Rapid Dephosphorylation of the TorR Response Regulator by the TorS Unorthodox Sensor in *Escherichia coli*

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Two-component signal transduction systems are widespread in bacteria, archaebacteria, plants, and lower eucaryotes, and they enable cells to adapt to changing environments (3, 10, 26, 28). The simplest two-component systems contain a sensor protein and a cognate response regulator (11, 23). The sensor protein is usually a transmembrane protein whose N-terminal domain, oriented toward the exterior, detects specific environmental stimuli and whose C-terminal domain, located in the cytoplasm, is a histidine autokinase domain called the transmitter. Once stimulated, the sensor autophosphorylates and transphosphorylates its cognate response regulator on a conserved aspartate of the receiver domain (D1), respectively (Fig. 1). We have previously shown that activation of TorR involves the following four-step phosphorelay: His443 → Asp723 → His850 → Asp(TorR). In this study, we provide genetic evidence that TorS can dephosphorylate phospho-TorR when TMAO is removed. Dephosphorylation probably occurs by a reverse phosphorelay, Asp(TorR) → His850 → Asp723, since His850 and Asp723 are both essential in this process. By using reverse transcriptase PCR, we also show that TMAO removal results in shutoff of *tor* operon transcription in less than 2 min. Based on our results and on analogy to other phosphorelay signal transduction systems, we propose that reverse phosphotransfer could be a rapid and efficient mechanism to inactivate response regulators.

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Transcription of the torCAD operon is rapidly stopped when TMAO is removed from the medium. To monitor the effect of TMAO exhaustion on torCAD expression, we used strain LCB620, which contains a torA-lacZ chromosomal fusion (20). Cells were first grown anaerobically in rich medium supplemented with 10 mM TMAO at 37°C. When the culture reached an optical density at 600 nm of about 0.7, the bacteria were pelleted and washed two times in cold medium. The bacteria then were resuspended in prewarmed rich medium supplemented or not with 10 mM TMAO, and expression of the fusion was determined after various incubation times. In the presence of TMAO, TorR is activated by phosphorylation via a four-step phosphorelay, as shown by the arrows overlined by a plus sign, leading to torCAD induction. The circled P corresponds to the phosphor group. The arrows underlined by minus signs indicate the reverse phosphorelay model for the dephosphorylation of TorR-P when TMAO is removed.

Figure 1: Schematic representation of the TorS-TorR phosphorelay system. TorS contains a N-terminal TMAO detector region, a primary transmitter domain (H1) with a histidine phosphorylation site (H443), a receiver domain (D1) with an aspartate phosphorylation site (D723), and a secondary transmitter domain (H2) with a histidine phosphorylation site (H850) (15). TorS is missing that TorS could dephosphorylate TorR-P when TMAO is removed. As many sensor proteins also play the role of phosphatase for their cognate response regulators ranges from seconds to hours depending on the response regulator (13, 33). To define the half-life of TorR-P, we incubated purified TorR with a constitutively active form of TorS (TorS726) in the presence of [γ-33P]ATP as previously described (15). Labeled TorR-P was then purified using a heparin column, and the half-life of the phosphoprotein was assessed after incubation in phosphorylation buffer (5 mM MgCl2) at room temperature and quantification of the remaining labeled TorR-P by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Surprisingly, the half-life of TorR-P was found to exceed 20 min (data not shown). The fact that the phospho-aspartyl bond of TorR-P is stable in vitro implies that another protein probably dephosphorylates TorR-P after TMAO removal.

Roles of sensor TorS and of its phosphorylation sites in the dephosphorylation of TorR-P in vivo. As many sensor proteins also play the role of phosphatase for their cognate response regulators in the absence of stimuli (5, 8, 30, 36), we hypothesized that TorS could dephosphorylate TorR-P when TMAO is missing. To study the role of TorS in vivo, we used strain LCB726 pcnB, which carries a constitutive allele of TorS (TorS726) and a torA-lacZ fusion (16). In this strain, TorS726...
phosphorylates TorR even in the absence of TMAO and, as a result, β-galactosidase activity is always very high. Production of a phospho-TorR phosphatase should lower the amount of TorR-P and thus decrease expression of the fusion. As shown in Fig. 3, production of the wild-type TorS protein from plasmid pSwt led to a 2.7-fold decrease of the β-galactosidase activity of the torA-lacZ fusion. This result indicates that in the absence of TMAO, TorS can down regulate the tor operon, most probably by dephosphorylating TorR-P.

As TorS contains three phosphorylation sites, we investigated the possible role of each site in the dephosphorylation of TorR-P. From Fig. 3 it is clear that TorSH443Q encoded by pSH443Q still affected expression of the fusion, whereas the presence of either TorSD723A (pSD723A) or TorSH850Q (pSH850Q) only slightly decreased the fusion activity. Furthermore, production from pΔS of a C-terminally truncated form of TorS, devoid of the D1 and H2 domains but containing an intact H1 domain, did not modify torA-lacZ expression. We concluded from these data that the primary phosphorylation site (H443) of TorS is not involved in the dephosphorylation of TorR-P whereas the other two are involved. Alternatively, mutations in the TorS phosphorylation sites D723 and H850 might result in structural changes in the TorS protein. However, this situation is reminiscent of that reported for the ArcB-ArcA system (8). Indeed, ArcA-P dephosphorylation occurs via a reverse phosphotransfer mechanism involving the phosphorylation sites of the secondary transmitter and the receiver domains of ArcB. Similar to the case for TorS, the histidine phosphorylation site of the primary transmitter is not involved in this process.

The TorS C-terminal D1-H2 region alone can dephosphorylate TorR-P in vivo. To confirm that the TorS receiver (D1) and secondary transmitter (H2) domains are responsible for the dephosphorylation of TorR-P, we constructed the pΔS plasmid series by cloning different alleles of the torS 3′-end sequence (positions 2001 to 2712), encoding the D1-H2 region, downstream from the tac promoter of plasmid pFJ19EH (7). The fragments were PCR amplified from chromosomal, pSD723A, or pSH850Q DNA with converging primers. The downstream primer (BamSH2) contains a BamH1 site, whereas the upstream primer (EcoSD1) contains an EcoRI site, a methionine codon instead of the codon for Arg666, and a strong ribosome-binding site (AAGGAG) upstream of the start codon. The cloned regions of the resulting plasmids were sequenced to verify the absence of unexpected mutations. As shown in Fig. 3, production of the C-terminal region (D1-H2) of TorS from pΔS decreased the activity of the torA-lacZ fusion. Strikingly, the β-galactosidase activities are similar in the presence of either the entire TorS protein or the TorS C-terminal region. This suggests that the D1-H2 region and intact TorS dephosphorylate TorR-P with equal efficiencies. When either phosphorylation site of the TorS C-terminal region was mutated, expression of the fusion was almost unaffected. This confirms the key role of D723 and H850 in the dephosphorylation of TorR-P.

The fact that the D1-H2 region alone can dephosphorylate TorR-P in vivo gave us the opportunity to study the dephosphorylation process in a more relevant genetic context, i.e., a torS wild-type strain. When plasmid pΔS was introduced into strain LCB640 (torS− torA-lacZ penB) (32), the β-galactosidase activity decreased fivefold in the presence of TMAO, whereas plasmids pΔSD723A or pΔSH850Q did not affect the activity of the fusion (Fig. 4). The negative effect of the D1-H2 region on the expression of the tor operon is thus more apparent in a torS wild-type context than in a torS constitutive strain. This difference probably originates from the fact that the phosphorylation capacity of TorS726 is higher than that of the wild-type TorS protein (compare the β-galactosidase activities in the absence of any plasmid in Fig. 3 and 4). In contrast, the dephosphorylation capacity of the D1-H2 domains probably remains constant in both strains. In conclusion, the D1-H2 region of TorS can efficiently dephosphorylate TorR-P in vivo, and this activity requires both phosphorylation sites of the D1-H2 domains.

**Dephosphorylation of response regulators by reverse phosphotransfer in phosphorelay systems: a general mechanism?**

The data presented here together with our previous findings (15) strongly suggest that the TorS sensor catalyzes both the phosphorylation and dephosphorylation of its cognate response regulator, TorR. Under inducing conditions, TorS transphosphorylates TorR via a four-step phosphorelay in which His443, Asp723, and His850 of TorS are sequentially

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FIG. 3. Effect of plasmid-borne TorS derivatives on expression of the torA-lacZ chromosomal fusion of strain LCB726 penB. Cells were grown aerobically on minimal medium supplemented with glucose (0.2%). No TMAO was added to the growth medium since LCB726 penB carries a TMAO-constitutive chromosomal mutation (torS726) (16). The construction of the pS plasmid series has been described previously (15), whereas that of the pΔS plasmid series is described in the text. The representation of each TorS allele encoded by the plasmids is shown. To avoid artifactual effects due to overproduction of the plasmid-borne TorS alleles, a penB mutation was transduced into strain LCB726 to lower the plasmid copy number (18). Moreover, as the plasmidic torS alleles are under the control of the leaky tac promoter, only 0.005 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was used for the pS-plasmid series and no IPTG was added for the pΔS plasmid series. β-Galactosidase activities were measured on whole cells by the method of Miller (21); values represent the averages of at least three determinations with a variation of no more than 15% from the mean.
phosphorylated and phospho-His850 is the phosphodonor site for TorR. When TMAO is removed, TorR behaves as a phosphatase for TorR-P, but this activity requires only His850 and Asp723. As in vitro studies have clearly shown that certain unorthodox sensors can dephosphorylate their cognate response regulators by reverse phosphotransfer (8), we propose a similar pathway from TorR D53 to TorS H850 and then TorS D723 (Fig. 1).

In several phosphorelay signal transduction systems, the H1-D1 domains and the H2 domain are found on distinct proteins (3, 27, 29). Interestingly, in Vibrio harveyi, the receiver (D1) domain of hybrid (H1-D1) sensor LuxN is again involved in the dephosphorylation of response regulator LuxO (6). This additional example reinforces the idea that reverse phosphotransfer is a strategy used by response regulator to the receiver of the hybrid or unorthodox sensor partner is a general mechanism of phosphorelay systems. A striking feature in at least several of these systems is that the half-lives of the response regulators are very long, whereas those of the phospho-receivers are quite short (8, 13, 34). These findings could explain the reason for the existence of reverse phosphorylases. Indeed, as the response regulator cannot dephosphorylate easily by itself, the reverse phosphotransfer allows the transfer of the phospho-group to a receiver domain from which the phosphate can readily be released. Finally, our kinetic analysis revealed that, at least for the TorS-TorR system, the proposed reverse phosphotransfer is both efficient and rapid, since expression of the target genes was shut off in less than 2 min after stimulus removal. Therefore, in our model, based on our results and on analogy to other phosphorelay signal transduction systems, reverse phosphorelay is a general and powerful mechanism to dephosphorylate stable phospho-response regulators.

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