel, for phosphotransfer to each purified, full-length response regulator encoded in the genome of interest. Each phosphotransfer reaction is incubated for an identical period of time, with reaction products resolved by SDS-PAGE and analyzed by phosphor-imaging. Autophosphorylated kinase alone is included as a control and forms a single intense band. Efficient phosphotransfer to a response regulator can be manifested in one of two ways. First, a high-intensity band corresponding to the phosphorylated response regulator will appear at a position corresponding to the regulator's molecular weight. Alternatively, efficient phosphotransfer can deplete radiolabel from both the histidine kinase and response regulator, resulting in a blank lane. The latter case can result from a high autophosphatase activity intrinsic to some response regulators. Some histidine kinases are also bifunctional and can act as phosphatases for their cognate regulators (Igo *et al.*, 1989b). In either case, autophosphatase activity of the response regulator or phosphatase activity from the histidine kinase, the net result is nearly complete depletion of radiolabel from the reaction components (Fig. 1B). Thus, to identify kinase-regulator phosphotransfer relationships, each reaction in a profile experiment is inspected for (i) a band corresponding to the response regulator or (ii) a decrease in intensity of the kinase band relative to the kinase-only control. Because this profiling method relies on the comparison, in parallel, of all potential phosphotransfer substrates for a given kinase, it is independent of the specific activity of the kinase being tested.

Although phosphotransfer profiling is an *in vitro* technique, the *in vivo* targets can be identified because histidine kinases exhibit a kinetic preference for their *in vivo* cognate response regulators. In other words, the substrate specificity of a histidine kinase appears to be intrinsic to the

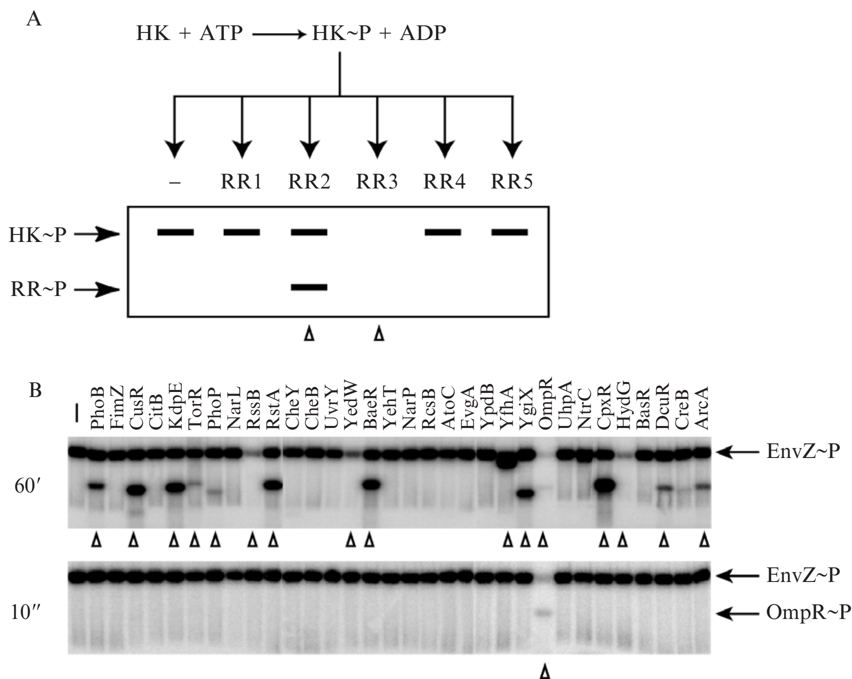

FIG. 2. Phosphotransfer profiling of two-component signaling proteins. (A) Diagram of the phosphotransfer profiling assay. A purified histidine kinase domain (HK) is incubated in the presence of radiolabeled ATP and then incubated with each of the purified response regulators (RR1–RR5) encoded in a genome of interest. Reactions are analyzed by SDS-PAGE and phosphorimaging. Each lane, including a control lane containing no response regulator, shows a band corresponding to the autophosphorylated histidine kinase. Phosphotransfer (lanes indicated by open arrowheads) is manifested as a second band at a position corresponding to the response regulator or by the depletion of radiolabel. The latter results from a high rate of phosphotransfer followed by rapid dephosphorylation of the response regulator. (B) An example of using phosphotransfer profiling to identify the kinetically preferred phosphoacceptor of a histidine kinase. The cytoplasmic kinase domain of EnvZ was profiled against each of the 32 *E. coli* response regulators with phosphotransfer reaction times of either 10 s or 60 min. Whereas multiple regulators are phosphorylated at the long timepoint, only the in vivo cognate substrate OmpR is phosphorylated at the short timepoint. Profiling data taken from Skerker *et al.*, 2005.

protein and not dependent on cellular context. Hence, by examining the phosphotransfer profile of a histidine kinase at multiple timepoints, one can quickly ascertain the preferred substrate. This *in vitro* kinetic preference of a histidine kinase for phosphotransfer to its *in vivo* cognate regulator has been demonstrated for kinase-regulator pairs in the phylogenetically distant organisms *E. coli* and *Caulobacter crescentus* (Skerker *et al.*, 2005).

This suggests that kinetic preference is a general property of two-component signal transduction systems, and that the technique can be applied to any organism that utilizes these signaling proteins.

An example of a typical phosphotransfer profile is shown in Fig. 2B. In *E. coli*, the histidine kinase EnvZ, upon stimulation, autophosphorylates and then transfers a phosphoryl group exclusively to OmpR (Forst *et al.*, 1989; Igo *et al.*, 1989a; Skerker *et al.*, 2005). As seen in Fig. 2B, the systematic profiling of EnvZ~P at a long timepoint of 1 h identifies many different response regulator partners, including OmpR. Although this may seem to suggest that EnvZ has multiple targets, a profile using just 10 s phosphotransfer incubation times demonstrates that EnvZ has a single, preferred target, the known cognate regulator OmpR. Additional kinetic analysis showed that EnvZ has at least a 2000-fold preference, in terms of relative k_{cat}/K_M ratios for phosphotransfer to OmpR relative to a noncognate substrate, CpxR (Skerker *et al.*, 2005). Similar profile experiments have identified the preferred substrates for CheA, CpxA, PhoQ, and PhoR in *E. coli*. Detailed kinetic studies of a small number of two-component signaling pathways have also demonstrated *in vitro* kinetic preference of a histidine kinase for its *in vivo* cognate substrate (Burbulys *et al.*, 1991; Fisher *et al.*, 1996; Grimshaw *et al.*, 1998; Igo *et al.*, 1989b). For example, in *B. subtilis*, the careful measurement of kinetic parameters showed that the kinase KinA has a 57,000-fold preference for phosphorylating Spo0F relative to Spo0A (Grimshaw *et al.*, 1998).

The phosphotransfer profiling technique is an extended, scaled-up version of the standard kinase assays that have been used for many years to study two-component signaling systems; for an earlier *Methods in Enzymology* chapter, see Hakenbeck and Stock, 1996. The protocols that will be described are derived from these earlier studies, but have been modified and, in some cases, streamlined to facilitate the scaling of these assays to a system-wide level. At the end of the chapter, we discuss the interpretation and pitfalls specific to the system-wide phosphotransfer profiling approach.

Detailed Protocols

Preparation of Sequence-Verified Clones and Expression Vectors

Response regulators and histidine kinases in a genome of interest can be identified by consulting databases such as SMART (http://smart.embl-heidelberg.de/) or PFAM (http://www.sanger.ac.uk/Software/Pfam/). However, the automated annotation procedures used by these databases often incorrectly identify some genes as encoding two-component signaling proteins and fail to identify others. A more tedious but reliable approach is to

use BLAST analysis of a complete genome followed by manual inspection of each hit. The query sequences used should contain only the receiver domain of a known response regulator and the kinase domain of a histidine kinase to avoid finding proteins with homology to other parts of the response regulator, such as the DNA-binding domain, or the histidine kinase, such as a PAS domain. During manual inspection of the BLAST results, each potential response regulator and histidine kinase should be evaluated for the presence of all major conserved residues involved in phosphotransfer (for a detailed analysis of the conserved sequence features of two-component signaling proteins, see Hoch and Silhavy, 1995, or Grebe and Stock, 1999).

Once a comprehensive list of two-component signaling genes has been established, genes corresponding to each response regulator must be cloned into expression vectors for protein purification. Expression vectors for histidine kinases of interest must also be generated. For organisms with large numbers of response regulators and/or histidine kinases, the generation of expression vectors can be greatly facilitated by using the Invitrogen Gateway high-throughput recombinational cloning system (for a detailed description, see http://www.invitrogen.com/ and Walhout *et al.*, 2000). In this system, clones generated by PCR can be rapidly inserted into expression vectors using highly efficient site-specific recombination between sites present in the vector and sites incorporated into the clone. This system does not require restriction enzymes or ligase and so eliminates much of the time-consuming subcloning that would otherwise be necessary. In the following text, we briefly outline the use of the Gateway system to generate expression clones for the protein purifications required for phosphotransfer profiling.

1. For each regulator, amplify the entire open reading frame by PCR with a proofreading DNA polymerase such as Pfu. Ideally, primers should be purified by reverse-phase cartridge, PAGE, or HPLC because unpurified (desalted-only) primers often contain a significant fraction of incorrect sequences, such as single base deletions.
2. Clone PCR amplicons into the pENTR/D-TOPO vector (available from Invitrogen) according to the manufacturer's protocol and transform into competent *E. coli* cells.
3. Screen 5 to 10 kanamycin-resistant colonies by PCR using M13F (5′-TGTAAAACGACGGCCAGT-3′) and M13R (5′-TCACACAGGAAACAGCTATGAC-3′) primers to verify vectors with inserts of the correct size. Even using a proofreading polymerase, positive clones should be further confirmed by sequencing to ensure that the protein that is ultimately produced will have no mutations that affect phosphotransfer behavior.

Once a set of sequence-verified pENTR clones is generated, they can be rapidly and efficiently mobilized into a wide range of expression vectors, called destination vectors, using the Gateway system's LR reaction. The products of these LR reactions are expression vectors in which each gene is fused to an affinity tag and under the control of an IPTG-inducible promoter. A wide range of destination vectors is commercially available for producing fusions to His_6, GST, and other common affinity tags. Alternatively, one can easily convert customized expression vectors into destination vectors (see http://www.invitrogen.com/). The protocol described here uses a customized destination vector that produces thioredoxin-His_6-TEV-tagged proteins (details on the tag used will be described later).

4. For each clone, set up a 5 μl LR reaction containing 75 ng destination vector, 30 ng pENTR plasmid DNA (equimolar ratio of destination: pENTR DNA), 1$\times$ LR buffer, 0.75 U topoisomerase I, and 0.5 μl LR clonase enzyme mix (Invitrogen).
5. Incubate each LR reaction overnight at room temperature, then transform 2 μl into chemically competent *E. coli* DH5α cells, and plate on LB with the appropriate antibiotic, depending on the resistance cassette carried by the chosen destination vector.
6. Colonies recovered should be retested, by patching onto appropriate plates, for presence of the antibiotic resistance marker carried on the destination vector and for kanamycin sensitivity to ensure no carryover of pENTR DNA. Positive colonies bear the desired expression vector and are ready for protein purification.

As an alternative to cloning full-length response regulators, the phospho-accepting receiver domains alone can be cloned and used for phosphotransfer profiling. Since some response regulators are large, multidomain proteins, the purification of receiver domains alone can often produce higher yields and more soluble protein. Moreover, the specificity of kinase interaction is conferred by the receiver domain alone and, hence, is sufficient for the mapping of kinase–regulator pairings. The receiver domains of hybrid histidine kinases can also be individually cloned and purified for profiling the specificity of histidine phosphotransferases (see later).

It should be noted that once sequence-verified clones are generated in pENTR vectors, these clones can be rapidly moved into a wide range of destination vectors using the highly efficient LR reaction. Destination vectors exist that create fusions to a number of popular affinity tags or fluorescent reporters such as GFP. The generation of pENTR clones thus makes possible a range of systematic studies (Walhout *et al.*, 2000).

Protein Purification by Affinity Chromatography

Response regulators can be easily purified in the native state by overexpression in *E. coli* followed by affinity chromatography. There are a wide variety of affinity tags (and corresponding Gateway destination vectors) available, but the protocol described here uses a thioredoxin-His_6-TEV tag. The N-terminal thioredoxin domain improves folding and solubility in *E. coli* and, hence, substantially increases the success rate and yield of the protein purifications. The inclusion of a TEV protease site allows for removal of the affinity tag, if necessary or desired.

1. Transform expression vector DNA for each response regulator independently into *E. coli* BL21-Tuner cells and select on LB plates supplemented with the appropriate antibiotic.
2. Inoculate 4 to 5 colonies into 500 ml of LB supplemented with the appropriate antibiotic and grow at 37° to an $OD_{600} \sim 0.6$ (~4–5 h).
3. To induce expression of the His_6-tagged constructs, add IPTG to 300 μM and grow the culture at 30° for 4 h.
4. Harvest cells by centrifugation at 10,800*g* for 5 min and store cell pellets at −80° until needed.
5. Resuspend each cell pellet in 10 ml lysis buffer (20 m*M* Tris-HCl, pH 7.9, 0.5 *M* NaCl, 10% glycerol, 20 m*M* imidazole, 0.1% Triton X-100, 1 m*M* PMSF, 1 mg/ml lysozyme, 125 units benzonase nuclease (Novagen)), transfer to a 50 ml conical tube, and incubate at room temperature for 20 min to allow cell lysis.
6. Sonicate cells (2 × 30 s in a Fisher Model 550 with microtip) to complete cell lysis, add fresh PMSF, and then centrifuge for 60 min at 30,000*g* to generate a cleared lysate. Transfer the lysate to a clean 50 ml conical tube, add 1 ml of Ni-NTA agarose slurry (Qiagen), which has been pre-equilibrated in lysis buffer, and incubate at 4° for 30 min.
7. Wash (centrifuge and resuspend) the Ni-NTA beads twice with 50 ml wash buffer (20 m*M* HEPES-KOH, pH 8.0, 0.5*M* NaCl, 10% glycerol, 20 m*M* imidazole, 0.1% Triton X-100, 1 m*M* PMSF) and then load the slurry onto an Econo-column (Bio-Rad).
8. Add 2.5 ml elution buffer (20 m*M* HEPES-KOH, pH 8.0, 0.5*M* NaCl, 10% glycerol, 250 m*M* imidazole) and collect the eluate which contains the purified protein. Then, load the eluate directly onto a PD-10 column (Amersham Biosciences) that had been pre-equilibrated with kinase buffer (10 m*M* HEPES-KOH, pH 8.0, 50 m*M* KCl, 10% glycerol, 0.1 m*M* EDTA, 1 m*M* DTT). The purified protein is then eluted with 3.5 ml kinase buffer and ready for concentration and storage.
9. Concentrate eluted samples to ~1 to 10 mg/ml using Amicon Ultra 30K or 10K columns (Millipore), depending on the protein size. Filter each

sample through an Ultrafree-MC (0.22 μm) spin filter (Millipore) and aliquot for storage at -80°.

10. Estimate protein concentration using Coomassie Plus Protein Assay Reagent and a BSA standard (Pierce). An equal amount (500 ng) of each protein sample should also be analyzed by SDS-PAGE to verify molecular weight and purity. Prior to phosphotransfer profiling, normalize all response regulator concentrations against a 500 ng BSA standard using a ChemiImager 5500 and densitometry (Alpha Innotech).

Starting from cell pellets (step 5), one person can purify 8 to 10 proteins in parallel in one day. Hence, for most genomes, the entire set of response regulators can be purified in about a week. This protocol typically generates from 100 μg to 1 mg of protein, depending on its solubility, which is enough for at least 100 phosphotransfer profiling experiments.

Histidine kinases can be cloned, expressed, and purified as done for the response regulators, but there are several additional considerations. First, the phosphotransfer profiling technique is predicated on having a comprehensive set of response regulators, but histidine kinases can be cloned and tested as desired, not necessarily as a complete set. Second, it can be helpful to use a His_6-MBP affinity tag for the kinases rather than a thioredoxin-His_6 tag. The larger MBP tag helps to ensure that the molecular weight of the histidine kinase is greater than each of the TRX-His_6-tagged response regulators. This facilitates resolution of reaction components by SDS-PAGE and, hence, easier interpretation of the profile by phosphor-imaging, as will be described. Third, the forward primer used for PCR amplification of a histidine kinase should be designed to omit any known or predicted N-terminal transmembrane domains to allow the native purification of the soluble kinase domain. In addition, some kinases have large intervening portions between the end of the last transmembrane domain and the beginning of the conserved kinase domain. The inclusion of all or part of this region may or may not affect the autophosphorylation activity of the purified kinase. There is not yet, to the best of our knowledge, a simple way to determine *a priori* whether to include or omit this intervening region. Instead, the simplest strategy is to produce several versions with different N-terminal start points and empirically identify an active construct. For histidine kinases with a structure similar to EnvZ, active kinases can often be obtained with an N-terminal start point after the last transmembrane domain (I179) or just prior to the H-box domain that contains the active site histidine (M223) (Park *et al.*, 1998). Histidine kinases have been purified as full-length constructs and reconstituted in membrane vesicles (Jung *et al.*, 1997). Such full-length kinases could, in principle, be used for

phosphotransfer profiling, although this has not yet been reported. Some histidine kinases, however, may not be active in any form *in vitro*, since they may require additional factors *in vivo* for activity. These additional factors, if known, could be purified and may be sufficient, if added to the purified kinase, to drive autophosphorylation. This would enable phosphotransfer profiling, but such a scenario has not yet been demonstrated.

Phosphotransfer Profiling

Before starting a phosphotransfer profile, the histidine kinase of interest should first be tested for autophosphorylation and the time required for producing radiolabeled kinase optimized. To accomplish this, set up a reaction containing 5 μM histidine kinase, 2 mM DTT, 5 mM $MgCl_2$, 500 μM ATP, 5 μCi [γ^{32}P]ATP (~6000 Ci/mmol; Amersham Biosciences), and enough kinase buffer to bring the reaction volume to 30 μl. Premix the radiolabeled and cold ATP and add last to initiate the reaction. Immediately begin taking samples every 15 min for up to 3 h while the reaction is incubated at room temperature or 30°. Stop the reaction by adding SDS-PAGE loading buffer and place on ice. After all samples have been collected, the autophosphorylation kinetics can be examined by SDS-PAGE and phosphorimaging. Once the optimal autophosphorylation time has been optimized in this fashion, the reaction can be scaled up to provide enough material for phosphotransfer profiling. The optimal autophosphorylation time for histidine kinases can vary from a few minutes to a few hours.

1. Dilute each purified response regulator to 5 μM in kinase buffer supplemented with 5 mM $MgCl_2$.
2. For each phosphotransfer reaction, mix 5 μl of autophosphorylated, radiolabeled kinase, with 5 μl of response regulator prepared and diluted as has been described. A control reaction containing kinase alone must also be included. Each 10 μl phosphotransfer reaction thus contains a final concentration of 2.5 μM of both a response regulator and the histidine kinase of interest.
3. Incubate the reactions at 30° for a defined period of time and then stop by adding 3.5 μl of 4× sample buffer (500 mM Tris-HCl pH 6.8, 8% SDS, 40% glycerol, 400 mM β-mercaptoethanol), and place the reaction on ice.
4. Load the entire reaction for each regulator and the control reaction into a 10% Tris-HCl polyacrylamide gel followed by electrophoresis at 150 volts at room temperature for 50 to 60 min. For typical 15 lane gels, several gels may be necessary to run out every reaction, depending on the number of response regulators.

5. Following electrophoresis, disassemble the gel tank and remove one of the glass plates sandwiching the gel. Then, using a razor blade, carefully remove the dye front which includes unincorporated ATP and place the wet gel (still on the back glass plate) into a sealed plastic bag.

6. Expose gels to a storage phosphor screen for 1 to 3 h at room temperature. If necessary, gels can be frozen at -80° or dried and exposed for longer periods of time. Longer exposures are often necessary for kinases with low autophosphorylation activity. After exposure, scan the phosphor screen and stitch the gel images together for analysis and presentation using any standard image processing software.

Interpretation and Analysis

A typical profile is shown in Fig. 2B. Phosphotransfer, as noted earlier, is indicated by either the appearance of a band corresponding to the phosphorylated response regulator or by the disappearance of the histidine kinase band. To identify the kinetically preferred substrate often requires only running a comprehensive phosphotransfer profile at two timepoints. Using the reaction conditions described previously, an incubation time of 10 to 30 s usually reveals the kinetically preferred substrate(s). This may not always be the case and multiple profiles may be necessary. As each profile is labor-intensive, a more practical strategy is to run only a single profile at a long timepoint, such as 1 h, and then perform detailed kinetics only on the response regulators that are phosphotransfer targets at the long time-point. Although histidine kinases are typically quite promiscuous after 1 h phosphotransfer incubations, this approach will dramatically reduce the number of regulators being examined at shorter timepoints.

In general, detailed kinetic analysis should be performed after phosphotransfer profiling to confirm the kinetic preference of a substrate and to quantify the specificity. For enzymes, the specificity constant is often defined as k_{cat}/K_M and the ratio of specificity constants for two substrates quantifies the relative preference. For many histidine kinases, this ratio is on the order of 10^3 or 10^4 for the *in vivo* cognate response regulator relative to the next best substrates. For example, with EnvZ the k_{cat}/K_M ratio is at least 2000-fold higher for phosphotransfer to OmpR relative to one of the next best substrates, CpxR (Skerker *et al.*, 2005). Histidine kinases, such as EnvZ, with such large preferences probably exclusively phosphorylate the kinetically preferred substrate *in vivo*. However, the gap may not always be as large and certain histidine kinases may have multiple targets, each phosphorylated to a quantitatively different extent (Mika and Hengge, 2005). Detailed kinetic analysis can quantify the relative preferences and thus guide additional, *in vivo* experiments.

The autophosphorylated histidine kinase can be purified away from ATP before examining phosphotransfer, either in profiling experiments or during detailed kinetic characterization. This additional purification is often desirable so that one can examine phosphotransfer from a kinase to a regulator without the confounding effects of additional autophosphorylation of the histidine kinase. For such purifications, the entire autophosphorylation reaction can be loaded onto a Nanosep-10K column (Pall Corporation) and washed 3 to 4 times with kinase buffer by repeated dilution and centrifugation. The phospho-histidine is relatively stable compared to a phospho-aspartate so the histidine kinase will retain most of the initially incorporated radiolabel, and can be stored at $-20°$ for several days before use.

Although the protocol described previously uses equal concentrations of histidine kinase and response regulator in each phosphotransfer reaction, the concentration of phosphorylated histidine kinase is typically lower than the concentration of total histidine kinase. Autophosphorylation reactions are unlikely to go to completion, and during the steps prior to phosphotransfer, some of the phosphate incorporated can be lost to hydrolysis. The final ratio of HK~P:RR is thus likely much less than one. This does not, however, impact the ability to assess the preferred substrate as long as the same preparation of kinase is used for each phosphotransfer reaction. In fact, a ratio of HK~P:RR less than one may better mimic the *in vivo* stoichiometries. For the EnvZ-OmpR pathway, quantitative Western blotting estimates that EnvZ is present at approximately 100 molecules per cell (~100 n*M*) and OmpR at approximately 3500 molecules per cell (~3.5 μM) (Cai and Inouye, 2002). Therefore, even if all of the EnvZ in an *E. coli* cell were phosphorylated, the ratio of HK~P:RR would be approximately 1:20. Estimates of protein abundance for other two-component proteins are of similar orders of magnitude.

The phosphotransfer profiling technique is designed to test each response regulator in isolation. This is not, however, essential. Multiple response regulators can be combined and incubated with histidine kinase in a single reaction (J. M. S. M. T. L., unpublished data). By pooling regulators into small groups, a complete phosphotransfer profiling experiment can be done with a single gel. This pooling strategy will also set up a competition among the response regulators as phospho-acceptors because, as has been described, the concentration of phosphorylated histidine kinase is much lower than that of the response regulators. This competition will lead to an apparent enhancement of the kinase's specificity since phosphotransfer to the kinetically preferred substrate will deplete the reaction of phosphorylated histidine kinase; this, in turn, leads to even less phosphotransfer to noncognate substrates than would be observed if examined in isolation. The

major barrier to performing phosphotransfer profiling in this manner is the inability to resolve, by SDS-PAGE and phosphorimaging, multiple response regulators, which are often of similar molecular weights. This fact can be circumvented by carefully choosing which response regulators to combine. Alternatively, response regulators can be grouped into sets of 3 to 5 and, if there is evidence of phosphotransfer to a set, the individual regulators in that group could be subsequently examined in detail.

Phosphorelays and Histidine Phosphotransferases

Phosphotransfer profiling can also be used to analyze hybrid histidine kinases and to map phosphorelays (Fig. 3) (Biondi *et al.*, 2006). For hybrid kinases, the kinase domain can be purified and profiled against the entire set of soluble response regulators, exactly as done with canonical, non-hybrid histidine kinases. In addition, the kinase domain can be profiled against the entire set of receiver domains from the hybrid kinases in a genome of interest. Receiver domains can be individually cloned, expressed, purified, and used for profiling exactly as has been described for full-length, soluble response regulators. Such profiles typically reveal that the kinase domain of a hybrid kinase preferentially phosphorylates its own receiver domain, reflecting what is usually an intramolecular phosphotransfer reaction.

In a typical phosphorelay, a histidine phosphotransferase shuttles phosphate from the receiver domain of a hybrid histidine kinase to the receiver domain of a diffusible response regulator. Unlike histidine kinases and response regulators, histidine phosphotransferases are difficult to identify by sequence homology. However, once identified by other means, they are easily purified since they are usually small, highly soluble proteins. All of the protocols that have been described can be easily modified to enable the profiling of histidine phosphotransferases (HPTs). Radiolabeling of an HPT for profiling first requires purification of a kinase domain and a receiver domain from a hybrid kinase. A fused version of the hybrid kinase containing both domains can be purified and used, but we have found that for a number of hybrid kinases, the activity of the kinase domain increases significantly when it is separated from its receiver domain (J. M. S. M. T. L., unpublished observations). A reaction similar to that described for histidine kinase autophosphorylation is then set up with three components: 1 μM hybrid kinase domain, 1 μM hybrid receiver domain, and 10 μM of the HPT of interest. For uncharacterized histidine phosphotransferases, the hybrid kinase used for this reaction does not need to be the cognate kinase. The purpose of this initial reaction is simply to ''load'' the HPT with radioactive phosphate, and a significant level of phosphorylated HPT can be achieved

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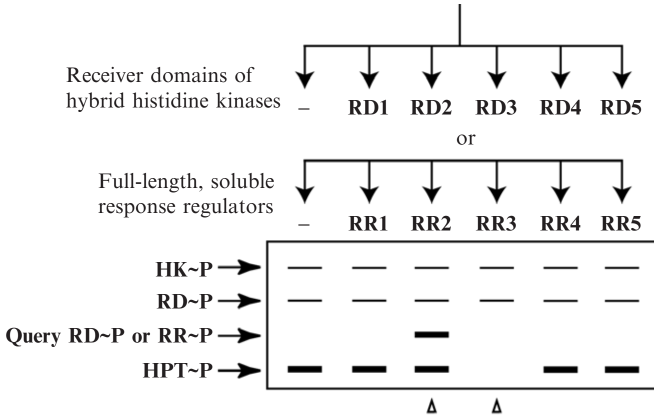

B

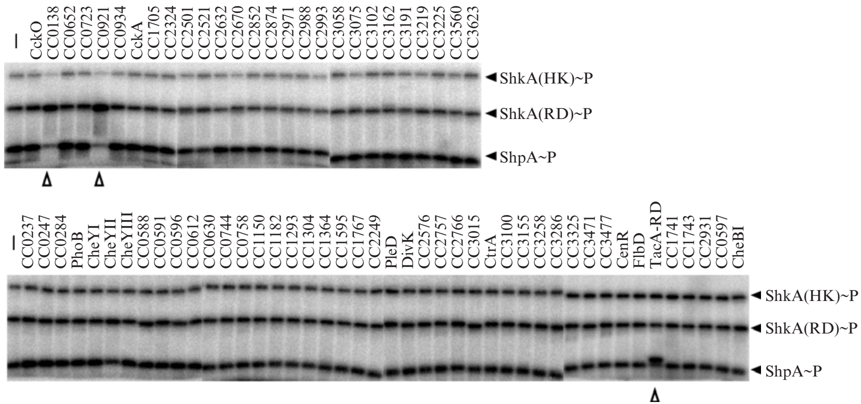

Fig. 3. Mapping phosphorelays. (A) Diagram of the phosphotransfer profiling assay for a histidine phosphotransferase (HPT). The kinase (HK) and receiver domains (RD) of a hybrid kinase are incubated with the HPT of interest and radiolabeled ATP, leading to phosphorylation of the HPT. The phosphorylated HPT is then mixed individually with the purified receiver domains from each hybrid histidine kinase or full-length, soluble response regulator from a genome of interest. As with phosphotransfer profiling of a histidine kinase (Fig. 2), reactions are analyzed by SDS-PAGE and phosphorimaging. The kinase and receiver domains of the hybrid kinase used for phosphorylating the HPT appear as bands in each lane. Phosphotransfer to a queried response regulator or receiver domain is manifested as a band at the appropriate position or by depletion of radiolabeled HPT (see Fig. 2 and text for details). (B) An example of using phosphotransfer profiling to identify the cognate partners of an HPT from *C. crescentus*, ShpA. Phosphorylated ShpA was profiled against the 27 receiver domains of *C. crescentus* hybrid kinases (top) and the 44 full-length response regulators (bottom). In each case, only a single timepoint of 2 min is shown. Open arrowheads indicate lanes with evidence of phosphotransfer. Data taken from Biondi *et al*., 2006.

with noncognate substrates by using an extended incubation time. Since all known histidine phosphotransferases share a common structural fold, extended incubation eventually leads to their phosphorylation by the hybrid kinase components. The exact concentrations, ratios of components, and reaction times can be empirically optimized. Moreover, for a given HPT, some hybrid kinases will be better than others at phosphorylating an HPT; as of now, this can only be determined empirically.

To profile an HPT:

1. Dilute the HPT phosphorylation reaction described previously 1:10 in kinase buffer.
2. Add 5 μl of the diluted reaction to 5 μl of each purified response regulator to be profiled. Final concentrations are thus 0.5 μM HPT and 2.5 μM of the query response regulator.
3. Subsequent steps are exactly as those described for histidine kinase phosphotransfer profiles. Phosphorimaging will reveal bands corresponding to the hybrid kinase components used to generate phosphorylated HPT. Phosphotransfer, however, is still easily assessed by examining gel images for a band corresponding to the query response regulator or the depletion of radiolabel from the HPT relative to a control lane containing no response regulator.

Because a histidine kinase domain and ATP are required for the initial phosphorylation of an HPT, the phosphorylation of a query response regulator during HPT profiling could, in principle, result from direct phosphotransfer by the kinase rather than from the HPT. This is unlikely because the kinase is present, after dilution, at a concentration of only 0.05 μM. Nevertheless, for each regulator identified in an HPT phosphotransfer profile, a control reaction should be run in which the histidine kinase domain is tested alone (at 0.05 μM) for phosphotransfer to the response regulator.

In vivo, HPTs can shuttle phosphate from the receiver domain of a hybrid kinase to a soluble response regulator, or vice versa, with the directionality probably dictated largely by mass-action (Georgellis *et al.*, 1998; Uhl and Miller, 1996). However, *in vitro*, HPTs can be examined for their ability to transfer to each soluble response regulator, as has been noted, or to the receiver domains of each hybrid histidine kinase encoded in a genome of interest. Receiver domains can be examined as phosphotransfer targets of an HPT just as with the full-length, soluble response regulators. The phosphotransfer profiling of an HPT against both receiver domains and soluble response regulators can thus allow the rapid mapping of complete phosphorelays (Biondi *et al.*, 2006).

Concluding Remarks

The phosphotransfer profiling technique enables the rapid, systematic mapping of two-component signaling pathways and phosphorelays. It should be emphasized, however, that phosphotransfer profiling is an *in vitro* technique and therefore only *suggests* the most probable *in vivo* target. All potential substrates identified by this *in vitro* methodology should be confirmed through the various tools and approaches of molecular biology. Thus far, no discrepancy between the preferred *in vitro* substrates and the *in vivo* relationships has been reported, but results should nevertheless be interpreted with caution and used as a precursor to further experimentation.

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