

Review

Bacterial Phosphoproteomic Analysis Reveals the Correlation Between Protein Phosphorylation and Bacterial Pathogenicity

Ruiguang Ge* and Weiran Shan

Laboratory of Integrative Biosciences, College of Life Sciences, Sun Yat-Sen University, Guangzhou 510275, China.

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Abstract

Increasing evidence shows that protein phosphorylation on serine, threonine and tyrosine residues is a major regulatory post-translational modification in the bacteria. This review focuses on the implications of bacterial phosphoproteome in bacterial pathogenicity and highlights recent development of methods in phosphoproteomics and the connectivity of the phosphorylation networks. Recent technical developments in the high accuracy mass spectrometry have dramatically transformed proteomics and made it possible the characterization of a few exhaustive site-specific bacterial phosphoproteomes. The high abundance of tyrosine phosphorylations in a few bacterial phosphoproteomes suggests their roles in the pathogenicity, especially in the case of pathogen–host interactions; the high abundance of multi-phosphorylation sites in bacterial phosphoprotein is a compensation of the relatively small phosphorylation size and an indicator of the delicate regulation of protein functions.

Key words: protein phosphorylation, bacterium, pathogenicity, phosphoproteomics

Introduction

Post-translational modifications are essential for the rapid and reversible modification of the physicochemical properties of a protein, resulting in the changes of enzyme activity, oligomerization state, protein–protein interaction, subcellular localization or half-life (1). Protein phosphorylation is the most abundant and biologically the most important post-translational modification on the tyrosine, serine and threonine residues, and is catalyzed reversibly by specific protein kinases and phosphatases. Bacteria and some plants rely on histidine autophosphorylation of the sensory kinases and aspartate phosphorylation

of the response regulators (thus two-component systems) (2). Protein phosphorylation is perhaps the best studied due to the close association between dys-regulated phosphorylation and human pathologies (3). Therefore, it is extremely important to determine the degree and the site of the *in vivo* protein phosphorylation. However, due to the very low stoichiometry, limited dynamic range, high complexity and quantitative difficulties of protein phosphorylations, highly selective enrichment procedures and sensitive mass spectrometry (MS) are required to decipher the phosphoproteome (4). Selective phosphopeptide enrichment has been accomplished in several ways by using anti-phosphotyrosine antibodies, immobilized metal affinity chromatography (IMAC), chemical modifications or strong cation exchange chromatography (5). The seamless combination of IMAC and nano-liquid chromatography en-

*Corresponding author.

E-mail: gerg@mail.sysu.edu.cn

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ables reproducible separation and identification of phosphopeptides in a low-femtomole range (6, 7), and thus it is the most frequently used method in the study of cellular phosphorylation.

For some time, protein phosphorylation was considered to exist exclusively in eukaryotes until the first observation of protein phosphorylation occurring in *Escherichia coli* (8, 9). Indeed, protein phosphorylation is fundamental to the regulation of all kinds of physiological processes for the bacteria, especially for several key steps in the infection process, such as adhesion to the host, triggering and regulation of pathogenic functions as well as biochemical warfare, scrambling the host signaling cascades and impairing its defense mechanisms (10, 11). The global phosphoproteome has been established in a number of bacteria (Table 1), including *Corynebacterium glutamicum* (12), *Campylobacter jejuni* (13), *Bacillus subtilis* (14-16), *E. coli* (17), *Latococcus lactis* (18), *Streptococcus pneumoniae* (19), *Klebsiella pneumoniae* (20), *Mycoplasma pneumoniae* (21), *Pseudomonas* species (22), *Mycobacterium tuberculosis* (23), *Streptomyces coelicolor* (24) and *Helicobacter pylori* (13, 25). Phosphorylation in the bacteria was biased toward threonine compared with serine, while serine phosphorylation may account for 80%-90% of total phosphorylation sites in the eukaryotes (26). *L. lactis*, *C. jejuni*, and *S. coelicolor* contain more phosphorylation sites of threonine than serine (Table 1).

The most abundant subset of phosphorylated pro-

teins is the enzymes involved in the central carbon/protein/nucleotide metabolism, and some other phosphorylated housekeeping proteins are helicases, chaperones, ribosomal proteins and amino acyl tRNA-synthetases (27). One quantitative phosphoproteomic analysis on the model bacterium *B. subtilis* has been performed with stable isotope labeling by amino acids in cell culture (SILAC) (28). The striking distinction between the known metazoan and bacterial phosphoproteomes are the extent: 30%-50% of proteins are phosphorylated in humans (29, 30), whereas it is at least one order of magnitude lower in bacteria (27). The only notable exception is *M. tuberculosis*, whose yield was significantly high: 516 phosphorylation sites in 301 phosphoproteins, accounting for >7% of *M. tuberculosis* proteins (23). Differential roles were proposed for the protein phosphorylations in eukaryotes and prokaryotes: protein phosphorylation in the eukaryotes is extensively used for transduction of signals inter- and intra-cellularly, whereas the function may be less central in the prokaryotes (16). Bacterial phosphorylation sites can be conferred by two major search algorithms NetPhosBac (31) and Diphos (32), which were summarized in a recent review paper (27). Here we will focus on an overview of the recent advances in the field of bacterial phosphoproteome, highlighting recent methods in phosphoproteomics, connectivity of the phosphorylation networks, as well as the correlation between pathological potentials and the known bacterial phosphoproteomes.

Table 1 The bacterial phosphoproteomes identified so far

Bacterium*	No. of phosphopeptides	No. of phospho sites	% of serine	% of threonine	% of tyrosine
<i>Escherichia coli</i> (17)	105	81	67.9	23.5	8.6
<i>Bacillus subtilis</i> (16)	103	78	69.2	20.5	10.3
<i>Latococcus lactis</i> (18)	102	79	46.5	50.6	2.7
<i>Pseudomonas putida</i> (22)	56	53	52.8	39.6	7.5
<i>Pseudomonas aeruginosa</i> (22)	57	55	52.7	32.7	14.5
<i>Campylobacter jejuni</i> (13)	58	35	30.3	72.7	9.1
<i>Streptococcus pneumoniae</i> (19)	102	163	47.2	43.8	9.0
<i>Streptomyces coelicolor</i> (24)	44	44	34.1	52.3	13.6
<i>Klebsiella pneumoniae</i> (20)	117	93	31.2	15.1	25.8
<i>Mycoplasma pneumoniae</i> (21)	15	15	53.3	46.7	0
<i>Helicobacter pylori</i> (25)	80	124	42.8	38.7	18.5

*Bacterial name followed by the reference in brackets.

Methods in Phosphoproteomic Analysis

The major challenges in phosphoproteomics are the generally very low stoichiometry and high complexity of phosphorylation, limited dynamic range of detection methods available, and quantitative difficulties (4). As for the complexity, the phosphosite database (www.phosphosite.org) lists over 99,000 non-redundant phosphorylation sites up to August 2011, and this number is expanding continually. The conservation of the phosphorylation sites in the bacterial phosphoproteome is extremely low, even in the case of proteins phosphorylated in most bacterial species, such as the enzymes of the central carbon metabolism (27), which suggests the diverse kinase specificities to adapt to different environmental niches. The dynamic range of the phosphoproteome is quite large ($\sim 10^9$), from hundreds of millions to a few copies per cell. Therefore, it is mission impossible to detect all the phosphopeptides upon proteolytic digestion of the whole cell lysates or tissue samples. Selective enrichment of the phosphoproteins/peptides is required and has been accomplished in several ways: anti-phosphotyrosine

antibodies (33), IMAC (34, 35), chemical modifications (36, 37) and strong cation exchange chromatography (SCX) (38). **Figure 1** summarizes several of the most significant work over the past decade (5, 39). For a global view of serine, threonine and tyrosine phosphorylations, IMAC may be the best choice (6), with peptide recovery up to 90% as determined by ^{32}P or ^{33}P -radioactivity measurements (40). Phosphopeptides are retained on nitriloacetic acid (NTA) or iminodiacetic acid (IDA)-linked hard metal ions, such as Fe^{3+} , Ga^{3+} , Al^{3+} (39) and Zr^{4+} (41), through their negatively charged phosphate group. Non-specific binding of peptides containing Glu and Asp could be minimized either by carefully controlling experimental conditions or through methyl-esterification of carboxylic acids by HCl-saturated dry methanol (42). Recently, titanium dioxide (TiO_2) chromatography has emerged as the most common method for the enrichment of global phosphoproteins/peptides (43). This technique requires shorter preparation time and has increased capacity compared to IMAC resins. Advances in the high accuracy MS allow for the identification of thousands of phosphorylation sites in a

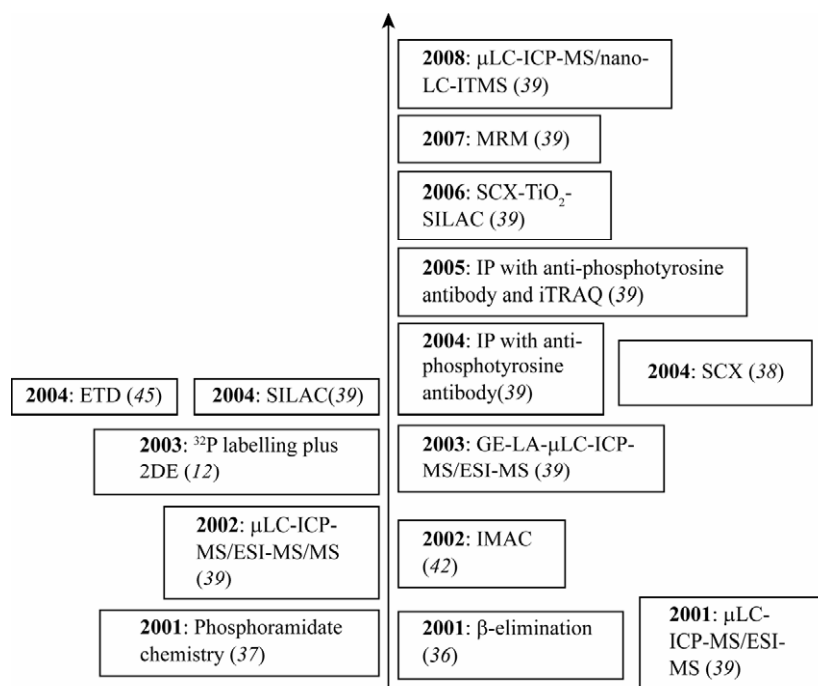


Figure 1 Timeline in phosphoproteomics with chosen milestones based on the implementation of a new method during the last decade. Abbreviations used are: μLC , micro-liquid chromatography; ICP, inductively coupled plasma; ITMS, ion trap mass spectrometer; MRM, multiple reaction monitoring; SCX, strong cation exchange chromatography; SILAC, stable isotope labeling by amino acids in cell culture; IP, immunoprecipitation; iTRAQ, isobaric tag for relative and absolute quantitation; ETD, electron-transfer dissociation; 2DE, two-dimensional gel electrophoresis; GE, gel electrophoresis; LA, laser ablation; ESI, electrospray ionization; IMAC, immobilized metal affinity chromatography.

single experiment (44). Both IMAC and TiO₂ columns can be coupled seamlessly with MS for reproducible separation, detection and identification of phosphopeptides in a low-femtomole range (7, 45-47). Upon the identification of the phosphoproteome, bioinformatics analysis through Gene Ontology (GO) annotation (48) or the bacterial localization prediction tool pSORTb (49) is often applied to get the relevant information of cellular function, localization and protein-protein interaction network (50).

Tyrosine Phosphorylation

As a Gram-negative, spiral-shaped bacterium that colonizes in the gastric mucosa of humans (51), *H. pylori* has been frequently associated with atrophic gastritis, peptic ulcer disease, functional dyspepsia and gastric carcinomas (52). Cytotoxin-associated antigen A (CagA) is a major pathogenicity protein in *H. pylori* and plays key roles in inducing gastric inflammation, ulcer and carcinogenesis. CagA protein is translocated from the bacterium, undergoes tyrosine phosphorylation at the Glu-Pro-Ile-Tyr-Ala (EPIYA) motif in the host cells and induces a cellular hummingbird phenotype of transformation (53). In addition, non-phosphorylated CagA interacts with host proteins, such as epithelial tight junction-scaffolding protein zonulin (ZO-1), cell adhesion protein E-cadherin, hepatocyte growth factor receptor c-Met, cadherin-associated protein β -catenin, adaptor protein GRB-2 and the kinase PAR1, leading to a loss of cell polarity and inducing pro-inflammatory and mitogenic responses (54). Besides the knowledge about CagA phosphorylation in the host cells, there is no information available about the *in vivo* phosphorylation state of *H. pylori* intracellular proteins. Bioinformatics indicated that the genome of *H. pylori* strain 26695 contains at least one protein kinase (HP0432) and one PPM-family protein phosphatase (HP0431) (55). Up to now, three independent studies intended to discover the intracellular phosphorylation in *H. pylori*: (1) eight proteins phosphorylated at serine residues through SDS-PAGE and autoradiography (56); (2) 57 proteins identified through Fe³⁺-IMAC enrichment, 2D gel electrophoresis and MALDI-TOF MS analysis, albeit without further phosphorylation site mapping

(13); and (3) 82 phosphopeptides from 67 proteins with 79 class I (with localization probability higher than 0.75) phosphorylation sites: 33 (42.8%) on serine, 31 (38.7%) on threonine and 15 (18.5%) on tyrosine (25).

Tyrosine phosphorylation has generally been regarded as an exclusively eukaryotic phenomenon and plays roles in multicellularity, and metazoans have a higher proportion of tyrosine phosphorylation than unicellular eukaryotes (57). This is based on the virtual absence of tyrosine phosphorylation in unicellular eukaryotes, such as yeasts. Most bacterial phosphorylation sites are on serine (70%) and threonine (20%), while tyrosine phosphorylation sites account for less than 10%. A noteworthy feature of *H. pylori* phosphoproteome is the significantly high overall abundance (~18.5%) of tyrosine phosphorylation. There are three bacterial phosphoproteomes with comparable tyrosine phosphorylation percentage: *S. coelicolor*, 13.6% (24); *P. aeruginosa*, 14.5% (22); and *K. pneumoniae*, 25.8% (20); while most other bacterial model organisms with known phosphoproteomes have less than 10% phosphorylation sites on tyrosine (Table 1). The level of tyrosine phosphorylation seems to be positively correlated with the pathogenicity. *P. aeruginosa* phosphoproteome has a much higher tyrosine phosphorylation level (14.5%) compared with the non-pathogenic *P. putida* species (7.5%) (22). In fact, various findings have supported the contribution of tyrosine phosphorylation to the bacterial pathogenicity, such as pedestal formation (Tir of enteropathogenic *E. coli* and *Citrobacter*) (58), cell elongation, scattering and inflammation (CagA of *Helicobacter*) (53, 59), capsular polysaccharide biosynthesis (20), cell invasion (Tarp of *Chlamydia*) (60), pro-inflammatory responses and cell proliferation (BepD-F of *Bartonella*) (61).

The association has been established between protein tyrosine phosphorylation and the control of surface polysaccharide production or transport (62), as well as between protein serine/threonine phosphorylation and cell envelope biosynthesis (63). Surface polysaccharides are believed to be involved in the early steps of the infection process and are considered potent virulent factors (64). Some proteins from various species are homologous to a family of enzymes involved in exopolysaccharide synthesis grouped to-

gether as BY-kinases (Bacterial tYrosine kinases) and show similar auto-phosphorylation activities (65, 66). BY-kinases, albeit only a few copies per bacterial genome, are widespread in bacteria (66) and are usually encoded by genes in the large operons involved in biosynthesis and export of sugar polymers (67). For instance, the tyrosine kinase Wzc of *E. coli* is essential for the synthesis of the exopolysaccharide colonic acid and the assembly of group 1 capsular polysaccharide (62). It is essential for the pathogenicity of *S. pneumoniae* to produce capsular polysaccharide (CPS). The CPS biosynthesis proteins CpsB, CpsC and CpsD regulate CPS production via tyrosine phosphorylation of CpsD, a homologue of Wzc. CpsC is required for CpsD tyrosine phosphorylation (68). The mutations in some key elements of CpsC produced wild-type levels of CPS, but were unable to cause bacteremia in mice upon intranasal challenge (69). It seems that the relatively high abundance of tyrosine phosphorylation in the phosphoproteomes of several pathogenic bacteria reflects in some way the pathogenicity of the bacteria, such as in the direct pathogen–host interactions.

Multiple Phosphorylation Sites

In eukaryotic cells, multiple phosphorylated proteins are common and serve as molecular switches for signal fine-tuning. For example, five phosphorylation sites are present in the eukaryotic initiation factor (eIF) 2B catalyzed by four different protein kinases (70): two conserved C-terminus serine sites are phosphorylated by casein kinase 2 and required for the *in vivo* interaction of eIF2B ϵ with eIF2 and *in vitro* eIF2B activity; the third site (Ser539) is required for the recruitment of the glycogen synthase kinase 3 (GSK3) to the fourth site (Ser535); the fifth site is phosphorylated by casein kinase 1 and lies out of the catalytic domain of eIF2B ϵ . One distinctive feature of *H. pylori* phosphoproteome is the high occurrence of multiple phosphorylation sites: 35 out of the identified 84 phosphopeptides contain at least two phosphorylation sites. One example is the peptide SAKANDASEITA LLNTIAYETISTLSK from the enzyme of alanine racemase, which has six phosphorylation sites. This phenomenon has been noted for proteins accumulated when the bacteria were under stress or with over-

loaded proteolytic systems (71), and was also reported in the characterization of the phosphoproteome of the Gram-positive pathogenic bacterium *S. pneumoniae* (19) as well as the non-pathogenic *L. lactis* (18). These observations indicated that one protein may be phosphorylated on multiple sites to fulfill differential roles or to function together to achieve delicate micro-regulations of the pathogenicity mechanisms, such as adhesion to the host, stimulation and regulation of pathogenic functions and impairing the host defense mechanisms (10). Multiple phosphorylation sites may have similar biological implications, at least for the species of *L. lactis* (18) and *H. pylori*: both *L. lactis* and *H. pylori* have smaller genome sizes, simpler transcriptional machinery and fewer (two and three, respectively) sigma factors compared with other model bacteria such as *E. coli*, suggesting that more regulations are needed through delicate post-translational modifications.

Protein–Protein Interaction Network of Phosphorylated Proteins

Protein–protein interaction map with the involvement of phosphoproteins is important to understand the regulatory mechanisms of post-translational modifications in the bacteria. As shown in **Figure 2**, the phosphoprotein interaction map of *H. pylori* consisted of a large network covering 163 proteins, among which there are 28 identified phosphoproteins (25). The major *H. pylori* pathogenicity factor VacA is centered on this interaction map. The possible partners for VacA in the A category include an outer membrane porin protein HopL, a hypothetical protein HP0699, a predicted ABC transporter HP1464 and a predicted ATPase/DNA transfer protein VirB4_5. VacA is secreted from *H. pylori* through the syringe-like VirB/VirD4-like type IV secretion apparatus. It is noteworthy that VirB4_5 is also identified to be phosphorylated, implicating the involvement of phosphorylation in the regulation of VacA secretion through the bacterial membrane. The two partner proteins GroEL and FlgB were determined to be phosphorylated and were predicted to interact directly with each other (25). The *in vivo* interaction between GroEL and FlgB was previously observed in

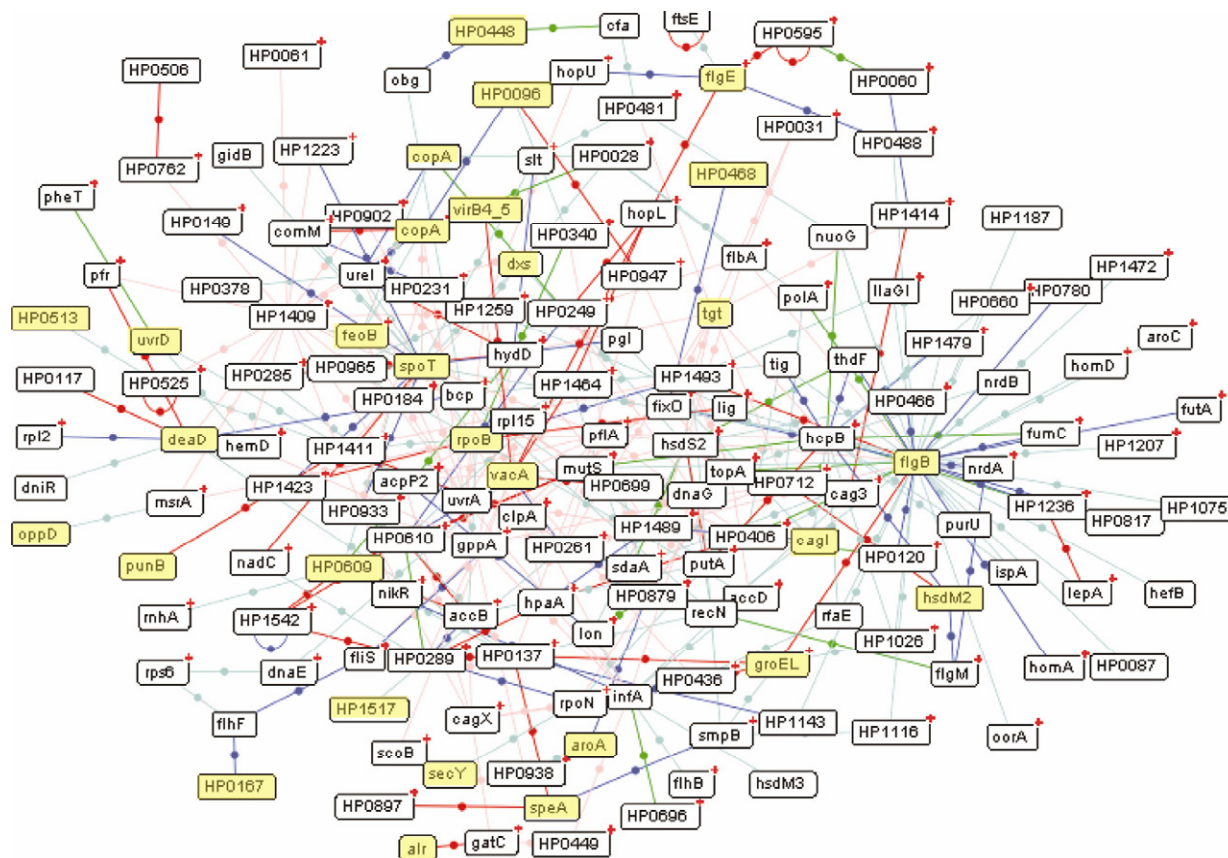


Figure 2 Protein-protein interaction network of the identified phosphoproteins in *H. pylori* constructed with PIMRider software (<http://pim.hybrigenics.com>) (76). Proteins in yellow represent the identified phosphoproteins. Interactions with different levels of reliability are assigned with different colors in the following sequence: red>blue>green>cyan>pink. A small red cross “+” is drawn on the top right of the protein whenever a protein contains partners that are not currently displayed within the map. The figure is reprinted with permission from Ref. 25.

B. subtilis (72), albeit without any assignment of the *in vivo* function for such interaction. *H. pylori* produces flagella to fulfill its requirement for the bacterial colonization in the human gastric mucosa. As a member in the HSP60 family, *H. pylori* GroEL has been shown to increase the risk of gastric carcinoma (73). Previous studies demonstrated that the phosphorylation and dephosphorylation of GroEL regulated its binding and dissociation from unfolded proteins (74), possibly through the switch between the oligomeric states mediated by phosphorylation (75). Further experimental investigations are needed to reveal the contribution of the GroEL and FlgB partners to the pathology of the bacterium with regards to how the phosphorylation of these two proteins regulates the bacterial colonization. Phosphoprotein interaction network through bioinformatics or experimental determinations provides some hints for the connectivity

of the phosphorylation networks and the correlation between pathological potentials and the known bacterial phosphoproteomes.

Conclusion

Phosphorylation is biologically the most important post-translational modification. It is essential to determine the degree and the site of the *in vivo* protein phosphorylation in order to understand the underlying mechanisms between dys-regulation of phosphorylation and human pathologies. Highly selective enrichment procedures and sensitive MS are required to decipher the phosphoproteome due to the very low stoichiometry, limited dynamic range, high complexity and quantitative difficulties of protein phosphorylations. Recent technical developments made it possi-

ble to uncover some whole-cell bacterial phosphoproteomes, which revealed the correlation between bacterial pathological potentials and phosphorylations: the high abundance of tyrosine phosphorylations in a few bacterial phosphoproteomes indicates their roles in the pathogenicity, especially in the case of pathogen–host interactions; the high abundance of multi-phosphorylation sites in bacterial phosphoprotein is a compensation of the relatively small phosphorylation size and an indicator of the delicate regulation of protein functions. With further development in the MS-based techniques, more information will be obtained about bacterial phosphoproteomes and the correlation between bacterial phosphorylations and potential pathogenicity. This will help us to develop protein phosphorylation-targeted prodrugs in the control of bacterial infections.

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