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Ser/Thr phosphorylation as a regulatory mechanism in bacteria

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Abstract

This review will discuss some recent work describing the role of Ser/Thr phosphorylation as a post-translational mechanism of regulation in bacteria. I will discuss the interaction between bacterial eukaryotic-like Ser/Thr kinases (eSTKs) and two-component systems as well as hints as to physiological function of eSTKs and their cognate eukaryotic-like phosphatases (eSTPs). In particular, I will highlight the role of eSTKs and eSTPs in the regulation of peptidoglycan metabolism and protein synthesis. In addition, I will discuss how data from phosphoproteomic surveys suggests that Ser/Thr phosphorylation plays a much more significant physiological role than would be predicted simply based on *in vivo* and *in vitro* analyses of individual kinases.

Regulatory phosphorylation in bacteria

Ser/Thr phosphorylation is the major mechanism of regulatory phosphorylation in eukaryotes. This mechanism was long considered the exclusive province of eukaryotes, despite that in 1969, one year after the initial observation of Ser/Thr kinases in eukaryotic cells, a cAMP-dependent Ser/Thr kinase was described in *Escherichia coli* [1]. Although the endogenous substrate of this kinase was not identified, *E. coli* isocitrate dehydrogenase was the first example of a protein phosphorylated on a Ser or a Thr residue in bacteria [2]. However, the kinase responsible for this modification lacked sequence homology with eukaryotic kinases, suggesting that different classes of enzymes mediated Ser/Thr phosphorylation in bacteria and eukaryotes. This view was challenged by the identification of a Ser/Thr kinase in *Myxococcus xanthus* with significant sequence homology to the catalytic domain of eukaryotic Ser/Thr kinases [3]. These so-called eukaryotic-like Ser/Thr kinases (eSTKs) exhibited remarkable structural homology with their eukaryotic counterparts [4]. At first, only relatively few bacterial species were shown to contain eSTKs, but whole-genome sequencing led to a virtual explosion in the diversity of bacteria containing predicted eSTKs and metagenomic approaches indicate that eSTKs are ubiquitous [5]. This diversity supports the hypothesis that eSTKs are the evolutionary predecessors of eukaryotic Ser/Thr kinases [6,7].

Two-component systems (TCS) are the predominant mechanism of regulatory phosphorylation in bacteria. TCS are composed of a Histidine kinase (HK), often a membrane protein, which responds to a ligand or a signal and undergoes autophosphorylation on a histidine residue. One activated, the HK transphosphorylates a response regulator, often a transcription factor, on an aspartate residue (Fig. 1A; [8]). This modification is relatively labile and a specific phosphatase is not necessary to remove it, although HKs can exhibit phosphatase activity. In contrast, Ser/Thr phosphorylations are stable, requiring active dephosphorylation to facilitate reversible regulation. Consistently, bacteria contain Ser/Thr phosphatases (eSTPs) with significant structural homology to eukaryotic PP2C-type Ser/Thr phosphatases that dephosphorylate eSTK substrates (Fig. 1B; [4]).

In eukaryotes, the many Ser/Thr kinases often function in large interacting networks, acting sequentially within a single signaling cascade as well as between different cascades [9]. Although eSTKs are much less abundant in bacteria, there is recent evidence that eSTKs also interact with each other and with tyrosine kinases [10]. In *Mycobacterium tuberculosis*, eSTKs interact functionally [11], thus forming a complex signaling architecture [12] that also appears to exist in the cyanobacterium *Synechococcus* sp. strain PCC 7002 [13]. In addition, as described in detail below, there are many examples of interactions between eSTKs and two-component systems.

Ser/Thr phosphoproteomic analysis

Many phosphoproteomic analyses of phylogenetically diverse bacteria have been published over the past decade [14]. While these studies reported numerous Ser/Thr phosphorylated proteins (>50), including a few important proteins such as FtsZ, DivIVA and EF-Tu, there is relatively little overlap in identified protein targets between bacteria. Surprisingly, this is even true for conserved proteins and closely related bacteria (*e.g.*, *Listeria monocytogenes* and *Bacillus subtilis* [15]). Whether this discrepancy reflects actual biological differences is unclear, as the phosphorylation of most of the reported substrates has not been confirmed *in vitro* or *in vivo*.

Recently, comparative phosphoproteomic studies have been used to gain insight into the physiological function of Ser/Thr phosphorylation. One strategy is to compare the phosphoproteome of a single organism under different physiological conditions. For example, a phosphoproteomic analysis of *E. coli* in different growth phases demonstrated intriguing dynamics in the phosphorylation of particular proteins [16]. Specifically, phosphorylation of EF-Tu and EF-Ts increased in stationary phase, suggesting that modifications of these key regulators of translation might be connected to the overall decreased rate of protein synthesis under these conditions. A similar, although more limited, analysis was recently reported in *S. aureus* [17]. A second strategy is to compare strains carrying deletions in specific eSTKs or eSTPs. For example, the differential phosphoproteomes of *B. subtilis* strains carrying mutations in the PASTA-domain-containing eSTK PrkC or its cognate eSTP PrpC were used to identify likely PrkC targets [18]. Although this approach cannot be applied to essential eSTKs like *M. tuberculosis* PknB, a similar strategy was used to obtain a phosphoproteomic analysis of a *M.*

tuberculosis strain lacking the eSTK PrkE [19]. Finally, in the future, innovative and more efficient methods such as high-throughput IMAC-based phosphoprotein enrichment [20], will greatly facilitate the generation of differential phosphoproteomes.

Interaction with Two-component Systems

The TCS HK produces changes in gene expression via the phosphorylation of the response regulator that directly binds to promoters of target genes. The linear structure of this signaling cascade facilitates rapid and specific changes in gene expression. eSTKs also have effects on global gene expression (*e.g.*, *S. aureus* Stk [21]). However, eSTK/eSTP systems do not include an explicit transcription factor, thus raising the question of how these transcriptional effects are mediated. One possibility is direct phosphorylation of non-TCS DNA-binding proteins and several examples are discussed below (**Other eSTK substrates)**. Another possibility is that the eSTK interacts with a TCS, as has been described in an excellent historical survey of these interactions [22]. Here, we will focus on more recent studies concerning the Ser/Thr phosphorylation of TCS response regulators (Fig. 2), although TCS histidine kinases have also been shown to be eSTK targets [23].

The CovRS TCS virulence regulators from Groups A and B streptococci influence global gene expression and pathogenesis. Changes in the expression of some of the same genes are observed in cells lacking the eSTK Stk, suggesting cross-talk between the systems. Consistent with this prediction, Stk phosphorylates Group B CovR on Thr-65, inhibiting DNA binding of CovR [24]. While Stk also phosphorylates Group A CovR on Thr-65, this modification has less of an effect on DNA binding than observed with Group B CovR. However, this modification does negatively influence CovR phosphorylation on Asp-53 by CovS [25]. Since Asp-53 phosphorylation is critical for dimer formation, and consequently for DNA binding, this suggests a model where Stk regulates CovR-dependent gene expression by interfering with CovR dimerization and subsequently DNA binding.

The *S. aureus* eSTK Stk1 phosphorylates regulators in two different cascades: the GraR response regulator in the GraSR TCS, and VraR regulator in the three-component VraTSR signal transduction system. GraSR mediates resistance to cationic antimicrobial peptides and vancomycin, probably through regulation of cell wall metabolism [26]. Stk1 phosphorylates GraR on several Thr residues in the DNA-binding domain, affecting the expression of the *dltABCD* operon [27] that encodes proteins responsible for incorporating D-ala into wall teichoic acids. The VraTSR three-component signal transduction system modulates the expression of genes in the cell wall stress regulon in response to a number of different cell wall active antibiotics. Stk1 phosphorylates VraR on multiple Thr residues, negatively affecting its DNA-binding properties [28]. Since Stk1 has a muropeptide-binding extracellular domain, these modifications connect cell wall sensing with the expression of genes that mediate peptidoglycan metabolism and antibiotic resistance.

Role in cell wall metabolism

As with eukaryotic kinases, identifying the activating ligand of an eSTK has provided useful hints for elucidating its physiological role. Perhaps the clearest example of this kind of insight has come from studying eSTKs that have PASTA (**P**eptidoglycan **a**nd **S**er/**T**hr kinase

associated) repeats in their extracellular domain. These eSTKs were hypothesized to sense cell wall related processes and subsequently mediate the regulation of cell-wall biosynthesis [29,30]. Subsequently, two studies confirmed the outline of this hypothesis. First, the *B. subtilis* PASTA-containing eSTK PrkC was shown to be required for the response of both dormant spores [31,32] and growing cells [33] to muropeptides. Second, metabolomic studies revealed that *S. aureus* strains lacking either the PASTA-containing eSTK PknB or its cognate phosphatase Stp exhibited significant changes in the levels of cell wall precursor biosynthesis [34]. Finally, biophysical and structural studies of the interaction of PASTA domains with muropeptides (reviewed in [35]) indicate that there is a direct and specific interaction between a single amino acid in the PASTA domain and the 3rd residue (*m*-DAP in the case of *B. subtilis*) of the peptidoglycan stem peptide [36].

Recent work has been directed at understanding how PASTA-containing eSTKs regulate peptidoglycan metabolism. A particular focus has been septal peptidoglycan synthesis since PASTA-containing eSTKs localize to the septum (*e.g.*, *S. pneumoniae* StkP [37]). This localization is dependent on the PASTA domain (*e.g.*, StkP [38] and *M. tuberculosis* PknB [39]) and is consistent both with the interaction of the PASTA domain with the stem peptide and with the ability of antibiotics that inhibit the last stages of peptidoglycan synthesis to delocalize StkP [38]. Presumably, availability of ligand affects not only localization but also kinase activity.

The identification of specific PASTA-containing eSTK substrates has been a prominent goal of recent efforts. For example, *S. pneumoniae* StkP phosphorylates the cell division protein DivIVA on Thr-221 [40] and cells expressing a phosphoablative mutant (DivIVA-T221A) exhibit changes in shape and patterns of peptidoglycan synthesis that are phenotypically similar to a Δ*stkP* strain. However, these changes are not precisely the same, suggesting that additional StkP substrates may be important [38]. Possibilities include penicillin-binding proteins (PBPs), since StkP is present in a large complex consisting of several PBPs [41], or GpsB, another conserved cell division protein required for the ability of StkP to phosphorylate DivIVA [42]. StkP also phosphorylates MapZ, a protein that is essential for the appropriate position of the Z-ring [43]. I*n vitro*, both StkP and *C. glutamicum* PknA phosphorylate MurC, the MurNAc:L-alanine ligase, suggesting that phosphorylation may be a conserved regulatory mechanism for Lipid II synthesis [44,45]. Interestingly, a *S. aureus* strain lacking PknB exhibit significantly reduced levels of the precursor UDP-MurNAc-Ala, suggesting that PknB is positively regulating MurC [34]. *B. subtilis* PASTA-containing eSTK PrkC phosphorylation of YvcK, a protein of unknown function, is necessary for proper PBP1 localization and function [46].

The apparent role of eSTKs in the regulation of peptidoglycan synthesis may have implications for the sensitivity of bacteria to inhibitors of this process. For example, inhibition of the *L. monocytogenes* PASTA-containing eSTK kinase PrkA increases β-lactam sensitivity [47]. This finding suggests that a possible strategy to increase the efficacy of β-lactam antibiotics is pharmacologic targeting of these kinases. Some PASTA-containing eSTKs are essential and thus have been the subject to intensive study as antibiotic targets (*e.g.*, *M. tuberculosis* PknB). Even in bacteria containing non-essential homologs, deletion mutations lead to severe distortions in cell shape. These changes, which

may permit viability under laboratory conditions, could result in a competitive disadvantage in the environment.

Role in protein synthesis

Other recently identified eSTK substrates lack an obvious link to cell wall metabolism but are instead involved in protein synthesis. The very abundant GTPase Elongation Factor Tu (EF-Tu) is observed to be phosphorylated in nearly all published phosphoproteomic studies. EF-Tu delivers the aminoacyl-tRNA to the ribosome during protein synthesis. *M. tuberculosis* PknB phosphorylates EF-Tu on several sites [48] thereby inhibiting its interaction with GTP. In addition, the eSTK *B. subtilis* YabT phosphorylates EF-Tu on a conserved Thr residue in the GTP binding pocket, thereby ablating the GTPase activity (Pereira, Gonzalez and Dworkin, in revision). The kinase YabT is only expressed in sporulation and this modification occurs with the maturing spore, suggesting that it plays a key role in entry into metabolic dormancy. A second example is *B. subtilis* CpgA, a ribosome-associated GTPase which is phosphorylated on Thr-166 by *B. subtilis* PrkC, thereby inhibiting its GTPase activity [49]. A final example is phosphorylation of the glutamyl-tRNA synthetase GltX by the *E. coli* Ser/Thr kinase HipA [50,51]. Mutations in *hipA* affect the frequency of persisters, bacteria that are phenotypically resistant to antibiotics, suggesting that the GltX phosphorylation is important for this very interesting phenotype.

Modification of transcription factors

In addition to the phosphorylation of TCS response regulators, eSTKs can directly phosphorylate other types of transcription factors. One example is *S. aureus* CcpA, a highly conserved regulator of carbon catabolite repression. CcpA is phosphorylated by the eSTK Stk1 on two Thr residues in the DNA binding domain, inhibiting CcpA DNA binding *in vitro*. *In vivo*, replacement of these residues with either phosphoablative or phosphomimetic residues results in changes in gene expression [52]. A second example is *B. subtilis* AbrB, an transition phase transcriptional regulator, which is phosphorylated on a single Ser residue, consequently impairing DNA binding and binding-induced cooperativity [53]. *In vivo*, expression of mutant AbrB proteins containing either phosphoablative or phosphomimetic substitutions leads to deregulation of many AbrB-target genes [53]. Finally, the *M. tuberculosis* eSTK PknD phosphorylates an anti-anti-sigma factor protein in response to changes in osmolarity, resulting in the activation of a sigma factor responsible for the transcription of osmotically regulated genes [54].

eSTKs can also indirectly affect DNA structure and thereby regulate gene expression. For example, *M. tuberculosis* HupB, a bacterial histone-like protein is phosphorylated *in vivo* and *in vitro* by several eSTKs including PknB, PknE and PknF [55]. Proteins of this family have large global effects on transcription and are thought to play a role in the overall down-regulation of transcription as they accumulate in stationary phase or dormancy. Since phosphorylation inhibits DNA binding, phosphorylation of HupB specifically during exponential phase would limit its interaction with DNA and ensure that its effects are

limited to stationary phase. In addition, the *B. subtilis* eSTK YabT phosphorylates the DNA recombinase RecA on Ser-2, with an effect on RecA foci formation [56].

Ser/Thr Phosphatases (eSTPs)

Ser/Thr phosphorylations are stable, so active dephosphorylation by eSTPs is necessary to facilitate reversible regulation. Genetic analysis of strains lacking eSTPs suggests that they have important physiological effects. A key question is how much these effects are due to direct dephosphorylation of the eSTK and how much results from substrate dephosphorylation. That is, *in vitro*, eSTPs often can directly dephosphorylate both the eSTK and the eSTK substrate, but *in vivo*, the relative contribution of each reaction to overall phosphorylation of the substrate is unclear. Interestingly, although many bacteria contain paired eSTKs and eSTPs that are co-transcribed, there are usually far fewer eSTPs than eSTKs in any given genome. For example, *M. tuberculosis* has 11 eSTKs but only one eSTP, suggesting that there is substantial promiscuity in the eSTP/eSTK interactions

Unlike their eukaryotic counterparts, eSTPs lack obvious regulatory domain(s). *In vitro*, the *M. tuberculosis* eSTP PstP is phosphorylated on several residues by the eSTKs PknA and PknB and these modifications appear to increase its activity [57]. However, these residues are not conserved in homologous eSTPs. The physiological role of eSTPs is less clear than for eSTKs, although there are numerous recent reports suggesting that they impact sensitivity to cell wall targeting antibiotics. For example, *E. faecalis* strains lacking the eSTP IreP have greatly increased resistance to cephalosporin [58], and *S. aureus* strains lacking the eSTP PP2C exhibit reduced susceptibility to vancomycin and daptomycin [59]. These changes may reflect alterations in the cell wall, and consistently, *E. faecium* StpA, a close homolog of IreP and PP2C plays an important role in peptidoglycan crosslinking [60]. One issue interpreting physiological studies of phosphatase mutants is the difficulty in disentangling the direct effect of dephosphorylation of a single substrate from indirect, presumably more global effects, resulting from dephosphorylation of the eSTK.

Conclusions

Phosphoproteomic surveys as well as the diversity of identified substrates suggest that eSTKs and eSTPs mediate a broad range of physiological responses. However, there are two fundamental questions raised by these studies. First, why is there so little overlap between the phosphoproteomes of relatively closely related bacteria? Second, what are the biologically significant substrates? This is perhaps easiest to answer in the case of PASTA-containing eSTKs, since the ligand is known, but for other eSTKs, we will likely be unable to answer this question until the ligand/stimulus is identified. The question of biological significance also touches on an issue that remains largely unresolved in comparatively well-studied eukaryotic systems: what is the actual *in vivo* stoichiometry of phosphorylated to unphosphorylated protein? For example, since phosphorylation can cause a protein to act as a dominant negative, a low stoichiometry may be sufficient to have physiological consequences. And, finally, are there kinases hidden in the dark matter of genomes? That is, given the low sequence homology of some eSTKs, often restricted to the key catalytic residues, are there as yet unidentified kinases among the un-annotated genes? Despite all

these unanswered questions and caveats, it is nonetheless clear that Ser/Thr phosphorylation is an important mechanism of signaling in bacteria.

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- Ser/Thr phosphorylation is involved in the regulation of cell wall synthesis.
- Ser/Thr kinases phosphorylate elements of two-component systems
- Ser/Thr kinases phosphorylate other known transcriptional regulatory mechanisms

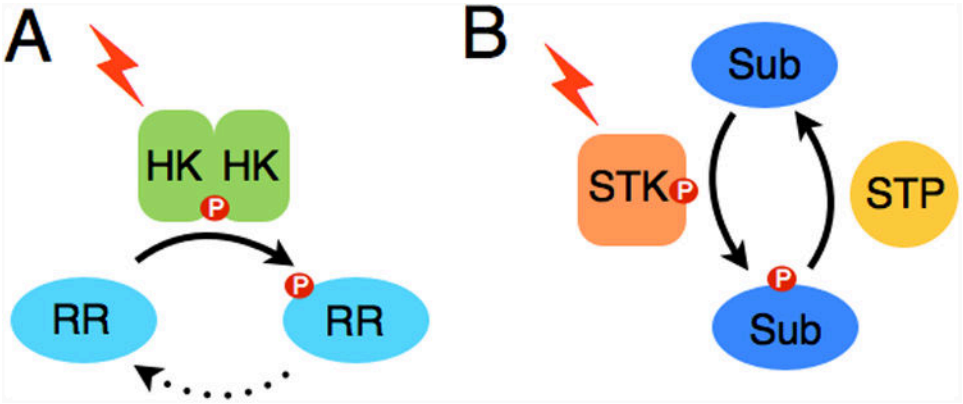

Fig. 1. Regulatory phosphorylation in bacteria

A. In Two-component signaling, a Histidine kinase (HK; green) autophosphorylates in response to a signal (↗) and transphosphorylates a response regulator (RR; blue) on an Asp residue. **B.** In Ser/Thr kinase signaling, the kinase (STK; orange) undergoes autophosphorylation, presumably in response to a signal (↗) and transphosphorylates a substrate (Sub; blue) on Ser/Thr residue(s). A phosphatase (STP; yellow) removes these stable modifications.

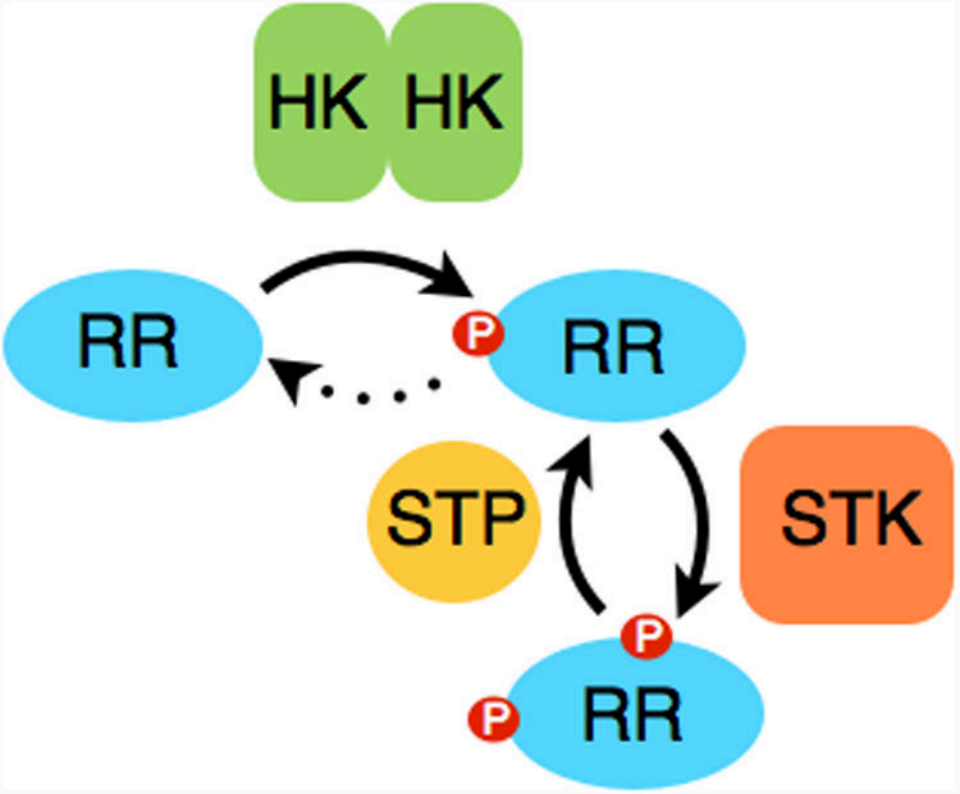

Fig. 2. Interaction between TCS and eSTK/eSTP systems

The response regulator (RR; blue) can be phosphorylated by the Histidine kinase (HK; green) on an Asp residue and then by a Ser/Thr kinase (STK; orange) on Ser/Thr residue(s). The Ser/Thr modifications can be reversed by a Ser/Thr phosphatase (STP; yellow).