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Intramolecular arrangement of sensor and regulator overcomes relaxed specificity in hybrid two-component systems

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Cellular processes require specific interactions between cognate protein partners and concomitant discrimination against noncognate partners. Signal transduction by classical two-component regulatory systems typically entails an intermolecular phosphoryl transfer between a sensor kinase (SK) and a cognate response regulator (RR). Interactions between noncognate partners are rare because SK/RR pairs coevolve unique interfaces that dictate phosphotransfer specificity. Here we report that the in vitro phosphotransfer specificity is relaxed in hybrid two-component systems (HTCSs) from the human gut symbiont *Bacteroides thetaiotaomicron*, which harbor both the SK and RR in a single polypeptide. In contrast, phosphotransfer specificity is retained in classical two-component regulatory systems from this organism. This relaxed specificity enabled us to rewire a HTCS successfully to transduce signals between noncognate SK/RR pairs. Despite the relaxed specificity between SK and RRs, HTCSs remained insulated from cross-talk with noncognate proteins in vivo. Our data suggest that the high local concentration of the SK and RR present in the same polypeptide maintains specificity while relaxing the constraints on coevolving unique contact interfaces.

phosphorylation | protein interactions | co-evolution

Molecular recognition between protein partners is critical to all cellular processes. The fidelity of these processes requires proteins to discriminate against spurious interactions with noncognate partners. Gene duplication and divergence give rise to protein families whose members exhibit structural similarities. These similarities could lead to interactions between noncognate partners, potentially with deleterious effects (1, 2). However, interactions between noncognate partners are rare, indicating that noncognate interactions are selected against as novel functions emerge. (3, 4). This process has been well characterized in systems in which interacting protein surfaces are arranged in two separate proteins (4–6). Yet, little is known about the molecular coevolution of interacting surfaces arranged within the same molecule. Here, we examine interaction specificity between partners that exist as a single or as two independent proteins in a class of bacterial signaling molecules (7, 8).

Two-component regulatory systems are signal-transduction modules that enable cells to sense particular stimuli and mount an adaptive response to the environment denoted by the stimuli. The classical two-component system (CTCSs) consists of a sensor kinase (SK) and a response regulator (RR). These proteins engage in a transient interaction that culminates in the transfer of a phosphoryl group from the SK to the RR, thereby modulating RR activity (9). A single bacterium can house dozens of CTCSs, each detecting a different signal and controlling a distinct set of genes or activities (3, 10). The fidelity of signal perception and response is maintained by the coevolution of the amino acid residues comprising the interfacial surfaces of cognate SK/RR pairs. These interfaces allow CTCS proteins to prevent cross-talk by discriminating against noncognate partners (3, 4, 6, 11).

Many bacterial species harbor a peculiar class of two-component systems, designated hybrid two-component systems (HTCSs), that incorporates all the domains found in SKs and RRs in a single polypeptide (8, 12). These proteins are distinct from the widely distributed hybrid histidine kinases because they also include an output domain that often facilitates DNA binding and regulation of target gene transcription. The intramolecular arrangement of SK and RR domains raises the possibility that canonical signal transduction paradigms from CTCSs may not apply to HTCSs. For example, a CTCS SK displays phosphotransfer fidelity whereby it distinguishes its cognate RR from the pool of noncognate RRs. However, tethering cognate SK/RR pairs in HTCSs increases their relative local concentration, which could reduce the need to maintain the determinants that dictate specificity. The expanded repertoires of both HTCSs and CTCSs in *Bacteroides thetaiotaomicron* allow us to examine directly how intramolecular and intermolecular interactions in this protein family affect specificity.

Here we report that SKs from HTCSs exhibit promiscuous phosphotransfer activity capable of phosphorylating noncognate RRs. This promiscuity is in stark contrast to the specificity exhibited by a cognate SK/RR pair from a CTCS in the same organism. We could rewire a HTCS in *B. thetaiotaomicron* successfully by assembling noncognate SK and RR pairs into a functional protein. Our findings suggest that specificity is maintained in the HTCS protein family by the high local concentration of intramolecularly arranged protein partners.

Results

SKs and RRs of HTCSs Exhibit Reduced Covariation at Predicted Interaction Interfaces. Computational analyses of covarying residues in cognate SK/RR pairs from bacterial CTCSs enabled the identification of specificity-determining positions (6, 11, 13). These positions govern phosphotransfer specificity between SK and RR pairs (5, 6, 13–15). The exchange of residues at these positions between two CTCS SKs enabled the successful rewiring of CTCSs whereby an SK activates a noncognate RR (6).

To examine the covariance in HTCSs, we obtained from various sequence databases 959 nonredundant amino acid sequences corresponding to HTCSs, which we defined as those containing a HisKA [Protein Families (pFam) ID: PF00512], ATPase_c (pFam ID: PF02518), Response_reg (pFam ID: PF00072), and

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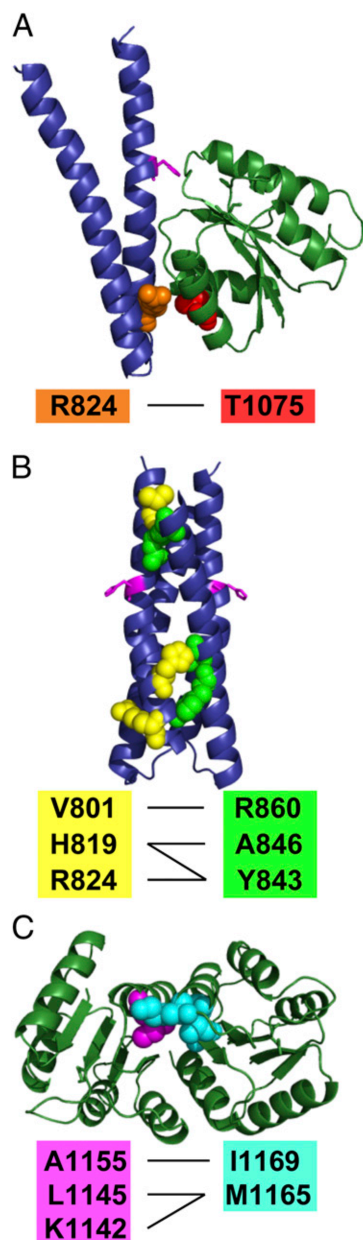

Fig. 1. HTCSs exhibit reduced covariation at positions that mediate specificity in CTCSs. (*A*) The SK (blue) and RR (green) domains of BT3172 have a single pair of covarying residues, which are displayed as spheres (SK, orange; RR, red). The phosphor-donating histidine is shown in magenta for reference. (*B*) The SK homodimerization interface has several covarying positions, which are displayed as spheres (monomer 1, green; monomer 2, yellow). The phosphor-donating histidine is shown in magenta for reference. (*C*) The HTCS RR from BT3172 was threaded through the structure of the CTCS RR ArcA homodimer [Protein Data Bank (PDB) ID, 1XHE] (48). Positions comprising the RR homodimerization interface that exhibited covariation are displayed as spheres (monomer 1, magenta; monomer 2, cyan). See also Fig. S1.

a DNA-binding domain. The sequences were aligned, positions with gaps greater than 10% were removed, and direct-coupling analysis was performed as previously described (Fig. S1, Dataset S1, and Dataset S2) (16). Positions critical for interfacial surfaces in HTCSs and CTCSs are comparable because these two classes of signaling molecules exhibit similarity in their amino acid sequences and their predicted secondary structures. Thus, we compared the mutual information values calculated for HTCSs with similar analyses carried out with CTCSs (6, 11, 13).

We detected covariation at a single position in the SK and the RR from HTCSs corresponding to the putative interface of these proteins (Fig. 1*A* and Fig. S2). This result is in contrast to CTCSs, where nine positions in the SK were found to covary with six positions in the corresponding RR (6, 11, 13). The inability to uncover extensive covariation in HTCS proteins cannot be ascribed to the method we used, because there was covariation between multiple positions that facilitate homodimerization of SKs and RRs (Fig. 1 *B* and *C*, and Fig. S2) (17). These results suggest that HTCSs may not be as reliant as CTCSs on distinct surface interfaces to distinguish cognate from noncognate partners.

CTCS SK BT0927 Exhibits Kinetic Preference for Its Cognate RR BT0928. Cells often house multiple, structurally similar CTCSs. However, they maintain fidelity in signal transduction because specific SK/RR interactions enable rapid phosphotransfer between cognate pairs (3). Although a given SK also can phosphorylate a noncognate RR in vitro, the rate at which this phosphorylation occurs is dramatically slower than the rate at which the SK phosphorylates its cognate RR (5, 6, 10). The disparity in phosphotransfer rates from an SK to cognate and noncognate RR domains observed in vitro—referred to as a "kinetic preference"—explains how a given SK specifically phosphorylates its cognate RR in the presence of noncognate RRs in vivo (10).

To determine whether the CTCSs of *B. thetaiotaomicron* exhibited a phosphotransfer fidelity similar to that displayed by CTCSs in *Escherichia coli* and *Caulobacter crescentus* (6, 10), we investigated the phosphotransfer specificity of the purified cytoplasmic domain from the CTCS SK encoded by *BT0927* (i.e., BT0927c) when incubated with a panel of purified RRs that included its putative cognate RR (i.e., BT0928) and 32 additional RR domains originating from all the annotated HTCSs in *B. thetaiotaomicron* (Fig. 2) and from four RRs from CTCSs in *B. thetaiotaomicron* (Fig. S3*A*) (12). We measured phosphotransfer as the reduction in the amount of phosphorylated SK in each reaction containing an RR. When incubations were carried out for 10 s, phosphotransfer was detected only to BT0928 (Fig. 2*A* and Fig. S3*A*). However, when the incubation was extended to 60 min, there was phosphotransfer to more than half the noncognate RRs (Fig. 2*B* and Fig. S3*A*). These data indicate that BT0927, and potentially other SKs from CTCSs in *B. thetaiotaomicron,* function similarly to SKs from CTCSs in other organisms, in that they exhibit kinetic preference toward their cognate RRs (6, 10).

SKs from HTCSs Lack a Kinetic Preference for Their Cognate RRs. We investigated phosphotransfer profiling with the RR domain from each of the 32 HTCSs that included half of the linker region that separates the SK and RR domains in the cytosolic portion of these proteins (7, 12, 18). We used a portion of the BT3172 SK spanning the first residue after the final transmembrane domain (E772) through the end of the annotated ATPase_c domain (K1020). The resulting construct encoded a dual-affinity N-terminal 6xHistidine-MBP tag that facilitates rapid purification and distinguishes the SK from the RR domain by molecular weight. This type of construct was used successfully in previously reported phosphotransfer-profiling experiments carried out with CTCSs (5, 6, 10).

To examine phosphotransfer specificity of HTCS SKs, we incubated phosphorylated BT3172 SK with each of the 32 HTCS RRs in individual reactions. We measured phosphotransfer as the reduction in the amount of phosphorylated SK in each reaction containing an RR. Each measurement was normalized to a control reaction in which the SK was incubated with BSA instead of an RR. This method is more accurate than measuring the total amount of phosphorylated RR in each reaction because

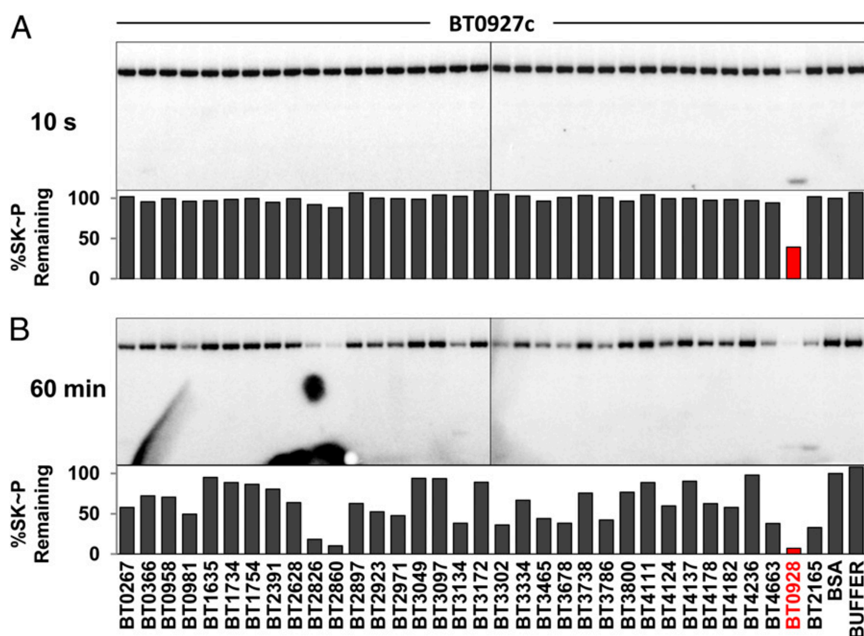

Fig. 2. The CTCS SK BT0927c exhibits kinetic preference for its cognate RR BT0928. Phosphotransfer between phosphorylated BT0927c and a panel of purified RRs was examined after incubation for 10 s (*A*) or 60 min (*B*). The histograms below the autoradiographs represent the percent reduction in phosphorylated BT0927c (SK~P) after incubation with RR domains relative to that resulting from incubation with BSA, which was used as a negative control. The data corresponding to incubation with the cognate RR BT0928 are shown by red bars.

SKs can exhibit phosphatase activity that could prevent accumulation of the phosphorylated RR (5). However, this method does not exclude the possibility that a given RR stimulates dephosphorylation of the phosphorylated SK in the absence of phosphotransfer. Phosphotransfer was examined after incubations for 1 and 60 min.

The levels of phosphorylated BT3172 SK decreased by more than 60% following a 1-min incubation with its cognate RR or with the noncognate RR domain from the HTCS BT2826 (Fig. 3*A*). The noncognate RRs from HTCSs BT0366, BT1635, and BT4137 also elicited reductions in phosphorylated BT3172 SK but did not exceed those resulting from incubation with the RR domains from BT3172 and BT2826. As expected, the levels of phosphorylated BT3172 SK decreased dramatically in reactions carried out with a variety of RRs for 60 min (Fig. 3*B*). Interestingly, the BT3172 SK did not exhibit promiscuity toward a small panel of CTCS RRs (Fig. S3*B*). Thus, the SK from BT3172 exhibits promiscuous phosphotransfer toward a subset of RRs from HTCSs.

To determine whether phosphotransfer promiscuity was particular to the BT3172 SK or also applied to other HTCS SKs, we performed similar experiments with the BT1635 SK. There was a 60% reduction in phosphorylated BT1635 SK following a 1-min incubation with the noncognate RRs from BT2826, BT3172, and BT4137 (Fig. 3*C*). Surprisingly, the decrease in phosphorylated BT1635 SK was faster during incubations with each of these three noncognate RRs than in incubations carried out with the cognate BT1635 RR (Fig. 3*C*). This result suggests that BT1635 SK can use noncognate substrates more efficiently than its cognate RR. (However, it is also possible that our preparations of the BT1635 RR contain less active protein than other HTCS RRs.) Like the BT3172 SK, the BT1635 SK did not exhibit promiscuous phosphotransfer to several investigated CTCS RRs (Fig. S3*C*), suggesting that promiscuity is limited to RR proteins from HTCSs. Therefore, our data demonstrated that HTCS SKs exhibit relaxed specificity for phosphotransfer substrates and suggest that phosphotransfer promiscuity could be a general property of HTCSs.

HTCS Chimeras Exhibit Phosphotransfer Promiscuity in Vitro. A possible explanation for the phosphotransfer promiscuity exhibited by the HTCS SKs is that the specificity determinants of the SK and RR were lost when these domains were separated into two different polypeptides. To evaluate this possibility, we examined whether a HTCS SK can transfer the phosphoryl group efficiently to a noncognate RR present in the same molecule. Thus, we engineered chimeric proteins consisting of the BT3172 SK tethered to the noncognate RRs BT1635 or BT2826, which are phosphorylated effectively in vitro, or to the noncognate RR domains from BT1754, which is not (Fig. 3*A*).

An HTCS can be phosphorylated on a histidine within the SK domain or an aspartic acid within the RR domain. Unfortunately, approaches previously used to examine phosphotransfer between an SK and an RR of CTCSs could not be used to distinguish between histidine- and aspartate-phosphorylated HTCSs because these two forms of phosphorylated HTCS could not be resolved by SDS/PAGE. Additionally, loss of the phosphoryl group from the RR could hinder detection of intramolecular phosphotransfer. Therefore, we used a variety of mutant proteins substituted in the amino acid residues predicted to be the sites of phosphorylation. We reasoned that replacing the conserved phospho-accepting aspartic acid within the RR domain with an alanine residue would prevent phosphotransfer to the RR domain. In this case, the amount of phosphorylated protein in a chimera should be similar to that displayed by the SK domain when incubated alone.

There was low accumulation of phospho-protein in reactions carried out with BT3172c or with the BT3172SK-BT1635RR and BT3172SK-BT2826RR chimeras (Fig. 4*A*). This result likely reflects efficient phosphotransfer followed by dephosphorylation of the RR domains. In contrast, the chimera containing the wild-type RR domain from BT1754 fused to the BT3172 SK domain accumulated phospho-protein at higher levels than the BT3172SK-BT1635RR and BT3172SK-BT2826RR chimeras (Fig. 4*A*). This result could indicate that phosphotransfer to the RR domain followed by dephosphorylation is not efficient in the BT3172SK-BT1754RR protein. In agreement with this notion, BT3172c, BT3172SK-BT1635RR, and BT3172SK-BT2826RR with the

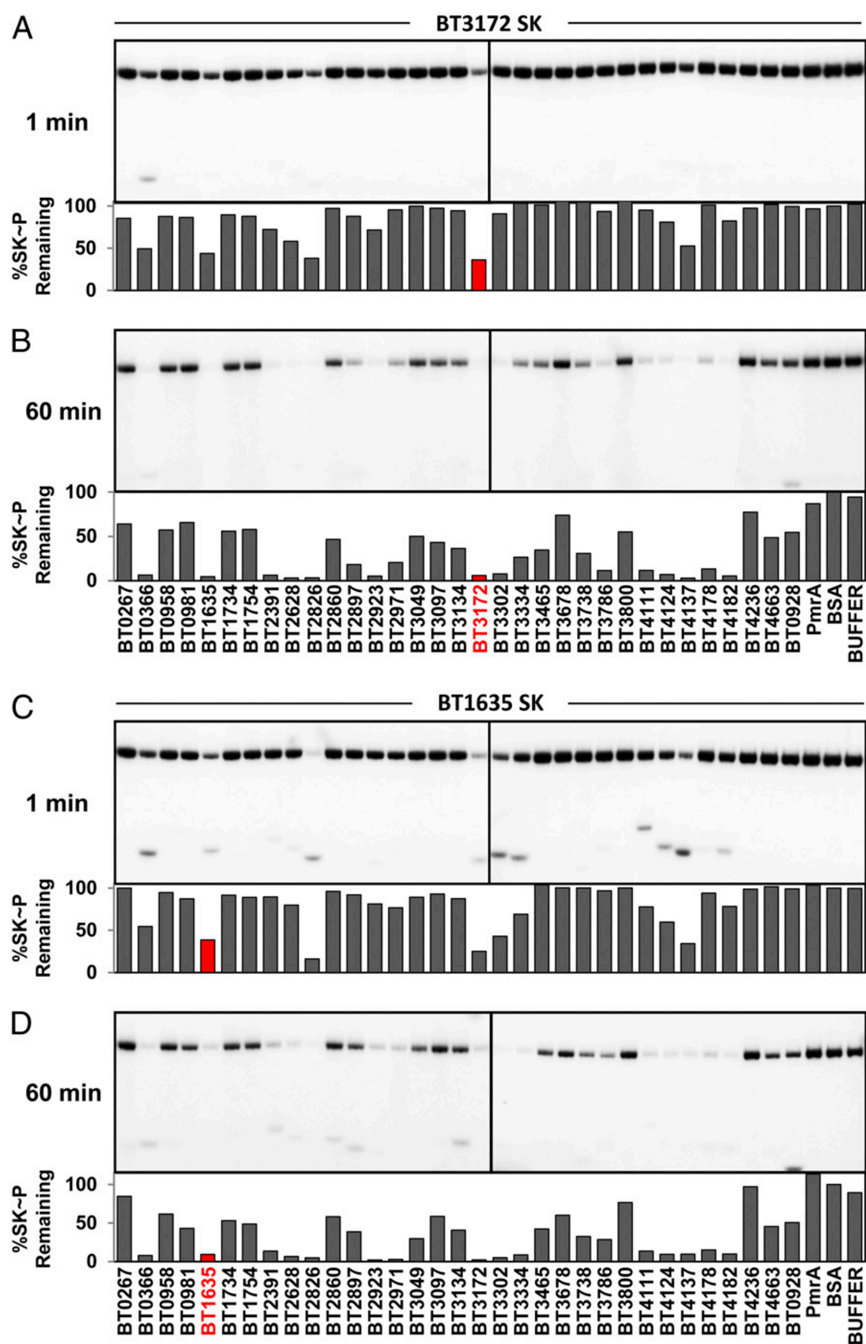

Fig. 3. HTCS SKs exhibit phosphotransfer promiscuity. Phosphotransfer between the HTCS SKs from BT3172 (*A* and *B*) and BT1635 (*C* and *D*) and the panel of RRs listed at the bottom of the figure after incubation for 1 (*A* and *C*) or 60 (*B* and *D*) min. The histograms below each autoradiograph represent the percent reduction in phosphorylated SK (SK~P) relative to the BSA control. Results corresponding to incubations of an SK with its cognate RR are denoted by red bars.

conserved aspartate replaced by alanine accumulated high levels of phospho-protein (Fig. 4*A*). Likewise, mutation of the conserved aspartate promoted increased accumulation of phosphoprotein in the BT3172SK-BT1754RR chimera (Fig. 4*A*). The behavior of the latter protein might reflect defective phosphotransfer; alternatively phosphotransfer might occur as in the other chimeras, but the phosphorylated BT1754 RR is not dephosphorylated by the BT3172 SK.

We took advantage of the inherent instability of phospho-histidine at low pH and phospho-aspartate at high pH (19–21) to examine which phosphorylated form was present in the reactions. Control reactions contained separated SK and RR domains from BT3172. As expected, treatment with 0.1M HCl eliminated phosphorylated SK (Fig. 4*B*) compared with treatment with 0.1 M Hepes (Fig. 4*C*), whereas treatment with 0.2 M NaOH eliminated phosphorylated RR (Fig. 4*D*). When the chimeras were treated with 0.2 M NaOH, there was minimal reduction in the levels of phosphorylated proteins (Fig. 4*D*). However, treatment with 0.1 M HCl reduced the levels of phosphorylated protein >90% (Fig. 4*B*). This result indicates that the majority of phospho-protein in the reactions contained phosphorylated SK rather than phosphorylated RR and held true whether the protein contained a cognate or a noncognate RR domain. Taken together, these data demonstrated that chimeras containing the BT3172

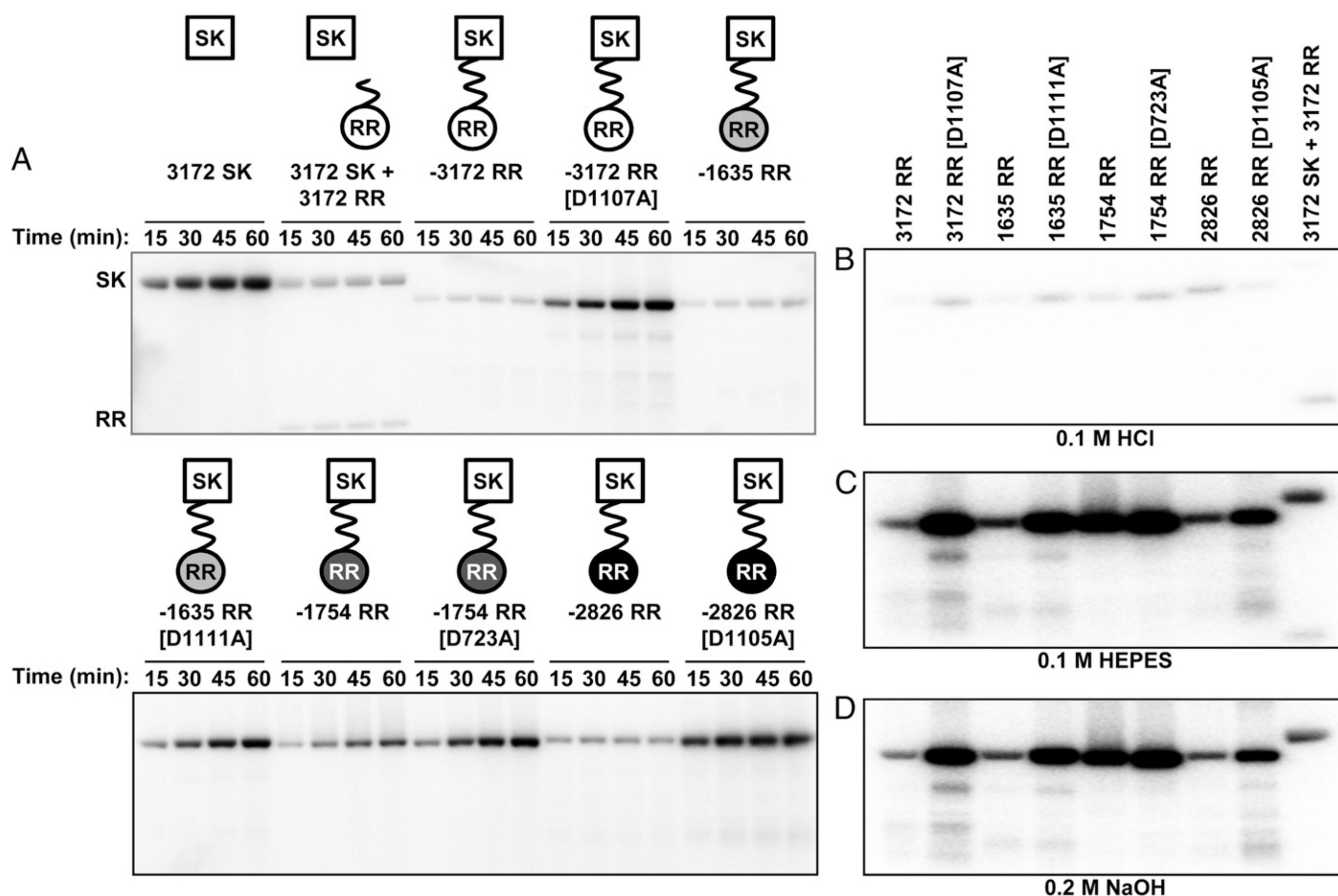

Fig. 4. Phosphotransfer properties of chimeric proteins comprising SK and RR domains from different HTCSs. (*A*) Accumulation of phosphorylated proteins for the indicated times after incubation of the chimeras with ^{32}P-γ-ATP. Samples were combined with sample buffer and fractionated by SDS/PAGE, and radiolabel incorporation was detected by autoradiography. Above each autoradiograph is a cartoon representation of the corresponding protein. (*B–D*) Acid/base sensitivity of phosphorylated chimeras. Chimeras were incubated with ^{32}P-γ-ATP for 60 min before each sample was combined with sample buffer and fractionated by SDS/PAGE. Radiolabel incorporation was detected by autoradiography. Identical SDS/PAGE gels containing fractionated phosphorylated chimeras were differentially treated with 0.1 M HCl (*B*), 0.1 M Hepes buffered to pH 7.0 (*C*), or 0.2 M NaOH (*D*). The position of the phosphor-accepting aspartic acid to alanine substitutions are denoted with respect to the full-length HTCS from which the RR originates.

SK fused to the RR domain from either BT1635 or BT2826 undergo intramolecular phosphotransfer, whereas those containing the BT1754 RR domain do not. Thus, an SK can transfer the phosphoryl group to a noncognate RR present in the same molecule of a chimeric HTCS.

In Vivo Rewiring of HTCSs. To determine whether intramolecular phosphotransfer between the BT3172 SK and noncognate RRs can occur in vivo, we examined the ability of bacteria expressing a chimeric protein to activate transcription of genes normally controlled by the HTCS whose RR domain was used to create the chimera. Expression of the BT3172SK-BT1635RR resulted in ~260-fold activation of *BT1631* (Fig. 5*A*), which is dependent on BT1635 (Fig. S4*A*). Similarly, the BT3172SK-BT2826RR chimera resulted in ~203-fold activation of *BT2818* (Fig. 5*A*), which is dependent on BT2826 (Fig. S4*B*). These chimeras promote expression in a specific manner, because they did not activate genes targeted by other HTCSs, including those regulated by BT3172 (Fig. 5*A*). Additionally, blocking phosphotransfer in these chimeras by replacing the conserved phosphor-accepting aspartic acid residue by alanine significantly reduced target gene activation (Fig. 5*A*), even though the relative protein levels of wild-type and mutant chimera were similar for each pair (Fig. 5*B*).

This in vivo rewiring is limited to chimeras harboring RR domains that serve as effective phosphotransfer substrates of the BT3172 SK domain in vitro because the BT3172SK-BT1754RR chimera induced the BT1754-dependent gene *BT1763* (7) only sixfold, which is 41-fold and 32-fold lower than the induction elicited by chimeras containing the RR domains from BT1635 or BT2826, respectively (Fig. 5*A*). This background level of induction appears to reflect expression of the chimera to nonphysiologically high levels, because similar levels of *BT1763* induction were displayed by a mutant BT3172 SK-BT1754 RR chimera substituted in the conserved aspartate in the RR domain (Fig. 5*A*). Taken together, these results demonstrate that particular noncognate RRs can serve as phosphotransfer substrates for the BT3172 SK in vivo and thereby permit rewiring of HTCS transcriptional responses.

Wild-Type HTCS BT3172c Exhibits Activation Fidelity in Vivo. The phosphotransfer promiscuity exhibited by noncognate HTCSs SK/RR pairs in vitro is in stark contrast to the specificity manifested by CTCS SK/RR pairs. This promiscuity raised the possibility that an SK from one HTCS might activate a noncognate RR(s) from another HTCS in vivo. Therefore, we examined whether activation of the HTCS BT3172 promoted transcription of genes that were induced in cells expressing chimeric HTCSs containing an RR domain that can serve as a phosphotransfer

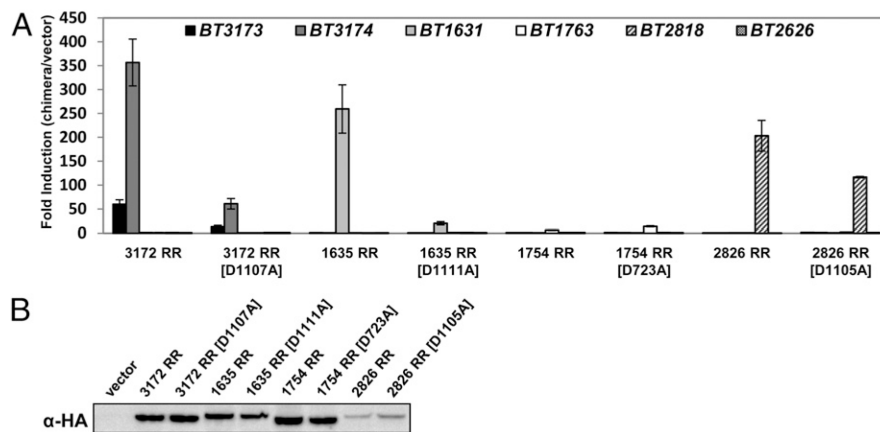

Fig. 5. Gene transcription by bacteria expressing chimeras comprising the BT3172 SK domain fused to cognate or noncognate RR domains. (*A*) Fold induction of BT3172-dependent (*BT3173* and *BT3174*) genes and BT3172-independent (*BT1631*, *BT1763*, *BT2818*, and *BT2626*) genes in cells expressing proteins comprising the BT3172 SK fused to the indicated RR domain. Fold induction reflects the ratio of the mRNA levels produced by cells harboring the HTCS relative to those carrying the plasmid vector. mRNA levels were measured by quantitative RT-PCR. Error bars represent SD calculated from two experiments performed in duplicate. (*B*) Western blotting of cell extracts prepared from an aliquot of the cultures described in *A*. The positions of the phosphor-accepting aspartic acid to alanine substitutions are denoted with respect to the full-length HTCS from which the RR originates.

substrate for the isolated BT3172 SK domain in vitro. We measured transcription of target genes for various HTCSs following expression from a multicopy number plasmid of BT3172c under the control of an inducible promoter. Because BT3172c exhibits constitutive autokinase and phosphotransfer activities in vitro, we could follow its transcriptional activity even though the ligand that activates the BT3172 HTCS remains unknown.

Control experiments demonstrated that expression of BT3172c induced the BT3172-dependent genes *BT3173* and *BT3174* by 60- and 357-fold, respectively (Fig. 5*A*). In contrast, transcriptional induction of *BT3173* and *BT3174* was five- and sixfold lower, respectively, in a strain with the BT3172c mutant [D1107A], which cannot phosphorylate its RR domain (Fig. 5*A*). The reduced gene activation indicates that the conserved aspartate in the RR domain is required for activity. We ascribe the remaining activation to overexpression of the mutant protein, which has been observed in *Salmonella* following overexpression of the CTCS RR PhoP mutated in the conserved aspartic acid residue (22, 23).

Expression of the BT3172c protein appears to induce BT3172-dependent genes specifically, because transcription of *BT1631*, *BT1763*, *BT2818*, and *BT2626* was not affected (Fig. 5*A*). Thus the genes regulated by the HTCSs BT1635 and BT2826 (i.e., *BT1631* and *BT2818,* respectively) were not activated upon BT3172c expression even though the noncognate RRs from BT1635 and BT2826 can serve as phosphotransfer substrates for the BT3172 SK in vitro (Fig. 3*C*). Furthermore, cells expressing the BT3172c [D1107A] mutant did not activate BT1635- and BT2826-dependent genes. The BT3172c [D1107A] mutant is unable to transfer the phosphoryl group to its tethered cognate RR, which would therefore enhance the possibility of phosphotransfer between different HTCSs. These data indicate that, in vivo, phosphotransfer does not occur between different HTCSs *in trans.* These findings are in agreement with a previous report that activation of individual HTCSs results in the transcription of specific target genes and not those regulated by other HTCSs (24).

Discussion

Protein families can expand by gene duplication and divergence, giving rise to new members that perform distinct cellular functions. Specific interactions between cognate protein partners occur despite the structural similarity with noncognate family members. Two distinct mechanisms are commonly used to confer specificity between cognate protein partners. On the one hand, the evolution of distinct interfacial surfaces simultaneously facilitates recognition of cognate partners and discrimination against noncognate partners. This mechanism is used by both eukaryotes and prokaryotes to maintain specificity between cognate protein partners involved in a variety of cellular processes including signal transduction, metabolism, and protein secretion (9, 25, 26). On the other hand, physically colocalizing protein partners can insulate a particular interaction from cross-talk with noncognate proteins by maintaining high local concentrations of cognate partners. Insulating protein partners from cross-talk can be achieved by compartmentalizing cognate partners using a scaffold to organize particular partners into a complex or by incorporating interacting domains into a single polypeptide (27, 28). Although these two mechanisms are not mutually exclusive a priori, the selective pressures that drive the evolution of distinct interaction interfaces might be diminished when partners are maintained at high local concentrations.

We determined that phosphotransfer specificity between SKs and RRs is different when these domains are found as two independent proteins (in CTCSs) or as a single polypeptide (in HTCSs) in the human gut bacterium *B. thetaiotaomicron*. We observed phosphotransfer specificity between a cognate SK/RR pair from a CTCS in vitro (Fig. 2), as is consistent with previous reports that CTCS proteins use unique interfaces to facilitate specific interactions (6, 11, 29). In contrast, SKs from HTCSs were promiscuous and effectively transferred a phosphoryl group to noncognate RRs in vitro (Figs. 3 and 4). This promiscuity permitted chimeric HTCSs consisting of noncognate SK/RR pairs to rewire signal-transduction pathways in vivo (Fig. 5*A*). These results indicate that HTCSs are less dependent on distinct interfaces for specific phosphoryl transfer between cognate SK/RR partners and that they may rely instead on the high local concentration of cognate SK/RR pairs resulting from the tethering of these two domains.

What dictates phosphotransfer from a HTCS SK to noncognate RRs? A possible explanation is the degree of amino acid identity between the noncognate RR and the cognate RR domains. However, the degree of identity cannot explain the efficient phosphotransfer from BT3172 SK to the noncognate BT2826 RR because the amino acid sequence of the BT3172 RR domain is 52.9% identical to the BT2826 RR domain but is 86.6% and 79.0% identical to the RRs from BT1635 and

BT4137, respectively, which are not as good phosphotransfer substrates for BT3172 SK (Fig. 3).

Our results suggest that the constraints governing the coevolution of unique interaction interfaces are relaxed when cognate protein partners are maintained at high local concentrations. This relaxation is facilitated by the linker region in HTCSs, which tethers cognate SK/RR pairs into a single polypeptide. In eukaryotes, multiple members of the MAPK signaling family regulate cell proliferation and differentiation in response to extracellular cues (30). The fidelity of MAPK-mediated signal-transduction cascades is aided by scaffolding proteins, which bind and position cognate partners. In the absence of these scaffolding proteins, cross-talk can occur between noncognate MAPKs (31). Furthermore, the introduction of novel scaffolds that colocalize noncognate MAPK partners can rewire signal transduction (32, 33). Thus, MAPK signaling proteins also can exhibit relaxed interaction specificity, although they use a different mechanism to facilitate high local concentrations of cognate partners.

The expansion of CTCSs repertoires by gene duplication and horizontal transfer events potentiates interactions between noncognate SK/RR pairs that could have substantial negative effects on fitness. Recent work has demonstrated that eliminating cross-talk between noncognate SKs and RRs is a strong selective pressure in the evolution of CTCSs (4) that has resulted in CTCSs evolving unique contact interfaces that not only facilitate interactions between cognate SK/RR partners but also prevent interactions with noncognate partners.

HTCSs appear to have relaxed selection on the specificity determinants that distinguish cognate from noncognate partners. However, the potential for promiscuous phosphotransfer between noncognate SK/RR pairs does not result in cross-talk between SKs and RRs of *B. thetaiotaomicron* HTCSs in vivo. The avoidance of cross-talk is essential for their physiological function, which is to promote expression of particular polysaccharide utilization loci in response to the presence of a specific carbohydrate in the bacterium's surroundings (24). Presumably cross-talk does not occur because the tethering of SK and RR domains in a single polypeptide favors intermolecular phosphotransfer as opposed to interactions with noncognate RR domains in a different polypeptide. Therefore, bacterial CTCSs and HTCSs have evolved two distinct mechanisms that facilitate specificity and insulate individual pathways with similar signaling modules.

The evolution of distinct mechanisms to facilitate specific interactions raises questions about the potential selective advantages of tethering cognate partners (as in HTCSs) over coevolving unique contact surfaces (as in CTCSs). First, the tethered SK/RR pairs of HTCSs exhibited phosphotransfer promiscuity, which permitted transcriptional rewiring by swapping RR domains between two systems (Fig. 5*A*). One possibility is that promiscuity promotes modularity, which could facilitate reorganization of preexisting domains resulting in novel protein functions that could confer a selective advantage (33–36). Modularity has been implicated in the evolvability of eukaryotic protein-interaction networks and can facilitate rapid adaptation (36, 37). Furthermore, this potential for modularity has been exploited by bacterial pathogens, which introduce novel scaffolding proteins into eukaryotic cells that reposition noncognate signaling proteins into new functional complexes that subvert host defenses (32, 38).

Second, the organization of cognate SK/RR pairs into an HTCS insulates the interaction from cross-talk between noncognate partners despite their increased propensity for promiscuity. This inherent insulation could allow the rapid expansion of HTCS repertoires by gene duplication or lateral transfer events without requiring the constant coevolution of divergent specificity-determining residues necessary to avoid cross-talk as described for CTCSs (4) and thereby could enable the rapid distribution of loci necessary for the utilization of various polysaccharides and their associated regulatory systems (often HTCSs) between members of the *Bacteroidetes* in the distal human gut (39). Thus, tethering cognate SK/RR partners may have evolved to facilitate the rapid acquisition and integration of new regulatory systems without potentiating cross-talk with preexisting systems.

Materials and Methods

Strains and Growth Conditions. *E. coli* strains were derived from S17-1 (40) or BL21 (DE3) (41) and were grown in LB medium containing 50 μg/mL ampicillin. *B. thetaiotaomicron* strains were derived from ATCC 29148 (VPI-5482) (12) and were grown in tryptone-yeast extract-glucose medium containing tetracycline (2 μg/mL), erythromycin (10 μg/mL), or gentamicin (200 μg/mL) when applicable. All strains and plasmids used in this study are listed in Table S1. All primers used in this study are listed in Table S2.

Protein Purification. The SK portion of BT3172 and BT1635 was N-terminally tagged with a tandem affinity tag comprising a 6xHis tag and the maltose-binding protein. All chimeras, BT3172c, and RRs from CTCSs or HTCSs were tagged with an N-terminal 6xHis tag. All proteins were purified from BL21 (DE3) by inoculating a single colony into 10 mL of LB broth containing ampicillin and subsequently grown for 16 h at 37 °C. Each strain was subcultured into 500 mL of LB broth supplemented with ampicillin. Cultures were grown to an OD_{600} 0.4– 0.6 before the addition of isopropylthio-β-galactoside (to a final concentration of 300 μM) and were incubated at 30 °C for 4 h. The cultures were centrifuged at 8,000 × *g* for 10 min, supernatant was decanted, and the pellet stored at −20 °C overnight. All proteins were purified from cell pellets using the Ni-NTA System (Qiagen) according to the manufacturer's instructions. The BT3172 and BT1635 SKs were purified further using amylose-conjugated agarose resin (New England BioLabs) according to the manufacturer's instructions. All SKs, chimeras, and BT3172c were stored in kinase buffer [5 mM MgCl2, 2 mM DTT, 50 mM KCl, in 10 mM Hepes (pH 8.0) with 10% glycerol (vol/vol)]. All RRs were stored in 50 mM Tris (pH 8.0) containing 20% glycerol (vol/vol). The amino acid segments of all purified proteins used in this study are presented in Table S3.

Phosphotransfer Profiling. Phosphotransfer profiling was performed as described (5) with the following modifications. Each purified SK (final concentration 5 μM) in kinase buffer [5 mM MgCl2, 2 mM DTT, 50 mM KCl, in 10 mM Hepes (pH 8.0) with 10% glycerol] was combined with a mixture of ^{32}P-γ-ATP (5 μCi/30 μL of the final reaction volume) and cold ATP (to a final concentration of 500 μM). The mixture was incubated at 30 °C for a period that had been predetermined to be the optimal incubation time for each SK. The phosphorylated SK subsequently was diluted into a reaction mixture (prewarmed to 37 °C) containing each individual RR so that both phosphorylated SK and RR were at a final concentration of 2.5 μM in 10% PEG-3350, 10% glycerol, 50 mM KCl, 5 mM $MgCl_2$, 2 mM DTT, and 10 mM Hepes (pH 8.0). The reactions were mixed thoroughly and placed at 37 °C for the indicated times before aliquots were removed and combined with SDS sample buffer and placed on ice while awaiting SDS/PAGE.

Acid/Base Sensitivity of Phosphorylated Proteins. The wild-type and mutant BT3172 cytoplasmic regions (BT3172c) and chimeras containing the BT3172 SK were subjected to autokinase/phosphotransfer conditions before being fractionated on 10% Bis-Tris Novex SDS/PAGE gels in 1× 3-(*N*-morpholino) propanesulfonic acid (Mops) buffer (Invitrogen) for 60 min at 180 V. Duplicate gels were treated as described (20). Gels were soaked in 40% isopropanol for 30 min before being immersed in one of the following solutions: 0.1 M Hepes (pH 7), 0.1 M HCl, or 0.2 M NaOH for 2 h at 55 °C. The gels were soaked in 40% isopropanol for 30 min before being dried and subjected to phosphorimaging to examine radiolabel incorporation.

Phosphotransfer Time Course. Each SK (final concentration 5 μM) in kinase buffer [5 mM MgCl2, 2 mM DTT, 50 mM KCl, in 10 mM Hepes (pH 8.0) with 10% glycerol (vol/vol)] was mixed with a mixture of ^{32}P-γ-ATP (5 μCi/30 μL of the final reaction volume) and cold ATP (to a final concentration of 500 μM) and was incubated at 30 °C for 60 min (BT3172 SKs) or 90 min (BT1635 SKs). Phosphorylated SK subsequently was combined with a solution containing RR (5 μM) prewarmed to 37 °C. The resulting mixture contained 2.5 μM SK∼P and RR in 5 mM MgCl2, 2 mM DTT, 50 mM KCl, 10 mM Hepes (pH 8.0), 10% glycerol (vol/vol), and 10% PEG-3350. Each reaction was mixed well and placed at 37 °C before 10-μL aliquots were taken at 10, 30, 60, 120, and 300 s and combined with 3.5 μL of sample buffer before being placed on ice. Each sample was fractionated by SDS/PAGE on 10% Bis-Tris Novex gels in 1× Mops

buffer (Invitrogen) for 60 min at 180 V. Gels were dried, and radiolabel incorporation was visualized by autoradiography. At each time point the band representing phosphorylated SK was quantified using ImageQuant 4.0 software (Molecular Dynamics). Phosphotransfer was measured as the percentage reduction in the intensity of this band relative to the intensity present at time 0.

Overexpression of HTCS Chimeras in *B. thetaiotaomicron*. BT3172c, BT1635c, and all chimeras encoded a C-terminal HA-tag expressed under control of the maltose-inducible *susB* promoter in the multicopy number plasmid pLYL01 (42). [Note that these chimeras consist of only the cytoplasmic domains following the last predicted transmembrane region. Thus, they differ from a recently reported chimera that contains the entire periplasmic carbohydrate sensing domain combined with the entire cytoplasmic region of two different HTCSs (43).] Plasmids encoding each chimera were introduced into a BT3172-deficient *B. thetaiotaomicron* strain by biparental conjugation (8, 44). Each strain was grown in minimal medium containing 0.5% glucose at 37 °C for 16 h under anaerobic conditions followed by 1:50 subculture into minimal medium containing 0.5% maltose as the sole carbon source. Each strain was grown to midlog phase (OD_{600} 0.3–0.6) before 2.0 mL of culture was collected for mRNA preparation and subsequent quantitative RT-PCR. An additional 3.0 mL of the cell culture was collected concomitantly for preparation of protein extracts for Western blot analysis.

Quantitative RT-PCR. Cell pellets representing 2.0 mL of culture were resuspended in 1.0 mL of a 1:2 dilution of RNA Protect (Qiagen) according to the manufacturer's directions, incubated for 5 min at room temperature, and pelleted by centrifugation for 10 min at 5,000 × *g*. Each pellet was stored at −80 °C until mRNA was purified by the RNeasy kit (Qiagen). cDNA was synthesized from 1 μg of purified RNA using the high-capacity RNA-to-cDNA master mix (Applied Biosystems). Expression of each gene was measured with primers listed in Table S2 and normalized against 16s ribosomal RNA.

Western Blot Analysis. Cell extracts were prepared by sonication of cell pellets containing 3.0 mL of midlog-phase culture. Cell debris was removed by centrifugation at 20,000 × *g* before total protein was quantified by absorbance at 280 nm. Then 50 μg of protein from each extract was combined with lithium dodecyl sulfate buffer and loaded onto a 10% Bis-Tris SDS/PAGE gel (Invitrogen). Gels were fractionated at 200V for 45 min in 1× Mes buffer (Invitrogen) before being transferred to a nitrocellulose membrane using an iBlot apparatus (Invitrogen). Membranes were blocked in TBS containing 3.0% skim milk (Sigma) before being probed sequentially with a rabbit α-HA anti-sera and HRP-conjugated α-rabbit monoclonal antibodies (Sigma). Proteins were detected by addition of Femto chemiluminescent substrate (Pierce) and imaged using an LAS-4000 imaging system (Fuji).

Covariation Analysis. Amino acid sequences from 960 hybrid two-component systems were subjected to multiple sequence alignment by the PAUP 4.0 software package (Sinauer Associates Inc.) with maximum likelihood (45–47). The aligned region representing the SK phosphoacceptor domain (HisKA), SK catalytic domain (HATPase_c), and RR receiver domain was subjected to direct coupling analysis as previously described (13, 16).

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