


Importance of protein Ser/Thr/Tyr phosphorylation for bacterial pathogenesis

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Protein phosphorylation regulates a large variety of biological processes in all living cells. In pathogenic bacteria, the study of serine, threonine, and tyrosine (Ser/Thr/Tyr) phosphorylation has shed light on the course of infectious diseases, from adherence to host cells to pathogen virulence, replication, and persistence. Mass spectrometry (MS)-based phosphoproteomics has provided global maps of Ser/Thr/Tyr phosphosites in bacterial pathogens. Despite recent developments, a quantitative and dynamic view of phosphorylation events that occur during bacterial pathogenesis is currently lacking. Temporal, spatial, and subpopulation resolution of phosphorylation data is required to identify key regulatory nodes underlying bacterial pathogenesis. Herein, we discuss how technological improvements in sample handling, MS instrumentation, data processing, and machine learning should improve bacterial phosphoproteomic datasets and the information extracted from them. Such information is expected to significantly extend the current knowledge of Ser/Thr/Tyr phosphorylation in pathogenic bacteria and should ultimately contribute to the design of novel strategies to combat bacterial infections.

Keywords: antibiotic kinase inhibitors; antibiotic resistance; BY-kinases; Hanks kinases; host–pathogen interactions; machine learning; omics integration; pathogenic bacteria; phosphoproteomics; Ser/Thr/Tyr phosphorylation

Abbreviations

BY-kinase, bacterial tyrosine kinase; CPS, capsular polysaccharide; DDA, data-dependent acquisition; DIA, data-independent acquisition; EHEC, enterohemorrhagic *Escherichia coli*; FHA, forkhead-associated; IMAC, immobilized metal affinity chromatography; LMW, low molecular weight; MRSA, methicillin-resistant *S. aureus*; MS, mass spectrometry; PASTA, penicillin binding protein and Ser/Thr kinase-associated; PTM, post-translational modification; PTP, protein Tyr-specific phosphatase; SH2, Src homology 2; SILAC, stable isotope labeling of amino acids in cell culture; T3SS, type III secretion system; T6SS, type VI secretion system; TCS, two-component system; TMT, tandem mass tag.

Major take-home messages from this review

- Serine, threonine, and tyrosine (Ser/Thr/Tyr) phosphorylation plays wide-ranging roles in bacterial pathogens affecting both basic physiology and pathogen-specific processes.
- Recent advances in bacterial phosphoproteomics have significantly increased our insight into the width of pathogen Ser/Thr/Tyr phosphorylation substrates.
- Further improvements in sample preparation, MS instrumentation, and data acquisition and analysis should enable time-resolved and spatially resolved analyses of both bacterial and host cell phosphoproteomes during *in vivo* infections.
- Automated methods to predict the functional implications of bacterial phosphosites should be developed.
- Omics integration and machine-learning methods will help to obtain a systems-level understanding of the phosphorylation networks driving bacterial pathogenesis.

Protein phosphorylation on serine (Ser), threonine (Thr), and tyrosine (Tyr) residues dynamically regulates the cellular activities in bacteria, archaea, and eukarya. By modulating the conformation and macromolecular interactions of proteins, phosphate attachment can change the activity, stability, or localization of proteins to guide cellular signaling. Although most thoroughly studied in eukarya, where it is the most common type of post-translational modification (PTM) [1], Ser/Thr/Tyr phosphorylation is increasingly recognized for playing wide-ranging and essential roles in the control of bacterial life, including the process of pathogenesis [2–5]. Other phosphorylation systems in bacteria include the phosphorylation of histidines and aspartates [such as in two-component systems (TCSs)] and, less commonly, of arginines and cysteines, with considerable crosstalk occurring between the different modification systems [6]. The phosphorylation reaction is catalyzed by distinct types of residue-specific kinases and is reversed by cognate phosphatases to terminate signaling events. Most bacterial Ser/Thr-specific kinases belong to the evolutionary conserved Hanks-type family, which are also present in eukaryotes [7–9]. In addition, bacteria contain a small group of atypical Ser/Thr kinases [10,11]. Most Tyr-directed kinases in bacteria do not, in contrast to eukarya, belong to the Hanks family, but are bacteria-specific and referred to as bacterial tyrosine kinases (BY-kinases) [10,12,13].

The catalytic domain of BY-kinases is characterized by the presence of Walker A, A' and B ATP/GTP-binding motifs [12,13]. All BY-kinases, as well as many Hanks kinases, are transmembrane or membrane-associated proteins that can relay signals from the extracellular environment to the cell interior [9,12,13]. Many Hanks kinases contain repeats of so-called penicillin binding protein and Ser/Thr kinase-associated (PASTA) domains in their extracellular portion that bind cell wall fragments [14,15]. Table 1 provides an overview of Ser/Thr- and Tyr-specific kinases identified in a selection of commonly studied pathogenic bacteria mentioned in this review [9,11,13,16–19]. Comparatively, much less is known about bacterial phosphatases, which are also fewer in number. The ones described to date include the phosphoprotein and metal-dependent phosphatases (PPPs and PPMs) acting on Ser/Thr residues, and the conventional and low molecular weight (LMW) protein Tyr-specific phosphatases (PTPs) [9,20]. Notably, there are also examples of kinases and phosphatases displaying dual amino acid specificity [5,21–23]. Detailed descriptions of bacterial kinases and phosphatases and their modes of regulation can be found in other reviews [9,10,12,13,24].

Bacterial pathogens are defined as any type of bacteria able to cause infection in a host organism. The infection process follows a sequence of events including pathogen host entry, immune evasion, replication, dissemination, and persistence. Literature on how bacterial Ser/Thr/Tyr phosphorylation is implicated in each of these steps is accumulating. We therefore start this review by presenting a selection of these functional studies. The current rise in antibiotic drug resistance poses an ever-increasing threat to human health and urgently calls for new therapeutic opportunities. As future therapies may involve targeted inhibition of the pathogenic signaling networks that sustain bacterial survival in the human host, research aiming at obtaining in-depth knowledge of bacterial phosphorylation systems should be prioritized. However, we are still far from having a complete picture of how phosphorylation networks contribute to pathogenesis. Mass spectrometry (MS)-based phosphoproteomics has become a key technology for obtaining global and systematic views of phosphorylation networks in biological systems. This review therefore places particular focus on the use of phosphoproteomics in the study of pathogenic bacteria. We provide a comprehensive overview of phosphoproteomic studies that have recorded phosphorylation sites at a global scale in different types of pathogens and describe the use of phosphoproteomics to understand kinase–substrate relationships and host–

pathogen interactions. Successively, a discussion on current and future technical advancements that should move the field forward with the ultimate goal of obtaining deep and quantitative views of the phosphorylation networks supporting bacterial infections is provided. Furthermore, based on insights obtained from eukaryal phosphorylation, we describe how protein structural and evolutionary information can facilitate qualified predictions of phosphosite functions. We then generally outline how machine learning can help reveal regulatory patterns embedded in complex phosphoproteomic datasets, and how 'omics' data integration should allow for a systems-level analysis of infectious disease. Finally, we discuss how knowledge of protein phosphorylation can be exploited in the design of new antibacterial therapies.

Control of bacterial pathogenesis by Ser/Thr/Tyr phosphorylation

It is becoming increasingly clear that protein phosphorylation has the potential to regulate most types of cellular processes, and hence, it is not surprising that phosphorylation also acts as an important regulator in the context of pathogenesis. To this attest, it has been shown for diverse types of pathogens that inactivation of specific kinases reduces or attenuates their virulence in animal models [14,25–33]. Kinase-dependent *in vivo* virulence has, for example, been well documented for species of *Streptococci* using different infection models. In *Streptococcus agalactiae* (group B streptococci), mutation of the PASTA-domain-containing Hanks kinase Stk1 diminishes virulence in a neonatal rat model of sepsis [29], and mutation of the homologous

kinase of *Streptococcus pneumoniae*, StkP, decreases the bacterial burden in the lungs and blood of intranasally or intravenously infected mice [30]. Furthermore, in *Streptococcus pyogenes* (group A streptococci) Stk1 has been found essential for disease development in a mouse myositis model of infection [31]. Kinase inactivation has also been seen to reduce the initial colonization of intestinal pathogens [19,34]. As shown in a hamster infection model, deletion of the Hanks kinase PrkC of *Clostridium difficile* leads to a delay in gut colonization, although it has no significant effect on overall virulence [34]. In some cases, kinase-mediated virulence may depend on the bacterial strain or the infection model. While deletion of the *Staphylococcus aureus* Stk1 Hanks kinase (also known as PknB) in the SH100 strain background causes a significant reduction of virulence in a mouse model of pyelonephritis [32], deletion of Stk1 in the Newman strain has no effect in a septicemia disease model [35]. What is more, a methicillin-resistant *S. aureus* (MRSA) USA300 Δ stk1 strain was actually seen to cause a more severe infection in a cutaneous mouse model compared to the USA300 wild-type strain [36]. Consistent with the dynamic nature of phosphorylation being important for cell function, phosphatase abrogation also frequently affects *in vivo* infection outcomes [22,35,37–39]. This includes the secreted LMW PtpA Tyr phosphatase of *S. aureus*, which enhances bacterial survival within macrophages and promotes mouse liver infectivity [38]. Similarly, the also secretory LMW but dual-specificity phosphatase SP-PTP of *S. pyogenes* is required for normal adherence to human lung cells and *in vivo* virulence in mice. Consistently, SP-PTP overexpression causes hypervirulence [22].

Table 1. Table listing reported Ser/Thr and Tyr kinases identified in a selection of commonly studied pathogenic bacteria. Additional uncharacterized kinases are likely to exist in some of these species. ND, no data.

Bacterium	Ser/Thr kinases	Tyr kinases	References
<i>Mycobacterium tuberculosis</i>	PknA; PknB; PknD; PknE; PknF; PknG; PknH; PknI; PknJ; PknK; PknL	PtkA	[9,13]
<i>Streptococcus pneumoniae</i>	StkP	CpsD; Ubk (only an autokinase?)	[9,13,16]
<i>Staphylococcus aureus</i>	Stk1 (PknB)	CapB	[9,13,32]
<i>Mycoplasma pneumoniae</i>	PrkC	ND	[9]
<i>Listeria monocytogenes</i>	PrkA	ND	[9]
<i>Escherichia coli</i>	HipA; YeaG; YihE (SrkA); Stk	Etk; Wzc	[8,9,11,13,17]
<i>Yersinia</i> spp.	YpkA (YopO)	ND	[9]
<i>Pseudomonas aeruginosa</i>	PpkA	WaaP	[9,13,26]
<i>Salmonella typhimurium</i>	SteC; RdoA	PutA	[11,13,162]
<i>Klebsiella pneumoniae</i>	KpnK	Wzc	[13,18]
<i>Enterococcus faecalis</i>	IreK (PrkC)	ND	[19]
<i>Clostridium difficile</i>	PrkC; CD2148	ND	[34]

The above-mentioned studies clearly demonstrate the importance of Ser/Thr/Tyr kinases and phosphatases for bacterial virulence. Yet the specific mechanisms by which these enzymes contribute to pathogenicity remain to be systematically elucidated. Individual kinases and phosphatases generally have multiple protein substrates operating in different cellular pathways. Thus, phosphorylation-based regulation is exerted on many levels of pathogen function and must be understood in a context- and species-specific manner. The literature on phosphorylation in the regulation of bacterial physiology and pathogenicity is vast and it is beyond the scope of this review to cover current knowledge in an all-encompassing manner. In this section, we nonetheless seek to illustrate the diversity by which Ser/Thr/Tyr phosphorylation can influence bacterial infections via the control of some critical pathogen functions.

Pathogen cell division and cell wall biosynthesis

Most bacteria harbor a peptidoglycan-based cell wall, which acts to protect the bacterium from lysis in its differing environments. Both cell elongation and the septum formed during cell division require peptidoglycan synthesis, making cell wall growth and division highly interlinked processes. Protein phosphorylation, especially by membrane-associated Hanks-type kinases with PASTA repeats, plays significant roles in regulating these processes. For instance, the Hanks kinase StkP is a key regulator of cell division in *S. pneumoniae* and controls the balance between elongated cell growth and septation [40]. In an *stkP* mutant, cell wall synthesis is perturbed resulting in elongated cells [40–42]. During cell division, StkP, together with its cognate phosphatase PhpP, localizes to the septum and regulates a number of cell division proteins [40]. One of these is DivIVA, and DivIVA phosphorylation negatively regulates cell elongation and promotes cell division [41,43]. StkP can also regulate cell division at the direct level of cell wall peptidoglycan synthesis. At the division site, the peptidoglycan synthase PBP2a forms a complex with the membrane-anchored cofactor MacP. MacP is in turn phosphorylated by StkP, leading to PBP2a activation [44]. A recent study aimed at dissecting the roles of the StkP PASTA repeats during *S. pneumoniae* cell division showed that while the three membrane-proximal repeats are required for kinase activation and septal cell wall thickness, the membrane-distal repeat ensures proper localization of StkP at the division site and effective cell separation [45]. Interestingly, StkP activation was seen not to depend on PASTA peptidoglycan binding and thus contrasts

the generally accepted view of PASTA-dependent kinase activation [9,45].

The *S. aureus* Stk1 Hanks kinase is also involved in the regulation of cell wall metabolism and division [32]. The cell wall precursor molecule Lipid II has been reported as the activating signal for Stk1, and binding to lipidII variants induces Stk1 autophosphorylation *in vitro* [46]. *In vivo*, Stk1 localizes to the septum following recruitment of the contractile Z-ring forming protein FtsZ. Phosphorylation reduces FtsZ GTPase activity to possibly modulate Z-ring dynamics. In addition, Stk1 phosphorylates the response regulator WalR, which controls the expression of a number of proteins involved in cell wall remodeling [46].

In *Mycobacterium tuberculosis*, we also find a PASTA-domain-containing Hanks kinase, PknB, involved in regulating cell wall metabolism. Here, a key PknB substrate is the peptidoglycan synthase CwlM [47,48]. In its phosphorylated form, CwlM interacts with the forkhead-associated (FHA)-domain protein FhaA in the cytosol to presumably control peptidoglycan synthesis. Conversely, nonphosphorylated CwlM localizes to the cell membrane and interacts with the lipid flippase MurJ important for peptidoglycan precursor export (Fig. 1A) [48]. Interestingly, in the related, but mostly nonpathogenic *Mycobacterium smegmatis*, phosphorylation of CwlM quickly diminishes when cells are starved, suggesting that CwlM phosphorylation acts to adjust peptidoglycan synthesis according to nutrient status [47].

Virulence gene expression

Pathogens produce a variety of virulence factors that enable them to colonize host cells and tissues and survive inside their host. Kinase or phosphatase inactivation often significantly alters bacterial gene expression profiles, including expression levels of virulence determinants [31,36,37,39,49–51]. These phenotypes are due to both direct and indirect effects of kinases and phosphatases on gene transcription. Inactivation of kinases or phosphatases may affect the transcription of genes regulated by direct kinase/phosphatase targets, but potentially also, indirectly, of genes part of the same gene regulatory networks. A common way by which phosphorylation directly regulates virulence gene expression is by modulating the DNA-binding properties of transcriptional regulators [52]. This is the case for the highly conserved catabolite control protein and virulence regulator CcpA in *S. aureus*. CcpA gets phosphorylated on two threonine residues in its DNA-binding domain by Stk1. By abrogating CcpA DNA binding, this phosphorylation event prevents the

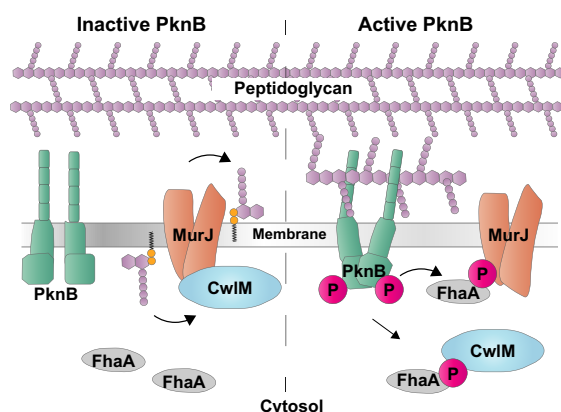
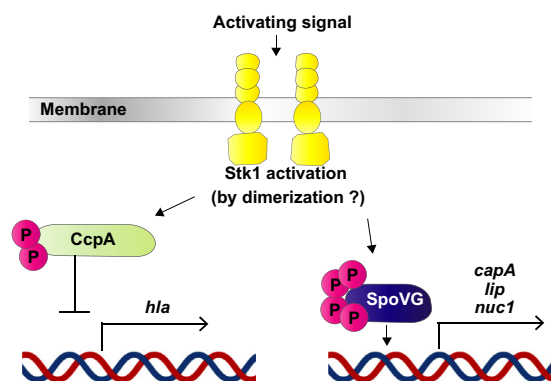
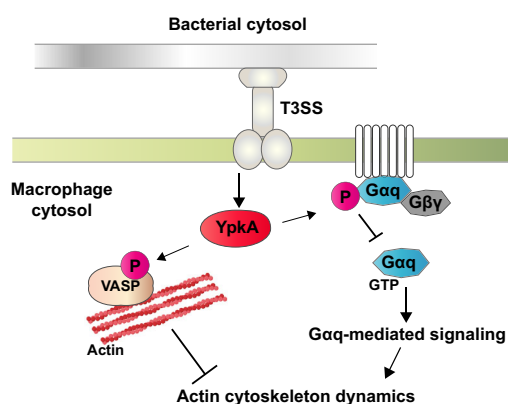
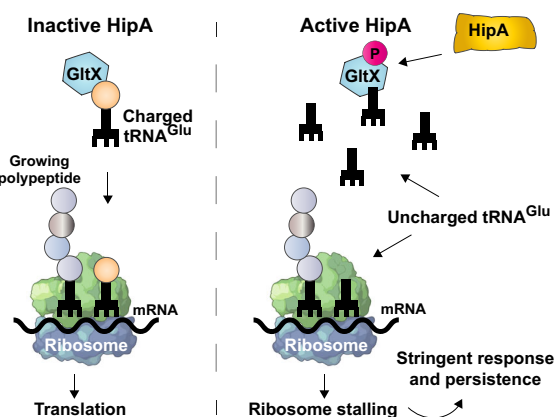
A *M. tuberculosis***B** *S. aureus***C** *Yersinia***D** *E. coli*

Fig. 1. Schematic illustrations of protein phosphorylation in the control of pathogen life cycle processes such as cell wall biosynthesis (A), virulence gene expression (B), host signaling interference (C) and host persistence (D). (A) *Mycobacterium tuberculosis* cell wall synthesis is controlled by the Hanks-type PASTA kinase PknB via phosphorylation of the peptidoglycan synthase CwlM. Left: Nonphosphorylated CwlM is localized at the cell membrane where it interacts with the lipid flippase MurJ, responsible for transporting peptidoglycan precursors across the membrane. Right: An accumulation of un-cross-linked extracellular peptidoglycan is sensed by the PknB PASTA repeats and leads to PknB activation. PknB in turn phosphorylates CwlM as well as MurJ, stimulating their interaction with the FHA-domain protein FhaA implicated in peptidoglycan biosynthesis [48]. (B) *Staphylococcus aureus* Stk1 regulates the expression of virulence genes via the phosphorylation of transcriptional regulators. Left: Stk1-mediated phosphorylation of the catabolite control protein CcpA within its DNA-binding domain prevents CcpA binding to its target DNA sequence and expression of the cytotoxic α -hemolysin protein (encoded by the *hla* gene) [53]. Right: Stk1 also phosphorylates the stage V sporulation protein SpoVG, which acts as a transcriptional regulator of virulence genes such as *capA*, *lip*, and *nuc1*. SpoVG gets phosphorylated on four N-terminal threonines, and in contrast to the situation for CcpA, SpoVG phosphorylation stimulates DNA promoter binding and hence virulence gene expression [54]. (C) A secreted *Yersinia* Hanks kinase obstructs host cell signaling. *Yersinia* bacteria secrete the kinase YpkA into host macrophage cells via its T3SS. Once inside, YpkA is able to phosphorylate host proteins such as the heterotrimeric G α q protein and the VASP. G α q is a G-protein-coupled receptor subunit and is phosphorylated by YpkA within its highly conserved diphosphate binding domain. This phosphorylation event inhibits G α q GTP binding and consequently G α q signaling functions such as actin cytoskeleton regulation [84]. Similarly, YpkA-mediated phosphorylation of VASP prevents VASP-driven actin polymerization. These effects on cytoskeleton dynamics impair effective macrophage phagocytosis [85]. (D) The HipA kinase promotes *Escherichia coli* host persistence via the glutamyl-tRNA synthetase GltX. When HipA is inactive, GltX exists in a nonphosphorylated and active state to promote protein translation. However, GltX phosphorylation by active HipA inhibits GltX catalytic activity, leading to an accumulation of uncharged tRNA^{Glu}, ribosome stalling, and ultimately, cell growth inhibition [109].

expression of the critical virulence protein α -hemolysin, a cytotoxin able to induce host cell death [53]. In contrast, Stk1-mediated phosphorylation of another *S. aureus* transcriptional regulator, SpoVG, enhances SpoVG binding to target promoters of virulence genes such as *lip* (lipase), *nuc1* (thermonuclease 1), and *capA* (capsular polysaccharide (CPS) biosynthesis protein; Fig. 1B) [54]. Exemplifying crosstalk between different phosphorylation-dependent signaling systems, *S. aureus* Stk1 is also seen to regulate the TCS response regulator GraR implicated in virulence and antibiotic resistance [55]. GraR phosphorylation increases its DNA-binding activity to proposedly induce expression of genes involved in the modulation of cell wall charge via wall teichoic acid (WTA) D-alanine incorporation. This is believed to have a global effect on *S. aureus* pathogenicity since WTA charge is important for diverse processes such as cell growth, host infection and attachment, biofilm development, and drug resistance [55]. Similar phosphorylation system crosstalk is seen in *Streptococci* between the CovR/CovS (control of virulence) TCS and the Stk1 Hanks kinase. In *S. agalactiae* and in *S. pyogenes*, the CovR transcriptional regulator can be phosphorylated on Thr65 by Stk1 [39,56,57]. CovR acts mainly as a repressor of virulence genes, and phosphorylation of CovR on Asp53 stimulates its DNA binding. On the other hand, a phospho-mimetic CovR-Thr65Glu variant reduces Asp53 phosphorylation and consequently CovR promoter regulation [56,57]. Consistently, in *S. pyogenes*, the phospho-silenced CovR-Asp53Ala and the phospho-mimetic CovR-Thr65Glu strains were seen to display similar profiles of gene repression to a CovR-deleted strain. Interestingly, compared to $\Delta covR$, CovR-Asp53Ala- and CovR-Thr65Glu-expressing strains were still able to positively influence the expression of some CovR-activated virulence genes, and in accordance caused hypervirulence in a mouse infection model [57]. Similarly, *in vivo* hypervirulence as well as increased blood–brain barrier penetration is seen for *S. agalactiae* CovR-Asp53Ala-, CovR-Thr65Glu-, and CovR-deleted strains [58]. Mechanistic insight into Hanks kinase and TCS crosstalk relevant to bacterial pathogenicity was also recently provided for *S. pneumoniae* [59]. During host invasion, *S. pneumoniae* has to adapt to local fluctuations in pH in both extra- and intracellular compartments. In response to acidic stress, *S. pneumoniae* may undergo acidic stress-induced lysis (ASIL), where virulence factors such as pneumolysin are released and cause tissue damage [60]. The response regulator ComE controls whether pro-survival or lytic responses are initiated at low pH [59,61]. More specifically, Piñas *et al.* demonstrated

that ComE phosphorylation on Thr128 by the StkP Hanks kinase stimulates ComE DNA binding, induction of genes involved in H₂O₂ production and cell lysis [59]. In contrast, StkP-deleted and ComE-Thr128Ala mutant strains were blocked in ASIL and displayed increased survival in pneumocytes [59].

Finally, an example of transcription factor regulation via Tyr phosphorylation has been elucidated in enterohemorrhagic *Escherichia coli* (EHEC). Tyr phosphorylation of the sugar-sensing transcription regulator Cra reduces its DNA binding and leads to downregulation of type III secretion system (T3SS) expression under glycolytic conditions, a condition where T3SS is usually not needed [62]. Recent phosphoproteomic studies have identified multiple transcriptional and post-transcriptional regulators phosphorylated on tyrosine residues in species such as *E. coli* and *Shigella flexneri* [63,64], and future characterization of these sites should allow us to uncover how Tyr phosphorylation affects virulence gene expression.

Regulation of pathogen exopolysaccharides

Besides governing virulence gene expression, Ser/Thr/Tyr phosphorylation regulates pathogen host invasion and survival through multiple other means. These include the regulation of cell surface capsular and non-capsular exopolysaccharides to facilitate host adherence, biofilm formation, immune system avoidance, and antibiotic resistance. Particularly, BY-kinases are recognized for their role in exopolysaccharide production and export [12,13]. A well-studied example is the Wzc BY-kinase of *E. coli*, which acts together with its cognate phosphatase Wzb in the synthesis of group 1 capsular and colonic acid polysaccharides [65–67]. The current model proposes that the cycling of Wzc autophosphorylation and Wzb-mediated dephosphorylation drives proper exopolysaccharide assembly [67]. Although the exact mechanism by which this influences exopolysaccharide production is not known, crystallographic studies suggest that Wzc phosphorylation and dephosphorylation cause Wzc to exist in mono- and oligomeric states, respectively [68]. Similarly, the other *E. coli* BY-kinase–phosphatase pair Etk–Etp promotes group 4 capsule formation in enteropathogenic strains [69]. In addition, Wzc and Etk can both phosphorylate the same tyrosine residue on the UDP-glucose dehydrogenase Ugd involved in the synthesis of exopolysaccharide precursors [70,71]. Whereas Ugd phosphorylation by Wzc regulates colanic acid production, Ugd phosphorylation by Etk is important for resistance to the antibiotic polymyxin [71]. In the

context of CPS synthesis, crosstalk between Tyr and Ser/Thr phosphorylation has interestingly been seen to coordinate capsular and cell wall synthesis in *S. aureus* by regulating the distribution of precursor molecules common to the two pathways [72]. While the CapA1B1 BY-kinase complex activates CPS synthesis via phosphorylation of the biosynthetic enzymes CapO [73], CapE, and CapM, the Ser/Thr kinase Stk1 promotes peptidoglycan synthesis and reduces CPS production by inhibiting CapA1B1 kinase and CapM glycosyltransferase activities [72]. BY-kinases are also able to coordinate capsule formation with cell division [74]. In *S. pneumoniae*, autophosphorylation of the BY-kinase CpsD, which forms part of a larger CPS assembly complex, is required for proper capsule production at the septum. Furthermore, by modulating the mobility of the chromosome partitioning protein ParB, phosphorylated CpsD acts to coordinate cell constriction with the encapsulation of daughter cells [74].

Host establishment

Upon infection with bacterial pathogens, the host initiates a variety of cellular signaling events that act to stimulate innate and adaptive immune defense mechanisms and ultimately restrict pathogen dissemination. A key virulence strategy utilized by pathogens to subvert these processes and thus promote their own survival is the injection of effector molecules into host cells. Effector translocation is mediated via specialized secretion systems and these are frequently regulated by phosphorylation. Initially, the reciprocal activity of the PpkA–PppA Thr kinase/phosphatase pair was seen to control secretion from a type VI secretion system (T6SS) in *Pseudomonas aeruginosa* [75]. In the opportunistic pathogen *Vibrio alginolyticus*, T6SSs are controlled by the PpkA2 Hanks kinase. Besides direct T6SS regulation, PpkA2 controls T6SS expression via a regulatory circuit involving the quorum sensing system [76]. T3SSs are also subjected to diverse modes of Ser/Thr/Tyr phospho-regulation. During infection, enterohemorrhagic *E. coli* uses T3SS both for host attachment and for injection of effector molecules [77]. In addition to transcriptional regulation, as mentioned above [62], EHEC T3SS is regulated by the multicargo chaperone CesT. CesT contains two highly conserved tyrosine phosphorylation sites and these mediate differential effector secretion [78]. In the closely related *S. flexneri*, Tyr phosphorylation seems to be a negative modulator of virulence and a Tyr-phospho-mimetic mutant of the T3SS-associated ATPase Spa47 displays impaired effector protein discharge [64].

Secreted effector molecules promote *in vivo* survival and propagation by manipulating host signaling and immune defense mechanisms. Many bacterial effectors are actually kinases and phosphatases that target host proteins [23,79–85]. In particular, there are several examples of secreted bacterial kinases and phosphatases disrupting host cytoskeleton structures and immunity [38,82,83,86,87]. Within the *Yersinia* genus, for example, secreted Hanks kinase YpkA (YopO), disrupts cytoskeleton dynamics via phosphorylation of host heterotrimeric G α q protein [82,84] and vasodilator-stimulated phosphoprotein (VASP) [85], obstructing effective macrophage phagocytosis (Fig. 1C). In *M. tuberculosis*, the secreted Tyr-specific PtkA kinase and PtpA phosphatase, which are encoded in the same operon, are both required for macrophage intracellular growth [83,88,89]. Inside macrophages, PtpA dephosphorylates and inactivates the host vacuolar protein sorting-associated protein 33B (VPS33B). This in turn blocks the trafficking of the vacuolar-H⁺-ATPase to the mycobacterial phagosome, impeding phagosome acidification, phagosome–lysosome fusion, and ultimately bacterial elimination [90]. The molecular mechanism behind PtkA-dependent *M. tuberculosis* macrophage survival is less understood but may occur via PtpA, as PtkA-mediated phosphorylation of PtpA enhances PtpA phosphatase activity [91]. In addition, PtkA is seen to downregulate the macrophage β -galactoside binding protein Galectin 3 involved in regulating apoptosis, and macrophages infected with a Δ ptkA strain undergo decreased apoptosis compared to a wild-type *M. tuberculosis* strain [89]. The secreted phosphatase PtpA of *S. aureus* is also required for intramacrophage survival, as well as for mouse infectivity, possibly via its interaction with the human cytoskeleton-associated protein coronin-A [38]. *Streptococcus pyogenes* encodes two secretory phosphatases, that is, the Ser/Thr phosphatase SP-STP and the Ser/Thr and Tyr dual-specificity phosphatase SP-PTP, which have both been seen to induce virulence in mouse peritonitis infection models [22,39]. In addition, both are upregulated upon human pharyngeal cell internalization [92]. SP-STP induces pharyngeal cell apoptosis through both extrinsic and intrinsic host cell stimulation in a manner dependent on its phosphatase activity. In accordance, a SP-STP knockout strain induces less host cell apoptosis in the lungs of infected mice. This suggests that SP-STP interferes with multiple host signaling pathways, and human pharyngeal cells exposed to ectopically expressed SP-STP display altered levels of various pro- and anti-apoptotic proteins as well as deregulated histone phosphorylation [92]. Lastly, to be mentioned is the interference of

bacterial effectors with host innate immune responses via the NF- κ B pathway. In *Legionella pneumophila*, the LegK1 Hanks kinase is secreted via the Dot/Icm type IV secretion system into host macrophages where it activates the NF- κ B pathway by functionally mimicking host I κ B kinase (IKK) proteins [93].

In some other cases, bacterial pathogens secrete proteins that upon becoming phosphorylated by a host kinase can suppress or hijack host protein function [94–96]. More specifically, Tyr-phosphorylated bacterial effectors can recruit a diverse array of host proteins containing pTyr-binding Src homology 2 (SH2) domains and thereby disrupt diverse host processes [97]. Moreover, bacterial SH2 domains were recently identified in a large group of *Legionella* effector proteins. These were shown to bind Tyr-phosphorylated host proteins with high affinity, proposing a novel mechanism by which bacteria can interfere with host cell signaling [98].

Finally, phosphorylation can guide the metabolic adaptation of pathogens to their changing host environment. Detailed mechanistic insight has been obtained for the regulation of glutamate metabolism by the soluble mycobacterial Hanks kinase PknG [27,99–101]. Curiously, PknG phosphorylation of GarA induces an intramolecular association in GarA between its phosphorylated N terminus and its pThr-binding FHA domain, which acts as a switch to relieve the inhibitory interaction with the α -ketoglutarate decarboxylase and NAD⁺-specific glutamate dehydrogenase [99,100]. PknG activation depends on nutrient status [102]. More precisely, periplasm amino acid levels are sensed by the solute binding protein GlnH and the signal is transmitted via the transmembrane protein GlnX to cytosolic PknG [101].

Pathogen persistence

In case of chronic disease, bacteria can employ different mechanisms for persistence, of which some are based on protein phosphorylation. Biofilm formation is a well-known strategy for development of persistent infection [103]. Biofilms involve the production of an extracellular matrix that consists of a mixture of proteins, lipids, DNA, and different exopolysaccharides. As described above, exopolysaccharide production is regulated by phosphorylation in various ways.

Interestingly, in the dental pathogen *Porphyromonas gingivalis*, the BY-kinase Ptk1 and the metal-dependent tyrosine phosphatase Php1 are required for extracellular polysaccharide synthesis as well as for interspecies community formation with the crucial oral biofilm constituent *Streptococcus gordonii* [104,105].

Dual-species assemblies are facilitated by the binding of the *P. gingivalis* fimbrial adhesion protein Mfa1 to the *S. gordonii* surface SspA/B proteins, and Ptk1 induces Mfa1 expression by suppressing transcription of the negative gene regulator CdhR [104], possibly via direct CdhR phosphorylation [106]. Ptk1 is itself a substrate for both Php1 and of the LMW tyrosine phosphatase Ltp1, with the latter having a positive effect on CdhR levels and a restrictive impact on *P. gingivalis*–*S. gordonii* community development [107,108]. Another important element of persistence is cell dormancy where the stochastic establishment of subpopulations of nonreplicating bacteria can confer resistance to antibiotics targeting proliferating cells. Upon drug withdrawal, reservoirs of dormant cells can resume normal growth and re-initiate infections. In *E. coli*, phosphorylation can induce the persistence phenotype via the toxin–antitoxin module HipAB. The toxin HipA is a Ser/Thr kinase that phosphorylates and inactivates the glutamyl-tRNA synthetase GltX. This leads to an increased pool of uncharged tRNA^{Glu}, which in turn leads to activation of the stringent response and inhibition of cell growth (Fig. 1D) [109]. Dormancy is also a key feature of *M. tuberculosis* infection. After the primary infection, immune-derived granuloma structures can form in which bacteria can reside for decades. Inside the granuloma, *M. tuberculosis* faces a hypoxic and nutrition-limited environment that leads to development of dormant cells. The TCS DosSR constitutes the main regulator of dormancy and is activated in response to hypoxia [110]. Thr phosphorylation of the response regulator DosR by the PknH kinase enhances DosR DNA binding and thus expression from the dormancy regulon [111]. The *M. tuberculosis* PknG kinase is similarly important for persistence. Indeed, PknG is required for metabolic adaptation to hypoxia and effective survival during latency-like conditions, a role that is mediated via its phosphorylation of the central metabolic regulator GarA [112].

In summary, the above examples demonstrate the importance of studying Ser/Thr/Tyr phosphorylation in order to understand how pathogenic bacteria invade, survive, and persist within their hosts. As shown, bacterial kinases can modify numerous and diverse proteins important for bacterial growth and pathogenicity. The secretion of bacterial effectors that can directly interfere with host phosphorylation signaling furthermore expands the repertoire of phosphorylation-dependent mechanisms driving bacterial infections. Notably, several of the discussed functional studies were inspired from phosphoproteomic datasets, emphasizing how unbiased phosphoproteomic profiling

can be used to acquire novel insight into the regulation of bacterial pathogenesis.

Phosphoproteomics in the study of bacterial pathogenesis

Major advancements in MS-based proteomic methodologies over the last two decades have allowed researchers to obtain global and unbiased insight into protein PTMs at the site-specific level. Consequently, it has revolutionized our knowledge of proteins and their regulation. Nowadays, phosphoproteomics is a mainstream laboratory technique used to identify regulatory events in all kinds of organisms. Mainly Ser/Thr/Tyr-type phosphorylations have been extensively studied in proteomic experiments. This is due to the chemical lability of other types of phospho-bonds, which challenge their detection by typical MS-based protocols. Initially, phosphoproteomic protocols were based on the separation of proteins on 2D gels where excised phosphorylated proteins were analyzed by MALDI-TOF MS. However, this strategy is biased toward only the most abundant phosphoproteins and can usually not determine the specific modification sites. Thus, it was not until the development of gel-free methods coupled to high-accuracy MS that high-throughput and site-specific phosphoproteomic analysis became feasible. The current state and future directions of phosphoproteomics in the study of bacterial pathogens will be discussed below.

Large-scale identification of phosphosites

A typical shotgun phosphoproteomic experiment involves the digestion of total cellular proteins followed by liquid chromatography–tandem MS analysis (LC-MS/MS) analysis of sorted phosphorylated peptides. As phosphorylations are typically substoichiometric and of low abundance, phosphopeptides only represent a tiny fraction of total protein digests [113]. Therefore, their isolation from the rest of the proteome prior to MS analysis is of paramount importance for their successful detection [114,115]. The most common enrichment methods are immobilized metal affinity chromatography (IMAC) and metal oxide affinity chromatography (most often with TiO₂) [116]. Both methods rely on the affinity of negatively charged phosphate groups for metals. Strong cation exchange chromatography can furthermore be used to fractionate peptides prior to phosphoenrichment to reduce sample complexity and thereby improve phosphopeptide MS/MS identifications. Alternatively, phosphopeptides can be enriched by phospho-specific

antibodies [115]. Another important step for maximizing phosphopeptide/site coverage is related to the fragmentation patterns of phosphopeptides during MS/MS. Current fragmentation methods have recently been discussed by Potel *et al.* [117]. Phosphoproteomic protocols have primarily been developed for eukarya, for which they are routinely used to identify tens of thousands of sites [118,119]. Comparatively, bacterial phosphoproteomes are much less explored. Bacterial Ser/Thr/Tyr phosphosites are generally of a much lower abundance compared to eukaryal sites [113,120,121], making their identification more challenging. Nevertheless, since the first studies in bacterial site-specific phosphoproteomics [122], our knowledge of bacterial phosphoproteomes has greatly expanded [2,114,123]. Phosphoproteomic studies in various types of bacteria have revealed that phosphorylation affects both basic bacterial physiology, such as central metabolism and gene expression, as well as more pathogen-specific functions [63,64,124–136]. A high proportion of studies have been conducted for *M. tuberculosis*, likely reflecting the high prevalence and mortality rates of disease caused by this organism [137,138]. Together, Tables 2 and 3 provide an overview of the untargeted and gel-free Ser/Thr/Tyr phosphoproteomic studies performed in pathogenic bacteria to date. While initial studies generally identified in the range of 50–500 phosphosites from several mg of starting protein (depending on the species), significant improvements in sample preparation have more recently been reported. For example, it is seen that the removal of interfering bacterial biomolecules, such as phospholipids, peptidoglycans, and nucleic acids, prior to phosphopeptide enrichment, can greatly increase phosphosite identifications [139–142]. Potel *et al.* [140] incorporated benzonase treatment and protein precipitation by methanol/chloroform in their workflow to obtain ultrapure phosphopeptide samples from which they identified 10 times as many *E. coli* phosphosites compared to a standard protocol. Another method that enhances sample purity is the prepurification of phosphopeptides by calcium phosphate precipitation (CPP) [143]. CPP treatment significantly expanded phosphosite coverage for the nonpathogenic *Streptomyces coelicolor* [142]. Improved phosphopeptide recovery may also be obtained by using IMAC resins in a column format rather than batch or microtip enrichment [140,144]. Moreover, species-specific optimization may be required to maximize protocol efficiencies [139].

The recent acquisition of large bacterial phosphoproteomes has allowed the assessment of phosphosite sequence features, representing putative kinase

recognition motifs. Phosphorylation consensus sequences have been identified in datasets obtained from *M. tuberculosis* [51,125], *Helicobacter pylori* [145], *S. flexneri* [64], and *E. coli* [63,146,147], including bacteria-specific N- and C-terminal phosphorylation preferences [139,147]. Interestingly, a phosphoproteomic dataset from the tick-transmitted *Ehrlichia ruminantium* pathogen was enriched in recognition motifs for several eukaryal kinases, suggesting significant host cell interaction [148]. Another general theme of bacterial phosphoproteomes is that they, similar to the eukaryal ones, are dominated by pSer sites, followed by pThr and, to a lesser extent, pTyr modifications. One exception is *M. tuberculosis* where pThr sites are more frequently reported [149–151]. However, pTyr sites are likely underrepresented in phosphoproteomic datasets obtained with metal affinity chromatography as their relatively low abundance compared to pSer and pThr sites can result in their disproportional enrichment. Instead, antibody-based pTyr-peptide isolation strategies can greatly improve pTyr identifications [115] with approximately 500 and 1000 pTyr sites identified in *E. coli* and *S. flexneri*, respectively [63,64]. An alternative strategy may be to use pTyr-binding SH2-peptide domains as a pTyr affinity reagent [152]. As the collection of bacterial phosphoproteomes continues to expand, it will allow more systematic analyses of phosphosite features across different species, potentially revealing interesting differences between pathogens and nonpathogens. To facilitate comparative and functional studies of bacterial phosphorylation, phosphosite data should be easily retrievable from public databases dedicated to collecting experimentally verified phosphosites. Yet such resources are currently underdeveloped for bacteria compared to eukarya. Curated databases that do include bacterial phosphosites are PHOSIDA, dbPTM, and the bacteria-specific dbPSP [153]. These were recently reviewed by Pagano *et al.* [154].

Assaying phosphoproteome dynamics

To obtain more than mere snapshots of cellular phosphoproteomes, phosphoproteomic workflows must be coupled to quantitative methods. Only then can the dynamics of phosphorylation events, and thus their regulatory implications, be understood. Quantification can be obtained either with labeled or nonlabeled approaches. Labeling techniques can be either metabolic, such as stable isotope labeling of amino acids in cell culture (SILAC), or chemical; for example, isobaric tag for relative and absolute quantitation, tandem mass tag (TMT) or dimethyl peptide

labeling [155,156]. The disadvantages of using labeling are that only a limited number of labels can be used simultaneously and, thus, the number of samples that can be compared is restricted. However, several labeled experiments can be combined via a common sample to obtain relative quantification comparing an increased number of samples. For instance, two triple-SILAC experiments were combined to record phosphorylation dynamics during *E. coli* culture growth [121]. Importantly, quantitative methods can be used to investigate infection-related phosphorylation. Verma *et al.* [125], for example, used a TMT-based quantitative approach to identify phosphorylation events specific to *M. tuberculosis* virulence by comparing virulent and attenuated substrains. Similarly, Marcelino *et al.* [148] used label-free quantification to compare the phosphorylation patterns of virulent versus attenuated variants of *E. ruminantium* that had been grown together with bovine aortic endothelial cells. Misra *et al.* used a hypervirulent strain of *Listeria monocytogenes*, harboring an activating mutation in the virulence gene activator PrfA, to identify virulence-associated phosphorylation events. This approach found the phosphorylation of especially glycolytic enzymes to be upregulated in the hypervirulent strain compared to a nonvirulent control; findings that are in line with altered glycolytic activity of *L. monocytogenes* during intracellular growth [157]. Finally, the phosphoproteome of a subspecies of *Francisella tularensis* (the causative agent of ‘rabbit fever’) was profiled in response to KCl, which induces T6SS formation required for immune evasion. Together with functional experiments, this study was able to establish Tyr phosphorylation of a T6SS sheath component to be essential for T6SS biogenesis [158]. Quantitative phosphoproteomics can also shed light on mechanisms of antibacterial drug resistance. For *E. coli*, label-free quantification was used to compare a clinically isolated antibiotic-resistant strain with wild-type cells grown with or without antibiotics for several time periods. This uncovered numerous cases of phosphorylation events potentially driving antibiotic resistance, such as on transcription factors that regulate resistance [147]. Another study aimed to understand the phenotypic mechanisms that contribute to *M. tuberculosis* tolerance to the first-line antibiotic rifampicin. By examining phosphoproteome dynamics of the Beijing strain B0/W148 in response to high-dose rifampicin, the authors identified upregulated phosphorylation of proteins involved in iron sequestration, which is believed to be linked to antibiotic resistance and dormancy [151].

Table 2. Table listing gel-free and site-specific shotgun phosphoproteomic studies performed in pathogenic bacteria. Quantitative studies are annotated, and total numbers of identified phosphosites/phosphoproteins are provided.

Bacterium	Quantitative	Phosphosites/phosphoproteins identified	Year	Reference
<i>Acinetobacter baumannii</i> (reference and invasive multidrug-resistant strains)	No	Strain ATCC17978: 48/41 Strain Abh12O-A2: 80/70	2014	[126]
<i>Acinetobacter baumannii</i> (clinical imipenem-resistant and sensitive strains)	No	Strain SK17-S: 410/248 Strain SK17-R: 285/211	2016	[124]
<i>Escherichia coli</i>	No	81/79	2008	[189]
<i>Escherichia coli</i>	Yes (Growth phase analysis)	108	2013	[121]
<i>Escherichia coli</i>	No	766/392	2015	[139]
<i>Escherichia coli</i>	Yes (Antibiotic treatment and resistance)	2509/1133	2018	[147]
<i>Escherichia coli</i>	Yes (Growth phase analysis)	1883 sites	2018	[141]
<i>Escherichia coli</i>	No	>1600 sites	2018	[140]
<i>Escherichia coli</i>	No	512/342 (pTyr only)	2013	[63]
<i>Ehrlichia ruminantium</i>	Yes (Comparing virulent and attenuated strains)	92/58	2019	[148]
<i>Francisella novicida</i>	Yes (KCl treatment)	103 phosphopeptides	2019	[158]
<i>Helicobacter pylori</i>	No	126/67	2011	[127]
<i>Helicobacter pylori</i>	No	Strain OK145: 50 sites Strain TN2GF4: 50 sites	2019	[145]
<i>Klebsiella pneumoniae</i>	No	93/81	2009	[128]
<i>Klebsiella pneumoniae</i>	No	559/286	2015	[139]
<i>Leptospira interrogans</i>	No	27 sites	2010	[129]
<i>Listeria monocytogenes</i>	No	143/112	2011	[130]
<i>Listeria monocytogenes</i>	Yes (PrfA WT versus PrfA* activated mutant strain)	242/191	2014	[157]
<i>Mycobacterium tuberculosis</i>	No	516/301	2010	[131]
<i>Mycobacterium tuberculosis</i>	No	414/214	2015	[132]
<i>Mycobacterium tuberculosis</i>	Yes (Antibiotic treatment)	191/132	2016	[151]
<i>Mycobacterium tuberculosis</i>	Yes (Comparing virulent and attenuated strains)	512/257	2017	[125]
<i>Mycobacterium tuberculosis</i>	No	30/17 (pTyr only)	2014	[21]
<i>Pseudomonas aeruginosa</i>	No	59/28	2014	[133]
<i>Pseudomonas aeruginosa</i>	No	55 sites	2009	[134]
<i>Staphylococcus aureus</i>	No	76/108	2014	[135]
<i>Shigella flexneri</i>	No	905/573 (pTyr only)	2016	[64]
<i>Streptococcus pneumoniae</i>	No	163/84	2009	[136]

Table 3. Table listing phosphoproteomic studies aiming at identifying kinase substrates in pathogenic bacteria. The table provides information on the quantitative method used and on the numbers of potential kinase substrates as well as total phosphosites/phosphoproteins reported in each study.

Bacterium	Kinase	Quantification method	Potential substrates: sites/proteins	Phosphosites/phosphoproteins identified	References
<i>Escherichia coli</i>	HipA/ HipA7	SILAC	Not easily extractable	1183/632	[146]
<i>Mycobacterium tuberculosis</i>	PknB	TMT peptide labeling	111/73	390 phosphopeptides /258	[150]
<i>Mycobacterium tuberculosis</i>	PknB	Label-free	17/13	ND	[48]
<i>Mycobacterium tuberculosis</i>	PknA/B	Label-free	68/48	1241/470	[51]
<i>Streptococcus agalactiae</i>	Stk1	Not quantitative	10/10	ND	[160]
<i>Vibrio alginolyticus</i>	PpkA2	Not quantitative	10/8	ND	[76]

Assigning substrates to kinases

To fully understand individual phosphorylation events, as well as entire phosphorylation networks, we need to characterize their regulation by kinases and phosphatases. Currently, only very few kinase–substrate pairs have been defined. By analyzing strains with altered levels of kinase activity, quantitative phosphoproteomics can identify phosphorylation events depending on a kinase of interest. Several groups have used this strategy to elucidate the substrates of Ser/Thr kinases in pathogens (see Table 3) [76,159,160]. For example, to shed light on the mechanisms behind HipA kinase-dependent *E. coli* persistence, HipA and its variant HipA7 were overproduced from ectopic promoters and their substrates sought out by SILAC experiments [146]. Though, the success of such a strategy will depend on the mechanism of kinase activation as kinase overexpression may not by itself upregulate kinase activity if the activating signal is concomitantly low. To identify substrates of the essential *M. tuberculosis* PknB kinase, Kaur *et al.* used a hyperactive mutant version (PknB-GM). This allowed them to identify 73 potential PknB substrates hyperphosphorylated in the PknB-GM background [150]. Another strategy in the search for *M. tuberculosis* PknB substrates was applied by Turapov *et al.* They used a conditional deleterious PknB mutant that could successfully grow in a specialized osmoprotective media. Label-free quantitative comparison of PknB-producing and PknB-depleted cells detected 13 potential PknB substrates, of which the peptidoglycan amidase homolog CwlM was characterized by further analysis [48]. An alternative to genetic manipulation is the use of chemical kinase inhibitors. A small-molecule inhibitor targeting both PknA and PknB was, for example, used by Carette *et al.* [51] to uncover *M. tuberculosis* PknA/B substrates by label-free quantification. Notably, among the 68 potential substrates identified in the Carette study, only nine overlap with the Kaur *et al.* dataset, and an overlap of only one and two substrates is seen for each of these studies with Turapov *et al.*, respectively. As this is likely to reflect the fact that dissimilar experimental conditions will reveal different substrate subpools, it highlights the need for extended and multifaceted screening to fully elucidate the substrate repertoire of any given kinase. Even though the above-mentioned studies are clearly invaluable in helping to suggest kinase substrate candidates, precautions must be made in their interpretation. More specifically, one must dissect phosphorylation events directly mediated by an investigated kinase from those mediated by downstream kinases operating within the same signaling cascade. Furthermore, kinases form

highly interconnected networks and perturbation of a single kinase is therefore likely to also influence other signaling nodes [1,161]. When using chemical inhibitors, one should additionally be aware of unspecific effects occurring due to off-target inhibitor binding. Therefore, complementary cross-validation such as by *in vitro* kinase or co-immunoprecipitation assays is essential to sort out direct from indirect targets.

Host–pathogen interactions at the phosphoproteome level

Knowledge of the molecular interactions that occur between pathogens and their hosts is critical not only for understanding the course of infectious diseases but also for discovering novel targets for future antibacterial therapies. A significant amount of host–pathogen crosstalk involves their PTMs. In addition to indirect effects exerted by pathogens on host phosphoproteomes via the activation of immune and stress responses, bacteria may, as described earlier, also interfere with host phosphorylation signaling in very direct ways. They may, for example, secrete effector kinases and phosphatases able to target host proteins, or they may interact with host phosphoproteomes via a family of phospho-binding domains present in both host and some bacterial cells [79,97,98]. The use of quantitative phosphoproteomics has become instrumental for elucidating pathogen-induced phosphorylation signaling in host cells. For example, the effect of the *Salmonella* pathogenicity island 2 (SPI2) on host phosphorylation was assessed in infected macrophage and epithelial cells by SILAC experiments [162]. SPI2 encodes a T3SS responsible for secreting a high number of virulent factors, including the *Salmonella* Hanks kinase SteC. Additional quantitative phosphoproteomic profiling of SteC-transfected host cells, in combination with an MS-based SteC binding partner screen, identified the Hsp27 host protein as a direct SteC substrate through which SteC induces actin cytoskeleton remodeling to promote *Salmonella* intracellular growth [162]. Pathogen-induced phosphoproteome alterations have also been investigated for *H. pylori*-infected human gastric epithelial cells [163,164]. Notably, temporal pTyr-specific phosphoproteomics revealed that changes in host phosphorylation mainly occurred at the early stages of infection compared to a 7-h postinfection time point. To further identify host signaling pathways affected by *H. pylori* infection, a wild-type strain was compared to mutant strains deleted for components of the *cag* pathogenicity island. This approach allowed dissection of host

signaling responses depending on the entire T4SS secretion system and the T4SS effector protein Cag4, respectively. While T4SS induced JNK and p38 kinase activation, Cag4 was required for ERK1 signaling [164]. Using a similar experimental design, time-specific phosphorylation was assessed for human cells infected with either wild-type or mutant enteropathogenic *E. coli* (EPEC) deficient for the virulence-associated T3SS secretion system [165]. This screen revealed activation of host mitogen-activated protein kinase (MAPK) signaling by EPEC infection and identified various T3SS-dependent phosphorylation events associated especially with immune responses, intracellular trafficking, and cytoskeletal regulation. Importantly, this study provided novel mechanistic insight into EPEC pathogenesis by identifying T3SS-mediated phosphorylation of the septin-9 protein to be critical for efficient EPEC–host cell adherence [165]. A study on *F. tularensis* also pointed to host MAPK signaling being disturbed upon pathogen interaction. Fabrik *et al.* used SILAC-based quantification to analyze the temporal dynamics of phosphorylation in primary dendritic cells (DC) at the early stages of infection by either virulent or attenuated *F. tularensis* versions. They found that the DC phosphorylation response underwent distinct phases, which differed between the two strains. Only the virulent strain induced later-phase MAPK signaling, believed to facilitate host survival and immune suppression [166].

Increasing the comprehensiveness of phosphoproteomic datasets

Despite significant recent progress in bacterial phosphoproteomics, as described above, there is still a long way to go before we have fully elucidated the complexity of pathogen phosphorylation networks. Optimizing phosphoproteomic protocols is essential if we are to obtain a deep and global understanding of the phospho-regulatory events that drive infectious diseases. For instance, to identify infection-relevant phosphorylation sites, phosphoproteomic analysis should be performed for bacteria isolated from *in vivo* infections. While experiments performed with free-living cells have shed important light on modification patterns under different growth and virulence conditions, phosphorylation sites should be recorded during live infections to increase the chance of identifying substrates important for pathogenesis. This could include either infected cell lines or mouse models [167]. Temporal changes in pathogen phosphoproteomes should then be systematically recorded over the course of the infection process. Separation of subcellular structures

would further allow for a spatial resolution of phosphoproteome dynamics [168]. Moreover, parallel analysis of samples obtained from the infected host would allow one to correlate bacterial and host phosphorylation patterns and obtain integrated insight into host–pathogen interactions. We propose that continued improvements in sample preparation, quantification methods, MS instrumentation, and data processing should make such experiments possible. A major challenge is associated with enriching low-abundance phosphopeptides from already low quantities of material obtainable from *in vivo* infection models. As bacterial proteins will be highly diluted in the more complex host proteome, the separation of bacteria from host cells must be optimized [169]. Continued developments in phosphopeptide preparation [139,140,141] and MS instrumentation [170] will further help to maximize the phosphosite coverage that can be obtained from these samples. Emergence of streamlined and time-effective sample preparation platforms that reduce sample requirements and optimize sample reproducibility [171] should in particular spur the generation of large-scale bacterial phosphoproteomic studies that require the parallel analysis of a high number of samples. Ideally, workflows should be fully automated to allow rapid and robust high-throughput sample processing. To this end, Leutert *et al.* [172] recently developed an end-to-end automated phosphoproteomic protocol (rapid-robotic phosphoproteomics or R2–P2) where proteins and phosphopeptides are captured on magnetic beads and processed in 96-well plates using a magnetic particle-processing robot.

The basis for obtaining meaningful biological information from phosphoproteomic datasets is the reproducible quantification of phosphopeptides. Phosphoproteomic quantification methods have traditionally involved labeling due to reproducibility problems of label-free methods where individual sample processing leads to high sample-to-sample variation. Even though SILAC is considered the most accurate quantification method [173] it is not suitable in cases where a high number of samples are to be compared, such as in extended time series experiments, or in cases where metabolic labeling is not feasible. Instead, multiplexing by TMT peptide labeling now allows comparative analysis of up to 11 samples and label-free quantification allows the comparison of an essentially unlimited number of samples. Furthermore, recent innovations in label-free methodologies have greatly justified their usage [174,175]. A detailed comparison of common phosphoproteomic quantification methods was recently produced by Högberg *et al.* [173]. Their conclusion was that even though label-free quantification and

SILAC are the most accurate techniques, MS2-based TMT-based quantification is at present the best method for identifying biologically meaningful regulatory phosphorylation events.

Another source for improving bacterial phosphoproteomics comes from advances in MS acquisition methods. New data-independent acquisition (DIA) setups can boost both the depth and reproducibility of shotgun proteomic analysis [167,170,176,177]. Compared to traditional data-dependent acquisition (DDA), where only the most abundant peptide ions in MS1 are selected for MS2 fragmentation in a semi-stochastic manner, all peptides within a predefined *m/z* range are cofragmented and measured together in DIA mode. This way DIA enables systematic measurement of all peptide ions including very low-intensity/abundance peptides. Better performance in terms of reproducibility and accuracy also makes DIA highly suitable for quantification purposes. Though, due to the high complexity of MS2 spectra resulting from peptide cofragmentation, DIA holds several analytical challenges and requires sophisticated processing tools. Typical spectral deconvolution methods rely on prerecorded spectral libraries but more streamlined methods based on *in silico* libraries or neural networks are now on the rise [178–180]. Although DIA has not yet been adapted to bacterial phosphoproteomics, an optimized DIA-based quantitative phosphoproteomic protocol was recently reported for human cells [177]. Using label-free quantification, the authors were able to quantify more than 10 000 phosphorylation sites across hundreds of samples. Other acquisition settings that avoid the random sampling problems of DDA are targeted approaches such as selected reaction monitoring (SRM) and parallel reaction monitoring (PRM) [181]. PRM and SRM are highly suitable for experiments that seek sensitive and reproducible analysis of already pre-specified phosphopeptides [182]. In summary, the field of phosphoproteomics is still undergoing profound advancements. The constant advent of methodologies improving the sensitivity, robustness, and quantification of phosphoproteomic profiling will undoubtedly expand our insight into infection-related signaling from both the host and pathogen perspective. Eventually, optimized protocols will allow us to chart pathogen phosphorylation signatures from a minimum of material, such as from infected patient samples, to suggest biomarkers for precision medicine of the future.

Making sense of phosphoproteomic data

While MS-based studies produce large lists of phosphorylated amino acids, characterizing the function of

each of these sites can so far not be performed in a high-throughput manner and thus represent a major bottleneck in phosphoproteomic research. This is, for example, reflected in the fact that less than 3% of identified human phosphosites have been designated a function [1]. In this section, we will present emerging concepts and methods, primarily arising from the study of eukaryal phosphoproteomes, that can propel the functional interpretation of phosphosites and phosphorylation networks at a global scale. Furthermore, the integration of phosphoproteomics with other types of omics data should eventually allow us to obtain a more holistic view of infectious diseases.

Functional prioritization of phosphosites

Addressing ways to predict functionally relevant phosphosites from big datasets has become a key point in phosphoproteomics as it allows researchers to prioritize phosphosites for further experimental exploration. Such prioritization is especially important given the suggestion that a noticeable proportion of phosphosites may be nonfunctional and possibly occur due to random kinase encounters and/or evolutionary neutral drift in regulatory interactions [183–185]. Exhaustive examinations of eukaryal phosphoproteomes have suggested that features such as phosphosite stoichiometry, condition-dependent dynamics, evolutionary conservation, PTM colocalization, and structural context can be used to indicate phosphosite functional relevance [1,185,186]. For instance, a large fractional stoichiometry is a good indication of phosphosite importance [119,187]. Though, the opposite is not always true as low-stoichiometry sites might mediate specialized functions for a minor subpool of a protein. Methods to systematically determine phosphosite stoichiometry from large-scale studies are becoming increasingly accessible [118,119,177].

Phosphosites are generally only slightly more conserved than their nonmodified counterparts, a theme seen for both eukarya and bacteria [63,183,185,188,189]. However, phosphosites with a known or predicted function show higher conservation rates [185,188]. Moreover, although phosphorylation might not be well conserved at an exact position, it may be conserved when considering a somewhat broader positional window. In fact, conserved so-called phosphorylation hotspots consisting of positionally less-constrained phosphosites can indicate areas of regulatory potential [185,190]. Eukaryal phosphosites are predominantly found in disordered regions [191] and phosphorylation hotspots situated herein are likely to regulate protein–protein interactions [1,190]. Hotspot regions are also

common in structural domains. By mapping known phosphosites from 40 eukaryal species onto Pfam domains, Strumillo *et al.* [186] identified 241 phosphorylation hotspots within 162 diverse domain families. Plotting these regions onto structural data exposed that they were often positioned at protein interfaces or nearby catalytic residues, consistent with phosphorylation regulating protein–protein contact and enzyme activity. In line with the notion that integrating multiple sources of information provides better predictive power compared to single features, computational machine-learning methods have recently been developed, as will be discussed later on, to systematically integrate multiple phosphosite attributes in the assessment of phosphosite function.

Because the above-mentioned structural and evolutionary patterns of phosphorylation have been extracted from eukaryal phosphoproteomes, they may not necessarily apply to bacteria. Similar comprehensive evolutionary analysis of bacterial phosphosites has so far not been performed due to the relative scarcity in recorded bacterial phosphoproteomes, but should become possible as we accumulate more and better-coverage datasets. In any case, the integration of phosphoproteomic data with structural information should be highly instructive for predicting the specific functional roles for bacterial phosphosites as well. In particular, the visualization of sites in their 3D environment can reveal phosphosites that are physically close to functional protein regions in 3D even though they are far in sequence. By suggesting regulatory potentials of phosphosites not deducible from sequence analysis alone, structural insight can greatly improve hypothesis-driven functional experiments. As manual mapping of phosphosites onto protein structures is a time-consuming process, some online PTM databases have started incorporating PDB (Protein Data Bank) structural information into their user interfaces [192]. A software tool to identify PTMs stored in 3D PDB structures has also been developed [193]. Recently a comprehensive PTM-structural database was launched (PRISMOID accessible through <http://prismoid.erc.monash.edu/>) in which protein modifications extracted from six different databases have been annotated onto matching PDB structures [194]. Since bacterial phosphosites are still underrepresented in Web-based repositories [154], their representation in PRISMOID is also minor. The shortage of adequately solved structures further compromises phosphosite 3D annotations. This notwithstanding, we propose that the next step will be to develop software that can integrate phosphosite-structural information with phosphoproteomic datasets in an automated

manner. By integrating information from different databases and prediction tools, the structural context of individual phosphosites, for example, disorder, domain, and interaction properties, could be extracted at a global scale. Additionally, phosphosites could be automatically mapped onto 3D structures if these are available and visual depictions could be generated. In cases where there is no structural data, it would be highly desirable if, in the future, protein structural prediction tools could alternatively provide some putative structural context [195].

How can phosphoproteomics benefit from machine learning

Machine learning is being increasingly used to make sense of the high-throughput and complex datasets produced in the ‘big data’ era. In short, machine learning is a type of artificial intelligence in which computer systems are set to learn and improve upon their own procedures through training datasets. Deep learning is a next-generation branch of machine learning based on multilayered neural networks and is best suited for analyzing highly dimensional and structured data available in very large quantities [196]. In the biological sciences, machine-learning methods can be used to identify cellular and molecular patterns embedded in the noise of biological systems [197,198]. In the field of phosphoproteomics, machine learning has especially been used for identifying contextual features of experimentally validated phosphosites with the aim to be able to predict unknown sites from protein sequences. Numerous different prediction methods have been published, including some that focus on kinase-specific phosphorylation sites [199,200]. A few prediction tools have been specifically tailored to bacteria [201–203]. The first bacteria-specific phosphosite predictor, Net-PhosBac, was developed using neural network algorithms [201]. At that time though, only about 150 bacterial phosphosites, from just two species, were available as a training dataset. The MPSite web resource is a more recent and improved machine-learning predictor trained on more than 3000 unique bacterial pSer and pThr sites from the dbPSP database [153,203]. While these and most other methods are solely based on primary, and maybe secondary, structural features, a small predictive performance gain may be obtained by including phosphosite 3D-context information [204]. Importantly, the use of deep-learning strategies particularly shows great promise for improving phosphosite predictions. In contrast to conventional machine-learning methods, which involve manual feature selection, deep-learning algorithms

allow automatic and unbiased discovery of phosphorylation patterns [205,206]. Owing to their improving performances, *in silico* predictions represent a valuable supplement to experimental phosphosite identification methods. For instance, in addition to being relatively labor- and resource-intensive, MS-based phosphoproteomic protocols also pose noteworthy technical limitations. In particular, they allow the identification of only a limited number of sites in any given experiment, that is, the sites modified to detectable degrees in the specific cellular condition. On top of that, the usage of just a few different amino acid-specific proteases (predominantly trypsin) in sample preparations, results in fractions of phosphopeptides that are either too short or too long for MS detection. This means that a significant portion of sites may persistently go undetected. For these reasons, the continued development of highly reliable phosphosite prediction algorithms could make a crucial contribution to mapping all possible phosphorylation substrates.

Another relevant application of machine learning is in functional prioritization of phosphosites, as it enables integrated evaluation of multiple distinct features that can predict functionality [207]. This was indeed showcased by Ochoa *et al.* who incorporated 59 phosphosite characteristics into a single functional score by training machine-learning algorithms on human phosphosites of already known function. Substantiating the applicability of their model, it was able to correctly categorize regulatory phosphosites from a comprehensive reference human phosphoproteome [208].

Finally, it is worth mentioning an example in which machine learning was used to facilitate the challenging task of identifying *bona fide* kinase substrates from *in vivo* studies [209]. As described earlier, quantitative phosphoproteomic screens are often used to elucidate kinase–substrate pairs by comparing kinase-perturbed cells with unperturbed cells. The interpretation of such datasets is however hampered by the fact that manipulation of a single kinase will alter the phosphorylation of both direct and indirect substrates. To address this issue, Kanshin *et al.* devised a machine-learning model that could dissect direct from indirect substrates from a large-scale quantitative phosphoproteomic experiment involving kinase inhibition. As a list of already known substrates was not available to use as a training set for conventional machine learning, they applied an approach related to positive and unlabeled learning. Interestingly, their model, which was validated for two different yeast kinases, found that direct kinase substrates display higher fold changes and faster dephosphorylation kinetics upon kinase inhibition compared to indirect substrates [209].

'Omics' data integration toward a systems-level understanding of infection

While phosphoproteomics can offer important insight into protein post-translational regulation, it only offers information on one aspect of a cell's biological state. In concert with massive developments within large-scale molecular biology techniques, such as genomics, transcriptomics, proteomics, and metabolomics, there has been an increasing interest in integrating different types of 'omics' data to explore bacterial physiology and pathogenicity in a more systemic manner [210,211]. Current studies include integration of two or more 'omic' layers. Deatherage Kaiser *et al.*, for example, used a multi-omics strategy that combined proteomics, metabolomics, glycomics, and metagenomics to elucidate interactions occurring between *Salmonella enterica*, its murine host, and the intestinal microbiome, revealing the capability of *S. enterica* to alter gut microbiome composition for its own advantage [212]. Ramos *et al.* [213] integrated genomic, transcriptomic, metabolic, and protein structural information to identify candidate targets for the development of novel antibiotics in *Klebsiella pneumoniae*. A few studies have included phosphoproteomic data in their multi-omics approach. A comprehensive integrated multi-omics dataset was prepared for *Mycoplasma pneumoniae*, comprising data from phosphoproteomics, lysine acetylomics, DNA methylomics, transcriptomics, proteomics, protein–protein interactions, metabolomics, and a genomewide essentiality map [171,214]. Using this resource to explore the regulatory layers of protein abundance, Chen *et al.* [214] uncovered that antisense noncoding RNAs, lysine acetylation, and protein phosphorylation were all better predictors of protein abundance than mRNA levels. A different study used multi-omics profiling, incorporating quantitative phosphoproteomics, proteomics, transcriptomics, lipidomics, and metabolomics, to achieve a deeper understanding of the essential roles played by the PknA and PknB Hanks kinases in *M. tuberculosis* pathogenicity. Together with chemical PknA/B inhibition, this multisystem analysis uncovered a role for PknA/B in the regulation of cell envelope and protein secretion processes, proposedly via phosphorylation of the essential TCS response regulator MtrA [51]. Similar integration of different omics assays is also being used to elucidate bacterial pathogenesis from the perspective of the host [211,215]. However, successful integration of heterogeneous multi-omics data under a mathematical model is still facing significant challenges. Differences in data file formats, nomenclatures, dimensionality, and quality

constitute some of the key obstacles for computational data integration and call for community-wide data standardization [216]. Further improvements in omics data preprocessing, integration, and machine-learning analysis are essentially needed to fully capitalize on the huge amount of information embedded in large-scale omics datasets. Looking ahead, multi-omics integration together with machine-learning modeling will likely revolutionize our knowledge on bacterial pathogenesis. In particular, integrating data obtained from both host and bacteria should help to uncover the complex web of molecular interactions that dictates infectious outcomes and ultimately offer a systems-level understanding of infectious diseases.

Phosphorylation in antibacterial therapies

The continuous emergence and worldwide spreading of drug-resistant bacterial strains [217] places a high demand on discovery of new antibacterial compounds. Understanding the molecular basis behind both inherent and acquired drug resistance is evidently crucial to devise such novel types of therapeutic strategies. Increased awareness of the essential roles played by bacterial kinases in the control of bacterial growth, pathogenicity, and antibiotic resistance has led to their exploration as potential future drug targets. Inspired by drugs already used in the clinic to treat cancer, there is an increasing effort to identify bacterial kinase inhibitors that can obstruct key signaling pathways sustaining bacterial pathogenesis and antimicrobial resistance.

Phosphorylation in antibiotic resistance

Common mechanisms underlying antibiotic resistance include modification or degradation of the antimicrobial agent to render it ineffective, modification of target proteins to reduce drug binding, and altered drug influx/efflux. Several studies have found that antibiotic resistance can be driven by bacterial kinases. Particularly, transmembrane Hanks kinases with PASTA domains are seen to confer resistance to cell-wall-inhibiting β -lactam-type antibiotics in various Gram-positive species [14,19,34,36,218–221]. Although this may not be surprising given their role in cell division and cell wall homeostasis as described earlier, the mechanism(s) by which Hanks-type PASTA kinases promote β -lactam resistance are only poorly defined. In *S. pneumoniae* antibiotic-sensitive strains, including some clinical isolates, the response to the β -lactam drugs involves the StkP Hanks kinase and its cognate

phosphatase PhpP [218,222]. StkP and PhpP expression as well as StkP phosphorylation is induced upon sublethal exposure to penicillin or cefotaxime. Deleting PhpP augments StkP phosphorylation levels and confers drug resistance, suggesting that StkP inactivation could be a strategy for overcoming *S. pneumoniae* β -lactam resistance [218]. Similarly, *L. monocytogenes* PrkA, *Enterococcus faecalis* IreK, *C. difficile* PrkC, and MRSA Stk1 are all required for resistance to different types of β -lactams [19,34,36,219–221]. In *S. aureus*, β -lactams induce the phosphorylation of the β -lactam antibiotic-sensing protein BlaR1 and this initiates a cascade leading to expression of antibiotic resistance determinants [223]. In *E. faecalis*, β -lactams stimulate IreK autophosphorylation, and this in turn leads to phosphorylation of the TCS histidine kinase CroS required for antibiotic resistance [224]. *Staphylococcus aureus* Stk1 has also been linked to the resistance to vancomycin, which is another type of cell wall-acting antibiotic. More specifically, Stk1 was shown to negatively regulate the DNA-binding activity of the vancomycin-resistance-associated response regulator VraR of the VraTSR three-component system [225]. These latter two examples illustrate the sophisticated crosstalk that occurs between different phosphorylation systems in the orchestration of drug responses.

Other types of antibiotics work by interfering with DNA replication, such as the quinolones. In *M. tuberculosis*, phosphorylation of the global transcriptional regulator MgrA by PknB controls its differential induction of the NorA and NorB multidrug-resistance efflux pumps, correlating with resistance to different types of quinolone drugs [226]. Other *M. tuberculosis* Hanks kinases implicated in drug resistance are PknF and PknG. In fact, the soluble PknG kinase promotes natural resistance to a broad diversity of antibiotics, including ones that target transcription, translation, and cell wall biosynthesis [227], while PknF is believed to reduce the activity of the antitubercular drug ethionamide [228]. Ethionamide is a prodrug that needs to be activated by the monooxygenase EthA and EthA expression is regulated by the transcriptional regulator EthR. Notably, PknF phosphorylation of EthR negatively affects its DNA-binding activity, and further studies should determine how this affects *M. tuberculosis* ethionamide resistance [228].

Taken together, the above findings emphasize the importance of phosphorylation signaling in bacterial drug responses. Yet it is also clear that substantially more work is needed to fully delineate the diversity of molecular mechanisms behind kinase-dependent

drug resistance. Such work will be instrumental in developing new and efficient types of antimicrobials, as discussed below.

Drugging bacterial kinases

Due to their well-documented and multifaceted roles in pathogenesis and antibiotic resistance, bacterial kinases are currently being investigated as potential new drug targets. While drug discovery studies are also trying to find targets toward histidine kinases [229], these will not be the focus here. Small-molecule drugs that target human Ser/Thr and Tyr kinases are already being routinely used in the treatment of several types of cancers and new classes of human kinase inhibitors continue to be experimentally and clinically explored [230]. The majority of these drugs target the ATP binding pocket of the kinase catalytic domain and given their clinical success, it was suggested that similar compounds could be devised against bacterial kinases. Already established small-molecule kinase inhibitor libraries have accordingly helped guide bacterial drug studies. Many efforts have especially been made to pharmacologically target the essential PknB kinase of *M. tuberculosis* [231–235]. For example, Loughheed *et al.* first screened a commercial library of more than 50,000 compounds and then optimized on a group of inhibitor candidates by medicinal chemistry [233]. Chapman *et al.* [234] also performed high-throughput screening followed by optimization of an aminopyrimidine inhibitor. However, while these candidate drugs were very efficient in inhibiting PknB activity *in vitro* (in the nanomolar range), their potency *in vivo* was only modest with minimum inhibitory concentrations in the micromolar range. This did not appear to be due to poor cell wall permeability [233,234].

Another approach to identify kinase inhibitors is by *in silico* modeling. Wlodarchak *et al.* [236] exploited the general structural similarity of bacterial and human Ser/Thr kinases to identify PknB inhibitors by *in silico* screening of a human kinase inhibitor collection. Their *in silico* structural docking revealed a family of imidazopyridine aminofurazan lead compounds with high binding affinity toward PknB. Intriguingly, and consistent with the known role of PknB in cell wall homeostasis, these inhibitors were able to significantly potentiate the effect of β -lactams in several types of mycobacteria and in the related *Nocardia asteroides* [236]. This synergistic concept has also been reported for kinase inhibitors of the nonessential *L. monocytogenes* PrkA and *S. aureus* Stk1 Hanks kinases [219,223,237–240]. In *L. monocytogenes*, the broad-spectrum kinase inhibitor staurosporine, as well as some more selective repurposed human kinase inhibitors, was seen to inhibit PrkA

activity and sensitize cells to β -lactam drugs [219,237]. Similarly, several studies have identified Stk1-inhibiting molecules from small-molecule libraries that sensitize MRSA *S. aureus* strains to β -lactams [223,238–240]. Notably, Kant *et al.* optimized a quinazoline-based Stk1-inhibiting molecule (Inh2-B1) that potentiates the bactericidal activity of the cephalosporins ceftriaxone and cefotaxime on drug-resistant *S. aureus*, both *in vitro* and in an *in vivo* septicemia mouse model with negligible cytotoxic side effects [240]. Mechanistically, Inh2-B1 was shown to reduce the expression of cell wall hydrolase-encoding genes important for cell wall synthesis and biofilm formation. This effect is likely to occur due to reduced phosphorylation and promoter binding of the WalR response regulator upon Stk1 inhibition [240]. Thus, in summary, several observations suggest that combined β -lactam and Hanks kinase inhibition therapy could represent a novel strategy to combat clinical β -lactam resistance.

Another line of currently investigated mycobacterial inhibitors are targeted toward the cytosolic PknG kinase [86,241,242]. In contrast to PknA and PknB, PknG is not essential but is instead important for host intracellular survival by blocking macrophage phago-lysosomal fusion [86]. By only targeting intracellular mycobacteria, it may be expected that PknG inhibition would pose less selective pressure for the development of resistant strains [243]. Encouragingly, an initial drug screen found that chemical inhibition of PknG at its ATP-binding site allowed effective macrophage clearing of mycobacterial cells via the phago-lysosomal system [86,241]. Since PknG is also proposed to be crucial for the survival of mycobacterial dormant persisters, antitubercular combination therapies that include PknG inhibition could be a prospective means to prevent disease reactivation [112,243]. An extensive overview of presently investigated mycobacterial kinase inhibitors is provided in Khan *et al.* and Mori *et al.* [243,244].

Even though the above-mentioned studies clearly demonstrate the clinical potential of bacterial kinase inhibitors, there are, to best of our knowledge, so far no reports on Ser/Thr or Tyr kinase inhibitors displaying promising antibacterial activities for single-drug clinical use. Larger scale drug screening and optimization of compound leads is basically needed to make antimicrobial kinase drugs clinically relevant. As, for example, discussed in Pensinger *et al.* [14], differences in biochemical and antibacterial efficacy of currently tested drugs may be related to limited penetration across the bacterial cell envelope, not allowing drugs to reach their cytoplasmic targets. Another explanation for the discrepancy between *in vitro* and *in vivo* effects could be due to *in vitro* kinase function not properly reflecting kinase

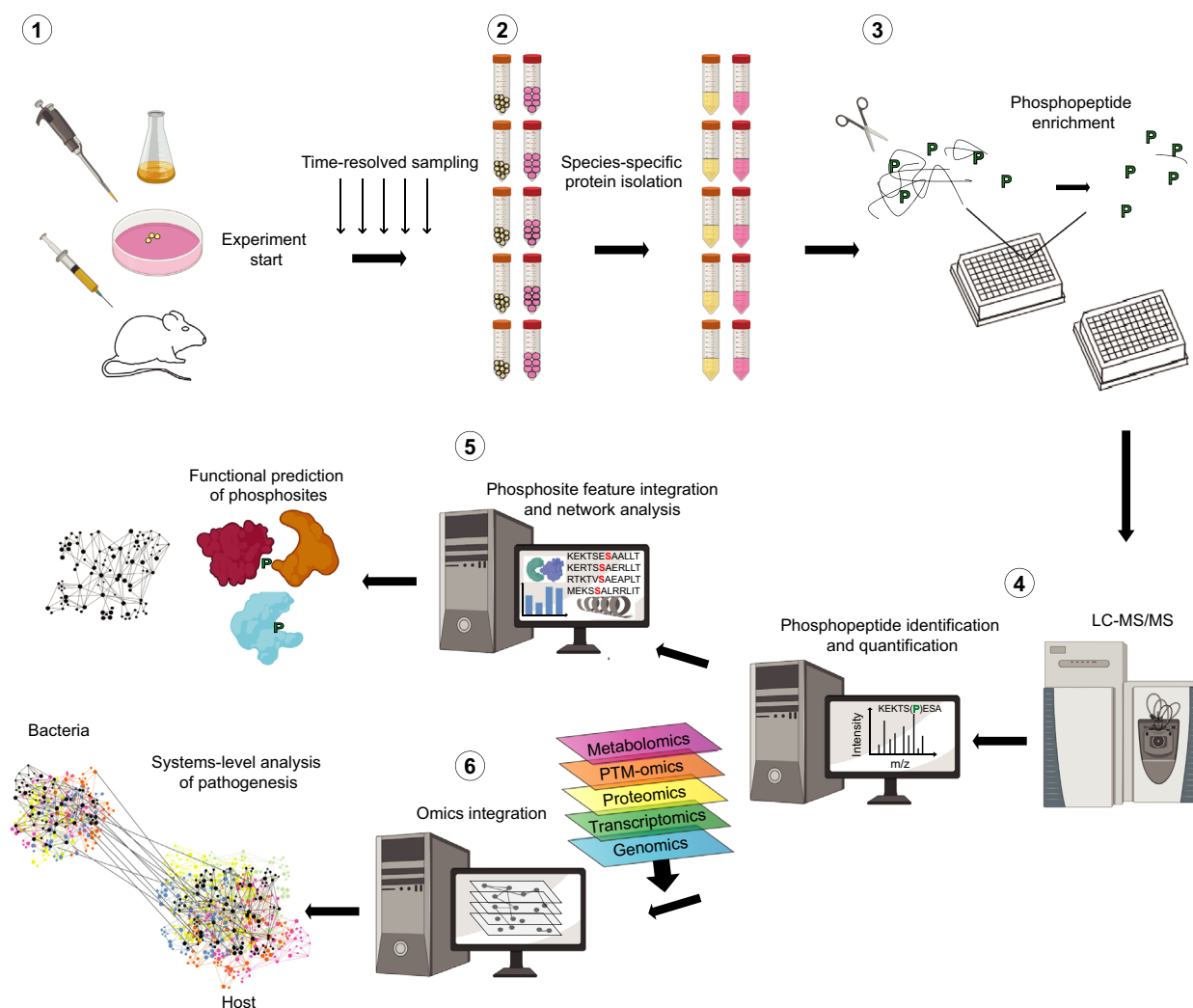


Fig. 2. Block chart presenting proposed future pipeline for phosphoproteomics in the study of bacterial infections. (1) Phosphoproteome dynamics should be studied during *in vivo* bacterial infections such as by cell culture or mouse models, and parallel analysis should ideally be performed on bacterial and host samples to facilitate an integrated understanding of host–pathogen signaling crosstalk. Furthermore, samples should be acquired at several time points during the course of infection to obtain time-resolved insight into phosphorylation dynamics. Quantification may be achieved by metabolic protein labeling, chemical peptide labeling, or label-free approaches. (2) Bacterial and host cells should be separated prior to species-specific lysis and protein extraction. In addition, subcellular and/or subpopulation fractionation may be performed to obtain spatially resolved sample analysis. (3) Reproducible and time-efficient analysis of a high number of parallel samples should be possible by automated procedures for phosphopeptide enrichment and cleanup in 96-well formats. (4) High-performance LC-MS/MS analysis of purified phosphopeptides together with optimized data acquisition methods, for example, DIA, should result in datasets of high phosphoproteome coverage and good quantitative reproducibility. (5) Phosphoproteomic data should subsequently be subjected to diverse types of bioinformatic and machine-learning analyses to get a systemic view of phosphorylation networks and to generate testable predictions of phosphosite functionality. (6) Finally, integration of phosphoproteomes with other types of omics data obtained from the same infection model should be analyzed by machine-learning algorithms to reveal molecular signatures of bacterial infections that could be therapeutically exploited.

activity in the bacterial setting where kinases may be tethered to the membrane and other molecular interaction partners are present. Thus, more knowledge of drug uptake mechanisms and *in vivo* kinase function is needed for optimized drug design. Moreover, to be clinically relevant, new drugs should be highly selective

toward only bacterial kinases and not show cross-reactivity with host enzymes. To improve target specificity of an initially identified PknA/B inhibitor, Wang *et al.* [245] produced compound–enzyme cocrystals and identified a pocket in the drug binding site of PknB that was unique to mycobacterial kinases. This allowed them to

modify their compound to achieve a 100-fold selectivity over mammalian kinases. As the structure of, for example, mycobacterial Hanks kinase domains displays only around 20–30% similarity to eukaryal kinases, there may be several options for designing highly bacteria-specific drugs [243,246]. Alternatively, inhibitors could be directed toward other kinase regions such as the extracellular PASTA domains, which would circumvent the problem of having to cross the cell envelope [14]. As PASTA domains are highly divergent [247] and not present in eukarya they might be suitable target candidates for drugs with high species specificity. Likewise, BY-kinases could be desirable future drug targets due to their absence in eukarya [248]. In any case, a thorough understanding of kinase structure and function as well as of the molecular basis for inhibitor specificity should be key to guide further development of both effective and species-specific drugs that could reach the clinic.

It should also be mentioned that another currently pursued antibacterial strategy involves the development of inhibitors directed toward secreted bacterial tyrosine phosphatases [249,250]. In addition, host-directed therapeutics targeting host kinases represent yet another exciting branch of explored anti-infectious medicines [251]. Host-directed therapeutics may, for example, inhibit pathogen-hijacked host pathways or work to boost host immune defenses. Importantly, drugs with such activities can be derived from already commercially approved drugs, a concept known as drug repurposing [252].

Conclusions and perspectives

While individual phosphoprotein studies have provided valuable information about the function of Ser/Thr/Tyr phosphorylation in specific contexts, a more systematic view of phosphorylation landscapes and their dynamics during different pathogenic stages is needed to fully grasp the phospho-regulatory mechanisms underlying bacterial pathogenesis. This includes the acquisition of spatial and time-resolved datasets and possibly, simultaneous analysis of both bacterial and host phosphoproteomes to detect phosphorylation-dependent host–pathogen interactions. In addition, future developments of single-cell phosphoproteomics will be useful for dissecting phosphorylation networks in heterogeneous cell populations. A proposed pipeline for future phosphoproteomic experiments in the study of bacterial pathogenesis is provided in Fig. 2. Basically, we need to move from a discovery mode to a quantification mode when it comes to studying bacterial phosphorylation. Quantitative phosphoproteomics techniques are routinely used to study phosphorylation dynamics in eukarya. However, their application in

bacterial studies is lagging far behind and bacterial phosphoproteomes remain in general largely understudied. This is partly due to detection difficulties related to lower levels of protein phosphorylation found in bacteria compared to eukarya. Thus, technical optimizations are urgently needed to achieve desired insights into bacterial phosphoproteomes. DIA MS represents one recent development that may benefit bacterial proteomics as it allows reproducible detection of low-abundance peptides. This strategy has to our knowledge not yet been applied in bacterial phosphoproteomics. Another major bottleneck in understanding the role of phosphorylation in bacterial pathogenesis is the functional characterization of phosphosites generated from MS analysis. To help predict phosphosite function, prediction strategies, relying on evolutionary and structural information of the phosphosite sequence, has been described for eukaryal phosphorylation. As we will gain more information on bacterial phosphorylation, it will be interesting to see whether bacterial phosphosites conform to the same localization patterns. We also argue that mapping of phosphosites onto protein 3D structures, or known domains, should greatly facilitate functional interpretations that can be tested experimentally. We propose that such a contextualization of sites generated from large screens should be automated in the future by the integration of phosphosite datasets with structural databases or predictions. The functional insights deriving from individual phosphorylation datasets could be enhanced by integrating them with other omics data, such as transcriptomics, proteomics, metabolomics, and acetylomics, to obtain a systems-level understanding of infectious diseases that can guide future antibacterial therapies. Developments in machine- and deep-learning algorithms are expected to facilitate information extraction from complex multi-omics data to unravel the contribution of protein phosphorylation to pathogenicity.

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References

- 1 Needham EJ, Parker L, Burykin T, James DE and Humphrey SJ (2019) Illuminating the dark phosphoproteome. *Sci Signal* **12**, <https://doi.org/10.1126/scisignal.aau8645>

- 2 Jers C, Soufi B, Grangeasse C, Deutscher J and Mijakovic I (2008) Phosphoproteomics in bacteria: towards a systemic understanding of bacterial phosphorylation networks. *Expert Rev Proteomics* **5**, 619–627.
- 3 Kobir A, Shi L, Boskovic A, Grangeasse C, Franjevic D and Mijakovic I (2011) Protein phosphorylation in bacterial signal transduction. *Biochim Biophys Acta* **1810**, 989–994.
- 4 Dworkin J (2015) Ser/Thr phosphorylation as a regulatory mechanism in bacteria. *Curr Opin Microbiol* **24**, 47–52.
- 5 Getz LJ, Runte CS, Rainey JK and Thomas NA (2019) Tyrosine phosphorylation as a widespread regulatory mechanism in prokaryotes. *J Bacteriol* **201**, <https://doi.org/10.1128/JB.00205-19>
- 6 Burnside K and Rajagopal L (2012) Regulation of prokaryotic gene expression by eukaryotic-like enzymes. *Curr Opin Microbiol* **15**, 125–131.
- 7 Hanks SK, Quinn AM and Hunter T (1988) The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science* **241**, 42–52.
- 8 Stancik IA, Šestak MS JB, Axelson-Fisk M, Franjevic D, Jers C, Domazet-Lošo T and Mijakovic I (2018) Serine/threonine protein kinases from bacteria, archaea and eukarya share a common evolutionary origin deeply rooted in the tree of life. *J Mol Biol* **430**, 27–32.
- 9 Janczarek M, Vinardell JM, Lipa P and Karaš M (2018) Hanks-type serine/threonine protein kinases and phosphatases in bacteria: Roles in signaling and adaptation to various environments. *Int J Mol Sci* **19**, 2872. <https://doi.org/10.3390/ijms19102872>
- 10 Mijakovic I, Grangeasse C and Turgay K (2016) Exploring the diversity of protein modifications: special bacterial phosphorylation systems. *FEMS Microbiol Rev* **40**, 398–417.
- 11 Zheng J, He C, Singh VK, Martin NL and Jia Z (2007) Crystal structure of a novel prokaryotic Ser/Thr kinase and its implication in the Cpx stress response pathway. *Mol Microbiol* **63**, 1360–1371.
- 12 Grangeasse C, Nessler S and Mijakovic I (2012) Bacterial tyrosine kinases: evolution, biological function and structural insights. *Philos Trans R Soc Lond B Biol Sci* **367**, 2640–2655.
- 13 Chao JD, Wong D and Av-Gay Y (2014) Microbial protein-tyrosine kinases. *J Biol Chem* **289**, 9463–9472.
- 14 Pensinger DA, Schaezner AJ and Sauer JD (2018) Do Shoot the messenger: PASTA kinases as virulence determinants and antibiotic targets. *Trends Microbiol* **26**, 56–69.
- 15 Shah IM, Laaberki MH, Popham DL and Dworkin J (2008) A eukaryotic-like Ser/Thr kinase signals bacteria to exit dormancy in response to peptidoglycan fragments. *Cell* **135**, 486–496.
- 16 Pelletier A, Freton C, Gallay C, Trouve J, Cluzel C, Franz-Wachtel M, Macek B, Jault JM, Grangeasse C and Guiral S (2019) The tyrosine-autokinase UbK is required for proper cell growth and cell morphology of *Streptococcus pneumoniae*. *Front Microbiol* **10**, 1942.
- 17 Figueira R, Brown DR, Ferreira D, Eldridge MJ, Burchell L, Pan Z, Helaine S and Wigneshweraraj S (2015) Adaptation to sustained nitrogen starvation by *Escherichia coli* requires the eukaryote-like serine/threonine kinase YeaG. *Sci Rep* **5**, 17524.
- 18 Srinivasan VB, Vaidyanathan V, Mondal A, Venkataramaiah M and Rajamohan G (2012) Functional characterization of a novel Mn²⁺ dependent protein serine/threonine kinase KpnK, produced by *Klebsiella pneumoniae* strain MGH78578. *FEBS Lett* **586**, 3778–3786.
- 19 Kristich CJ, Wells CL and Dunne GM (2007) A eukaryotic-type Ser/Thr kinase in *Enterococcus faecalis* mediates antimicrobial resistance and intestinal persistence. *Proc Natl Acad Sci USA* **104**, 3508–3513.
- 20 Bork P, Brown NP, Hegyi H and Schultz J (1996) The protein phosphatase 2C (PP2C) superfamily: detection of bacterial homologues. *Protein Sci* **5**, 1421–1425.
- 21 Kusebauch U, Ortega C, Ollodart A, Rogers RS, Sherman DR, Moritz RL and Grundner C (2014) *Mycobacterium tuberculosis* supports protein tyrosine phosphorylation. *Proc Natl Acad Sci USA* **111**, 9265–9270.
- 22 Kant S, Agarwal S, Pancholi P and Pancholi V (2015) The *Streptococcus pyogenes* orphan protein tyrosine phosphatase, SP-PTP, possesses dual specificity and essential virulence regulatory functions. *Mol Microbiol* **97**, 515–540.
- 23 Arora G, Sajid A, Arulanandh MD, Singhal A, Mattoo AR, Pomerantsev AP, Leppla SH, Maiti S and Singh Y (2012) Unveiling the novel dual specificity protein kinases in *Bacillus anthracis*: identification of the first prokaryotic dual specificity tyrosine phosphorylation-regulated kinase (DYRK)-like kinase. *J Biol Chem* **287**, 26749–26763.
- 24 Sajid A, Arora G, Singhal A, Kalia VC and Singh Y (2015) Protein phosphatases of pathogenic bacteria: role in physiology and virulence. *Annu Rev Microbiol* **69**, 527–547.
- 25 Galyov EE, Håkansson S, Forsberg A and Wolf-Watz H (1993) A secreted protein kinase of *Yersinia pseudotuberculosis* is an indispensable virulence determinant. *Nature* **361**, 730–732.
- 26 Wang J, Li C, Yang H, Mushegian A and Jin S (1998) A novel serine/threonine protein kinase homologue of *Pseudomonas aeruginosa* is specifically inducible within the host infection site and is required for full virulence in neutropenic mice. *J Bacteriol* **180**, 6764–6768.
- 27 Cowley S, Ko M, Pick N, Chow R, Downing KJ, Gordhan BG, Betts JC, Mizrahi V, Smith DA, Stokes

- RW *et al.* (2004) The *Mycobacterium tuberculosis* protein serine/threonine kinase PknG is linked to cellular glutamate/glutamine levels and is important for growth *in vivo*. *Mol Microbiol* **52**, 1691–1702.
- 28 Chawla Y, Upadhyay S, Khan S, Nagarajan SN, Forti F and Nandicoori VK (2014) Protein kinase B (PknB) of *Mycobacterium tuberculosis* is essential for growth of the pathogen *in vitro* as well as for survival within the host. *J Biol Chem* **289**, 13858–13875.
 - 29 Rajagopal L, Clancy A and Rubens CE (2003) A eukaryotic type serine/threonine kinase and phosphatase in *Streptococcus agalactiae* reversibly phosphorylate an inorganic pyrophosphatase and affect growth, cell segregation, and virulence. *J Biol Chem* **278**, 14429–14441.
 - 30 Echenique J, Kadioglu A, Romao S, Andrew PW and Trombe MC (2004) Protein serine/threonine kinase StkP positively controls virulence and competence in *Streptococcus pneumoniae*. *Infect Immun* **72**, 2434–2437.
 - 31 Bugrysheva J, Froehlich BJ, Freiberg JA and Scott JR (2011) Serine/threonine protein kinase Stk is required for virulence, stress response, and penicillin tolerance in *Streptococcus pyogenes*. *Infect Immun* **79**, 4201–4209.
 - 32 Débarbouillé M, Dramsi S, Dussurget O, Nahori MA, Vaganay E, Jouvion G, Cozzone A, Msadek T and Duclos B (2009) Characterization of a serine/threonine kinase involved in virulence of *Staphylococcus aureus*. *J Bacteriol* **191**, 4070–4081.
 - 33 Pensinger DA, Boldon KM, Chen GY, Vincent WJ, Sherman K, Xiong M, Schaezner AJ, Forster ER, Coers J, Striker R *et al.* (2016) The *Listeria monocytogenes* PASTA kinase PrkA and its substrate YvcK are required for cell wall homeostasis, metabolism, and virulence. *PLoS Pathog* **12**, e1006001.
 - 34 Cuenot E, Garcia-Garcia T, Douche T, Gorgette O, Courtin P, Denis-Quanquin S, Hoys S, Tremblay YDN, Matondo M, Chapot-Chartier MP *et al.* (2019) The Ser/Thr kinase PrkC participates in cell wall homeostasis and antimicrobial resistance in *Clostridium difficile*. *Infect Immun* **87**, <https://doi.org/10.1128/IAI.00005-19>
 - 35 Burnside K, Lembo A, de Los RM, Iliuk A, Binhtran NT, Connelly JE, Lin WJ, Schmidt BZ, Richardson AR, Fang FC *et al.* (2010) Regulation of hemolysin expression and virulence of *Staphylococcus aureus* by a serine/threonine kinase and phosphatase. *PLoS ONE* **5**, e11071.
 - 36 Tamber S, Schwartzman J and Cheung AL (2010) Role of PknB kinase in antibiotic resistance and virulence in community-acquired methicillin-resistant *Staphylococcus aureus* strain USA300. *Infect Immun* **78**, 3637–3646.
 - 37 Burnside K, Lembo A, Harrell MI, Gurney M, Xue L, BinhTran NT, Connelly JE, Jewell KA, Schmidt BZ, de los Reyes M *et al.* (2011) Serine/threonine phosphatase StpI mediates post-transcriptional regulation of hemolysin, autolysis, and virulence of group B *Streptococcus*. *J Biol Chem* **286**, 44197–44210.
 - 38 Gannoun-Zaki L, Pätzold L, Huc-Brandt S, Baronian G, Elhawry MI, Gaupp R, Martin M, Blanc-Potard AB, Letourneur F, Bischoff M *et al.* (2018) PtpA, a secreted tyrosine phosphatase from *Staphylococcus aureus*, contributes to virulence and interacts with coronin-1A during infection. *J Biol Chem* **293**, 15569–15580.
 - 39 Agarwal S, Pancholi P and Pancholi V (2011) Role of serine/threonine phosphatase (SP-STP) in *Streptococcus pyogenes* physiology and virulence. *J Biol Chem* **286**, 41368–41380.
 - 40 Beilharz K, Nováková L, Fadda D, Branny P, Massidda O and Veening JW (2012) Control of cell division in *Streptococcus pneumoniae* by the conserved Ser/Thr protein kinase StkP. *Proc Natl Acad Sci USA* **109**, E905–E913.
 - 41 Nováková L, Bezousková S, Pompach P, Spidlová P, Sasková L, Weiser J and Branny P (2010) Identification of multiple substrates of the StkP Ser/Thr protein kinase in *Streptococcus pneumoniae*. *J Bacteriol* **192**, 3629–3638.
 - 42 Giefing C, Meinke AL, Hanner M, Henics T, Bui MD, Gelbmann D, Lundberg U, Senn BM, Schunn M, Habel A *et al.* (2008) Discovery of a novel class of highly conserved vaccine antigens using genomic scale antigenic fingerprinting of pneumococcus with human antibodies. *J Exp Med* **205**, 117–131.
 - 43 Fleurie A, Manuse S, Zhao C, Campo N, Cluzel C, Lavergne JP, Freton C, Combet C, Guiral S, Soufi B *et al.* (2014) Interplay of the serine/threonine-kinase StkP and the paralogs DivIVA and GpsB in pneumococcal cell elongation and division. *PLoS Genet* **10**, e1004275.
 - 44 Fenton AK, Manuse S, Flores-Kim J, Garcia PS, Mercy C, Grangeasse C, Bernhardt TG and Rudner DZ (2018) Phosphorylation-dependent activation of the cell wall synthase PBP2a in *Streptococcus pneumoniae* by MacP. *Proc Natl Acad Sci USA* **115**, 2812–2817.
 - 45 Zucchini L, Mercy C, Garcia PS, Cluzel C, Gueguen-Chaignon V, Galisson F, Freton C, Guiral S, Brochier-Armanet C, Gouet P *et al.* (2018) PASTA repeats of the protein kinase StkP interconnect cell constriction and separation of *Streptococcus pneumoniae*. *Nat Microbiol* **3**, 197–209.
 - 46 Hardt P, Engels I, Rausch M, Gajdiss M, Ulm H, Sass P, Ohlsen K, Sahl HG, Bierbaum G, Schneider T *et al.* (2017) The cell wall precursor lipid II acts as a molecular signal for the Ser/Thr kinase PknB of *Staphylococcus aureus*. *Int J Med Microbiol* **307**, 1–10.

- 47 Boutte CC, Baer CE, Papavinasundaram K, Liu W, Chase MR, Meniche X, Fortune SM, Sassetti CM, Ioerger TR and Rubin EJ (2016) A cytoplasmic peptidoglycan amidase homologue controls mycobacterial cell wall synthesis. *Elife* **5**, e14590. <https://doi.org/10.7554/eLife.14590>
- 48 Turapov O, Forti F, Kadhim B, Ghisotti D, Sassine J, Straatman-Iwanowska A, Bottrill AR, Moynihan PJ, Wallis R, Barthe P *et al.* (2018) Two faces of CwlM, an essential PknB substrate, in *Mycobacterium tuberculosis*. *Cell Rep* **25**, 57–67.e5.
- 49 Sasková L, Nováková L, Basler M and Branny P (2007) Eukaryotic-type serine/threonine protein kinase StkP is a global regulator of gene expression in *Streptococcus pneumoniae*. *J Bacteriol* **189**, 4168–4179.
- 50 Donat S, Streker K, Schirmeister T, Rakette S, Stehle T, Liebeke M, Lalk M and Ohlsen K (2009) Transcriptome and functional analysis of the eukaryotic-type serine/threonine kinase PknB in *Staphylococcus aureus*. *J Bacteriol* **191**, 4056–4069.
- 51 Carette X, Platig J, Young DC, Helm M, Young AT, Wang Z, Potluri LP, Moody CS, Zeng J, Prisc S *et al.* (2018) Multisystem analysis of *Mycobacterium tuberculosis* reveals kinase-dependent remodeling of the pathogen-environment interface. *MBio* **9**, <https://doi.org/10.1128/mBio.02333-17>
- 52 Kalantari A, Derouiche A, Shi L and Mijakovic I (2015) Serine/threonine/tyrosine phosphorylation regulates DNA binding of bacterial transcriptional regulators. *Microbiology* **161**, 1720–1729.
- 53 Leiba J, Hartmann T, Cluzel ME, Cohen-Gonsaud M, Delolme F, Bischoff M and Molle V (2012) A novel mode of regulation of the *Staphylococcus aureus* catabolite control protein A (CcpA) mediated by Stk1 protein phosphorylation. *J Biol Chem* **287**, 43607–43619.
- 54 Bischoff M, Brelle S, Minatelli S and Molle V (2016) Stk1-mediated phosphorylation stimulates the DNA-binding properties of the *Staphylococcus aureus* SpoVG transcriptional factor. *Biochem Biophys Res Commun* **473**, 1223–1228.
- 55 Fridman M, Williams GD, Muzamal U, Hunter H, Siu KW and Golemi-Kotra D (2013) Two unique phosphorylation-driven signaling pathways crosstalk in *Staphylococcus aureus* to modulate the cell-wall charge: Stk1/Stp1 meets GraSR. *Biochemistry* **52**, 7975–7986.
- 56 Lin WJ, Walthers D, Connelly JE, Burnside K, Jewell KA, Kenney LJ and Rajagopal L (2009) Threonine phosphorylation prevents promoter DNA binding of the Group B *Streptococcus* response regulator CovR. *Mol Microbiol* **71**, 1477–1495.
- 57 Horstmann N, Saldaña M, Sahasrabhojane P, Yao H, Su X, Thompson E, Koller A and Shelburne SA (2014) Dual-site phosphorylation of the control of virulence regulator impacts group A streptococcal global gene expression and pathogenesis. *PLoS Pathog* **10**, e1004088.
- 58 Lembo A, Gurney MA, Burnside K, Banerjee A, de los Reyes McConnelly JE, Lin WJ, Jewell KA, Vo A and Renken CW *et al.* (2010) Regulation of CovR expression in Group B *Streptococcus* impacts blood-brain barrier penetration. *Mol Microbiol* **77**, 431–443.
- 59 Piñas GE, Reinoso-Vizcaino NM, Yandar Barahona NY, Cortes PR, Duran R, Badapanda C, Rathore A, Bichara DR, Cian MB, Olivero NB *et al.* (2018) Crosstalk between the serine/threonine kinase StkP and the response regulator ComE controls the stress response and intracellular survival of *Streptococcus pneumoniae*. *PLoS Pathog* **14**, e1007118.
- 60 Piñas GE, Cortes PR, Orio AGA and Echenique J (2008) Acidic stress induces autolysis by a CSP-independent ComE pathway in *Streptococcus pneumoniae*. *Microbiology* **154**, 1300–1308.
- 61 Cortes PR, Piñas GE, Cian MB, Yandar N and Echenique J (2015) Stress-triggered signaling affecting survival or suicide of *Streptococcus pneumoniae*. *Int J Med Microbiol* **305**, 157–169.
- 62 Robertson CD, Hazen TH, Kaper JB, Rasko DA and Hansen AM (2018) Phosphotyrosine-mediated regulation of enterohemorrhagic *Escherichia coli* virulence. *MBio* **9**.
- 63 Hansen AM, Chaerkady R, Sharma J, Díaz-Mejía JJ, Tyagi N, Renuse S, Jacob HK, Pinto SM, Sahasrabuddhe NA, Kim MS *et al.* (2013) The *Escherichiacoli* phosphotyrosine proteome relates to core pathways and virulence. *PLoS Pathog* **9**, e1003403.
- 64 Standish AJ, Teh MY, Tran ENH, Doyle MT, Baker PJ and Morona R (2016) Unprecedented abundance of protein tyrosine phosphorylation modulates *Shigella flexneri* virulence. *J Mol Biol* **428**, 4197–4208.
- 65 Wugeditsch T, Paiment A, Hocking J, Drummelsmith J, Forrester C and Whitfield C (2001) Phosphorylation of Wzc, a tyrosine autokinase, is essential for assembly of group 1 capsular polysaccharides in *Escherichia coli*. *J Biol Chem* **276**, 2361–2371.
- 66 Paiment A, Hocking J and Whitfield C (2002) Impact of phosphorylation of specific residues in the tyrosine autokinase, Wzc, on its activity in assembly of group 1 capsules in *Escherichia coli*. *J Bacteriol* **184**, 6437–6447.
- 67 Obadia B, Lacour S, Doublet P, Baubichon-Cortay H, Cozzzone AJ and Grangeasse C (2007) Influence of tyrosine-kinase Wzc activity on colanic acid production in *Escherichia coli* K12 cells. *J Mol Biol* **367**, 42–53.
- 68 Bechet E, Gruszczyk J, Terreux R, Gueguen-Chaignon V, Vigouroux A, Obadia B, Cozzzone AJ, Nessler S and Grangeasse C (2010) Identification of structural

- and molecular determinants of the tyrosine-kinase Wzc and implications in capsular polysaccharide export. *Mol Microbiol* **77**, 1315–1325.
- 69 Nadler C, Koby S, Peleg A, Johnson AC, Suddala KC, Sathiyamoorthy K, Smith BE, Saper MA and Rosenshine I (2012) Cycling of Etk and Etp phosphorylation states is involved in formation of group 4 capsule by *Escherichia coli*. *PLoS ONE* **7**, e37984.
 - 70 Grangeasse C, Obadia B, Mijakovic I, Deutscher J, Cozzzone AJ and Doublet P (2003) Autophosphorylation of the *Escherichia coli* protein kinase Wzc regulates tyrosine phosphorylation of Ugd, a UDP-glucose dehydrogenase. *J Biol Chem* **278**, 39323–39329.
 - 71 Lacour S, Bechet E, Cozzzone AJ, Mijakovic I and Grangeasse C (2008) Tyrosine phosphorylation of the UDP-glucose dehydrogenase of *Escherichia coli* is at the crossroads of colanic acid synthesis and polymyxin resistance. *PLoS ONE* **3**, e3053.
 - 72 Rausch M, Deisinger JP, Ulm H, Müller A, Li W, Hardt P, Wang X, Li X, Sylvester M, Engeser M *et al.* (2019) Coordination of capsule assembly and cell wall biosynthesis in *Staphylococcus aureus*. *Nat Commun* **10**, 1404.
 - 73 Soulat D, Grangeasse C, Vaganay E, Cozzzone AJ and Duclos B (2007) UDP-acetyl-mannosamine dehydrogenase is an endogenous protein substrate of *Staphylococcus aureus* protein-tyrosine kinase activity. *J Mol Microbiol Biotechnol* **13**, 45–54.
 - 74 Nourikyan J, Kjos M, Mercy C, Cluzel C, Morlot C, Noiro-Gros MF, Guiral S, Lavergne JP, Veening JW and Grangeasse C (2015) Autophosphorylation of the bacterial tyrosine-kinase CpsD connects capsule synthesis with the cell cycle in *Streptococcus pneumoniae*. *PLoS Genet* **11**, e1005518.
 - 75 Mougous JD, Gifford CA, Ramsdell TL and Mekalanos JJ (2007) Threonine phosphorylation post-translationally regulates protein secretion in *Pseudomonas aeruginosa*. *Nat Cell Biol* **9**, 797–803.
 - 76 Yang Z, Zhou X, Ma Y, Zhou M, Waldor MK, Zhang Y and Wang Q (2018) Serine/threonine kinase PpkA coordinates the interplay between T6SS2 activation and quorum sensing in the marine pathogen *Vibrio alginolyticus*. *Environ Microbiol* **20**, 903–919.
 - 77 Mellies JL and Lorenzen E (2014) Enterohemorrhagic *Escherichia coli* virulence gene regulation. *Microbiol Spectr* **2**, <https://doi.org/10.1128/microbiolspec.EHEC-0004-2013>
 - 78 Runte CS, Jain U, Getz LJ, Secord S, Kuwae A, Abe A, LeBlanc JJ, Stadnyk AW, Kaper JB, Hansen AM *et al.* (2018) Tandem tyrosine phosphosites in the enteropathogenic *Escherichia coli* chaperone CesT are required for differential type III effector translocation and virulence. *Mol Microbiol* **108**, 536–550.
 - 79 Canova MJ and Molle V (2014) Bacterial serine/threonine protein kinases in host-pathogen interactions. *J Biol Chem* **289**, 9473–9479.
 - 80 Park BC, Reese M and Tagliabracchi VS (2019) Thinking outside of the cell: secreted protein kinases in bacteria, parasites, and mammals. *IUBMB Life* **71**, 749–759.
 - 81 Humphreys D, Hume PJ and Koronakis V (2009) The Salmonella effector SptP dephosphorylates host AAA+ ATPase VCP to promote development of its intracellular replicative niche. *Cell Host Microbe* **5**, 225–233.
 - 82 Juris SJ, Rudolph AE, Huddler D, Orth K and Dixon JE (2000) A distinctive role for the Yersinia protein kinase: actin binding, kinase activation, and cytoskeleton disruption. *Proc Natl Acad Sci USA* **97**, 9431–9436.
 - 83 Bach H, Papavinasasundaram KG, Wong D, Hmama Z and Av-Gay Y (2008) *Mycobacterium tuberculosis* virulence is mediated by PtpA dephosphorylation of human vacuolar protein sorting 33B. *Cell Host Microbe* **3**, 316–322.
 - 84 Navarro L, Koller A, Nordfelth R, Wolf-Watz H, Taylor S and Dixon JE (2007) Identification of a molecular target for the Yersinia protein kinase A. *Mol Cell* **26**, 465–477.
 - 85 Ke Y, Tan Y, Wei N, Yang F, Yang H, Cao S, Wang X, Wang J, Han Y, Bi Y *et al.* (2015) Yersinia protein kinase A phosphorylates vasodilator-stimulated phosphoprotein to modify the host cytoskeleton. *Cell Microbiol* **17**, 473–485.
 - 86 Walburger A, Koul A, Ferrari G, Nguyen L, Prescianotto-Baschong C, Huygen K, Klebl B, Thompson C, Bacher G and Pieters J (2004) Protein kinase G from pathogenic mycobacteria promotes survival within macrophages. *Science* **304**, 1800–1804.
 - 87 Hervet E, Charpentier X, Vianney A, Lazzaroni JC, Gilbert C, Atlan D and Doublet P (2011) Protein kinase LegK2 is a type IV secretion system effector involved in endoplasmic reticulum recruitment and intracellular replication of *Legionella pneumophila*. *Infect Immun* **79**, 1936–1950.
 - 88 Wong D, Li W, Chao JD, Zhou P, Narula G, Tsui C, Ko M, Xie J, Martinez-Frailes C and Av-Gay Y (2018) Protein tyrosine kinase, PtkA, is required for *Mycobacterium tuberculosis* growth in macrophages. *Sci Rep* **8**, 155.
 - 89 Jaiswal S and Srivastava KK (2018) Protein tyrosine kinase A modulates intracellular survival of mycobacteria through Galectin 3. *Biochem Biophys Res Commun* **498**, 884–890.
 - 90 Wong D, Bach H, Sun J, Hmama Z and Av-Gay Y (2011) *Mycobacterium tuberculosis* protein tyrosine phosphatase (PtpA) excludes host vacuolar-H⁺-

- ATPase to inhibit phagosome acidification. *Proc Natl Acad Sci USA* **108**, 19371–19376.
- 91 Zhou P, Li W, Wong D, Xie J and Av-Gay Y (2015) Phosphorylation control of protein tyrosine phosphatase A activity in *Mycobacterium tuberculosis*. *FEBS Lett* **589**, 326–331.
 - 92 Agarwal S, Jin H, Pancholi P and Pancholi V (2012) Serine/threonine phosphatase (SP-STP), secreted from *Streptococcus pyogenes*, is a pro-apoptotic protein. *J Biol Chem* **287**, 9147–9167.
 - 93 Ge J, Xu H, Li T, Zhou Y, Zhang Z, Li S, Liu L and Shao F (2009) A legionella type IV effector activates the NF-kappaB pathway by phosphorylating the IkkappaB family of inhibitors. *Proc Natl Acad Sci USA* **106**, 13725–13730.
 - 94 Selbach M, Moese S, Hauck CR, Meyer TF and Backert S (2002) Src is the kinase of the *Helicobacter pylori* CagA protein *in vitro* and *in vivo*. *J Biol Chem* **277**, 6775–6778.
 - 95 Backert S and Selbach M (2005) Tyrosine-phosphorylated bacterial effector proteins: the enemies within. *Trends Microbiol* **13**, 476–484.
 - 96 Phillips N, Hayward RD and Koronakis V (2004) Phosphorylation of the enteropathogenic *E. coli* receptor by the Src-family kinase c-Fyn triggers actin pedestal formation. *Nat Cell Biol* **6**, 618–625.
 - 97 Selbach M, Paul FE, Brandt S, Guye P, Daumke O, Backert S, Dehio C and Mann M (2009) Host cell interactome of tyrosine-phosphorylated bacterial proteins. *Cell Host Microbe* **5**, 397–403.
 - 98 Kaneko T, Stogios PJ, Ruan X, Voss C, Evdokimova E, Skarina T, Chung A, Liu X, Li L, Savchenko A *et al.* (2018) Identification and characterization of a large family of superbinding bacterial SH2 domains. *Nat Commun* **9**, 4549.
 - 99 O'Hare HM, Durán R, Cerveñansky C, Bellinzoni M, Wehenkel AM, Pritsch O, Obal G, Baumgartner J, Vialaret J, Johnsson K *et al.* (2008) Regulation of glutamate metabolism by protein kinases in mycobacteria. *Mol Microbiol* **70**, 1408–1423.
 - 100 England P, Wehenkel A, Martins S, Hoos S, André-Leroux G, Villarino A and Alzari PM (2009) The FHA-containing protein GarA acts as a phosphorylation-dependent molecular switch in mycobacterial signaling. *FEBS Lett* **583**, 301–307.
 - 101 Bhattacharyya N, Nkumama IN, Newland-Smith Z, Lin LY, Yin W, Cullen RE, Griffiths JS, Jarvis AR, Price MJ, Chong PY *et al.* (2018) An aspartate-specific solute-binding protein regulates protein kinase G activity to control glutamate metabolism in Mycobacteria. *MBio* **9**, <https://doi.org/10.1128/mBio.00931-18>
 - 102 Rieck B, Degiacomi G, Zimmermann M, Cascioferro A, Boldrin F, Lazar-Adler NR, Bottrill AR, le Chevalier F, Frigui W, Bellinzoni M *et al.* (2017) PknG senses amino acid availability to control metabolism and virulence of *Mycobacterium tuberculosis*. *PLoS Pathog* **13**, e1006399.
 - 103 Costerton JW, Stewart PS and Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. *Science* **284**, 1318–1322.
 - 104 Wright CJ, Xue P, Hirano T, Liu C, Whitmore SE, Hackett M and Lamont RJ (2014) Characterization of a bacterial tyrosine kinase in *Porphyromonas gingivalis* involved in polymicrobial synergy. *Microbiologyopen* **3**, 383–394.
 - 105 Jung YJ, Miller DP, Perpich JD, Fitzsimonds ZR, Shen D, Ohshima J and Lamont RJ (2019) *Porphyromonas gingivalis* tyrosine phosphatase Php1 promotes. Community development and pathogenicity. *MBio* **10**, <https://doi.org/10.1128/mBio.02004-19>
 - 106 Liu C, Miller DP, Wang Y, Merchant M and Lamont RJ (2017) Structure-function aspects of the *Porphyromonas gingivalis* tyrosine kinase Ptk1. *Mol Oral Microbiol* **32**, 314–323.
 - 107 Chawla A, Hirano T, Bainbridge BW, Demuth DR, Xie H and Lamont RJ (2010) Community signalling between *Streptococcus gordonii* and *Porphyromonas gingivalis* is controlled by the transcriptional regulator CdhR. *Mol Microbiol* **78**, 1510–1522.
 - 108 Maeda K, Tribble GD, Tucker CM, Anaya C, Shizukuishi S, Lewis JP, Demuth DR and Lamont RJ (2008) A *Porphyromonas gingivalis* tyrosine phosphatase is a multifunctional regulator of virulence attributes. *Mol Microbiol* **69**, 1153–1164.
 - 109 Germain E, Castro-Roa D, Zenkin N and Gerdes K (2013) Molecular mechanism of bacterial persistence by HipA. *Mol Cell* **52**, 248–254.
 - 110 Peddireddy V, Doddam SN and Ahmed N (2017) Mycobacterial dormancy systems and host responses in tuberculosis. *Front Immunol* **8**, 84.
 - 111 Chao JD, Papavinasasundaram KG, Zheng X, Chávez-Steenbock A, Wang X, Lee GQ and Av-Gay Y (2010) Convergence of Ser/Thr and two-component signaling to coordinate expression of the dormancy regulon in *Mycobacterium tuberculosis*. *J Biol Chem* **285**, 29239–29246.
 - 112 Khan MZ, Bhaskar A, Upadhyay S, Kumari P, Rajmani RS, Jain P, Singh A, Kumar D, Bhavesh NS and Nandicoori VK (2017) Protein kinase G confers survival advantage to *Mycobacterium tuberculosis* during latency-like conditions. *J Biol Chem* **292**, 16093–16108.
 - 113 Krüger R, Kübler D, Pallissé R, Burkovski A and Lehmann WD (2006) Protein and proteome phosphorylation stoichiometry analysis by element mass spectrometry. *Anal Chem* **78**, 1987–1994.
 - 114 Mijakovic I and Macek B (2012) Impact of phosphoproteomics on studies of bacterial physiology. *FEMS Microbiol Rev* **36**, 877–892.

- 115 Leitner A (2016) Enrichment strategies in phosphoproteomics. *Methods Mol Biol* **1355**, 105–121.
- 116 Thingholm TE and Larsen MR (2016) Phosphopeptide enrichment by immobilized metal affinity chromatography. *Methods Mol Biol* **1355**, 123–133.
- 117 Potel CM, Lemeer S and Heck AJR (2019) Phosphopeptide fragmentation and site localization by mass spectrometry: an update. *Anal Chem* **91**, 126–141.
- 118 Sharma K, D'Souza RC, Tyanova S, Schaab C, Wiśniewski JR, Cox J and Mann M (2014) Ultradeep human phosphoproteome reveals a distinct regulatory nature of Tyr and Ser/Thr-based signaling. *Cell Rep* **8**, 1583–1594.
- 119 Olsen JV, Vermeulen M, Santamaria A, Kumar C, Miller ML, Jensen LJ, Gnad F, Cox J, Jensen TS, Nigg EA *et al.* (2010) Quantitative phosphoproteomics reveals widespread full phosphorylation site occupancy during mitosis. *Sci Signal* **3**, ra3.
- 120 Gnad F, Forner F, Zielinska DF, Birney E, Gunawardena J and Mann M (2010) Evolutionary constraints of phosphorylation in eukaryotes, prokaryotes, and mitochondria. *Mol Cell Proteomics* **9**, 2642–2653.
- 121 Soares NC, Spät P, Krug K and Macek B (2013) Global dynamics of the *Escherichia coli* proteome and phosphoproteome during growth in minimal medium. *J Proteome Res* **12**, 2611–2621.
- 122 Macek B, Mijakovic I, Olsen JV, Gnad F, Kumar C, Jensen PR and Mann M (2007) The serine/threonine/tyrosine phosphoproteome of the model bacterium *Bacillus subtilis*. *Mol Cell Proteomics* **6**, 697–707.
- 123 Yagüe P, Gonzalez-Quironez N, Fernández-García G, Alonso-Fernández S and Manteca A (2019) Goals and challenges in bacterial phosphoproteomics. *Int J Mol Sci* **20**, 5678.
- 124 Lai JH, Yang JT, Chern J, Chen TL, Wu WL, Liao JH, Tsai SF, Liang SY, Chou CC and Wu SH (2016) Comparative phosphoproteomics reveals the role of AmpC β -lactamase phosphorylation in the clinical Imipenem-resistant strain *Acinetobacter baumannii* SK17. *Mol Cell Proteomics* **15**, 12–25.
- 125 Verma R, Pinto SM, Patil AH, Advani J, Subba P, Kumar M, Sharma J, Dey G, Ravikumar R, Buggi S *et al.* (2017) Quantitative proteomic and phosphoproteomic analysis of H37Ra and H37Rv strains of *Mycobacterium tuberculosis*. *J Proteome Res* **16**, 1632–1645.
- 126 Soares NC, Spät P, Méndez JA, Nakedi K, Aranda J and Bou G (2014) Ser/Thr/Tyr phosphoproteome characterization of *Acinetobacter baumannii*: comparison between a reference strain and a highly invasive multidrug-resistant clinical isolate. *J Proteomics* **102**, 113–124.
- 127 Ge R, Sun X, Xiao C, Yin X, Shan W, Chen Z and He QY (2011) Phosphoproteome analysis of the pathogenic bacterium *Helicobacter pylori* reveals over-representation of tyrosine phosphorylation and multiply phosphorylated proteins. *Proteomics* **11**, 1449–1461.
- 128 Lin MH, Hsu TL, Lin SY, Pan YJ, Jan JT, Wang JT, Khoo KH and Wu SH (2009) Phosphoproteomics of *Klebsiella pneumoniae* NTUH-K2044 reveals a tight link between tyrosine phosphorylation and virulence. *Mol Cell Proteomics* **8**, 2613–2623.
- 129 Cao XJ, Dai J, Xu H, Nie S, Chang X, Hu BY, Sheng QH, Wang LS, Ning ZB, Li YX *et al.* (2010) High-coverage proteome analysis reveals the first insight of protein modification systems in the pathogenic spirochete *Leptospira interrogans*. *Cell Res* **20**, 197–210.
- 130 Misra SK, Milohanic E, Aké F, Mijakovic I, Deutscher J, Monnet V and Henry C (2011) Analysis of the serine/threonine/tyrosine phosphoproteome of the pathogenic bacterium *Listeria monocytogenes* reveals phosphorylated proteins related to virulence. *Proteomics* **11**, 4155–4165.
- 131 Pristic S, Dankwa S, Schwartz D, Chou MF, Locasale JW, Kang CM, Bemis G, Church GM, Steen H and Husson RN (2010) Extensive phosphorylation with overlapping specificity by *Mycobacterium tuberculosis* serine/threonine protein kinases. *Proc Natl Acad Sci USA* **107**, 7521–7526.
- 132 Fortuin S, Tomazella GG, Nagaraj N, Sampson SL, Gey van Pittius NC, Soares NC, Wiker HG, de Souza GA and Warren RM (2015) Phosphoproteomics analysis of a clinical *Mycobacterium tuberculosis* Beijing isolate: expanding the mycobacterial phosphoproteome catalog. *Front Microbiol* **6**, 6.
- 133 Ouidir T, Jarnier F, Cosette P, Jouenne T and Hardouin J (2014) Extracellular Ser/Thr/Tyr phosphorylated proteins of *Pseudomonas aeruginosa* PA14 strain. *Proteomics* **14**, 2017–2030.
- 134 Ravichandran A, Sugiyama N, Tomita M, Swarup S and Ishihama Y (2009) Ser/Thr/Tyr phosphoproteome analysis of pathogenic and non-pathogenic *Pseudomonas* species. *Proteomics* **9**, 2764–2775.
- 135 Bäsell K, Otto A, Junker S, Zühlke D, Rappen GM, Schmidt S, Hentschker C, Macek B, Ohlsen K, Hecker M *et al.* (2014) The phosphoproteome and its physiological dynamics in *Staphylococcus aureus*. *Int J Med Microbiol* **304**, 121–132.
- 136 Sun X, Ge F, Xiao CL, Yin XF, Ge R, Zhang LH and He QY (2010) Phosphoproteomic analysis reveals the multiple roles of phosphorylation in pathogenic bacterium *Streptococcus pneumoniae*. *J Proteome Res* **9**, 275–282.
- 137 Calder B, Albeldas C, Blackburn JM and Soares NC (2016) Mass spectrometry offers insight into the role

- of Ser/Thr/Tyr phosphorylation in the Mycobacteria. *Front Microbiol* **7**, 141.
- 138 Baros SS, Blackburn JM and Soares NC (2020) Phosphoproteomic approaches to discover novel substrates of Mycobacterial Ser/Thr protein kinases. *Mol Cell Proteomics* **19**, 233–244.
 - 139 Lin MH, Sugiyama N and Ishihama Y (2015) Systematic profiling of the bacterial phosphoproteome reveals bacterium-specific features of phosphorylation. *Sci Signal* **8**, rs10.
 - 140 Potel CM, Lin MH, Heck AJR and Lemeer S (2018) Defeating major contaminants in Fe³⁺-immobilized metal ion affinity chromatography (IMAC) phosphopeptide enrichment. *Mol Cell Proteomics* **17**, 1028–1034.
 - 141 Potel CM, Lin MH, Heck AJR and Lemeer S (2018) Widespread bacterial protein histidine phosphorylation revealed by mass spectrometry-based proteomics. *Nat Methods* **15**, 187–190.
 - 142 Manteca A, Ye J, Sánchez J and Jensen ON (2011) Phosphoproteome analysis of *Streptomyces* development reveals extensive protein phosphorylation accompanying bacterial differentiation. *J Proteome Res* **10**, 5481–5492.
 - 143 Zhang X, Ye J, Jensen ON and Roepstorff P (2007) Highly efficient phosphopeptide enrichment by calcium phosphate precipitation combined with subsequent IMAC enrichment. *Mol Cell Proteomics* **6**, 2032–2042.
 - 144 Ruprecht B, Koch H, Medard G, Mundt M, Kuster B and Lemeer S (2015) Comprehensive and reproducible phosphopeptide enrichment using iron immobilized metal ion affinity chromatography (Fe-IMAC) columns. *Mol Cell Proteomics* **14**, 205–215.
 - 145 Sugiyama N, Miyake S, Lin MH, Wakabayashi M, Marusawa H, Nishiumi S, Yoshida M and Ishihama Y (2019) Comparative proteomics of *Helicobacter pylori* strains reveals geographical features rather than genomic variations. *Genes Cells* **24**, 139–150.
 - 146 Semajski M, Germain E, Bratl K, Kiessling A, Gerdes K and Macek B (2018) The kinases HipA and HipA7 phosphorylate different substrate pools in *Escherichia coli* to promote multidrug tolerance. *Sci Signal* **11**.
 - 147 Lin MH, Potel CM, Tehrani KHME, Heck AJR, Martin NI and Lemeer S (2018) A new tool to reveal bacterial signaling mechanisms in antibiotic treatment and resistance. *Mol Cell Proteomics* **17**, 2496–2507.
 - 148 Marcelino I, Colomé-Calls N, Holzmüller P, Lisacek F, Reynaud Y, Canals F and Vachiéry N (2019) Sweet and sour *Ehrlichia*: glycoproteomics and phosphoproteomics reveal new players in *Ehrlichia ruminantium* physiology and pathogenesis. *Front Microbiol* **10**, 450.
 - 149 Banaei-Esfahani A, Nicod C, Aebersold R and Collins BC (2017) Systems proteomics approaches to study bacterial pathogens: application to *Mycobacterium tuberculosis*. *Curr Opin Microbiol* **39**, 64–72.
 - 150 Kaur P, Rausch M, Malakar B, Watson U, Damle NP, Chawla Y, Srinivasan S, Sharma K, Schneider T, Jhingan GD *et al.* (2019) LipidII interaction with specific residues of *Mycobacterium tuberculosis* PknB extracytoplasmic domain governs its optimal activation. *Nat Commun* **10**, 1231.
 - 151 de Keijzer J, Mulder A, de Beer J, de Ru AH, van Veelen PA and van Soolingen D (2016) Mechanisms of phenotypic rifampicin tolerance in *Mycobacterium tuberculosis* Beijing genotype strain B0/W148 revealed by proteomics. *J Proteome Res* **15**, 1194–1204.
 - 152 Bian Y, Li L, Dong M, Liu X, Kaneko T, Cheng K, Liu H, Voss C, Cao X, Wang Y *et al.* (2016) Ultra-deep tyrosine phosphoproteomics enabled by a phosphotyrosine superbinder. *Nat Chem Biol* **12**, 959–966.
 - 153 Pan Z, Wang B, Zhang Y, Wang Y, Ullah S, Jian R, Liu Z and Xue Y (2015) dbPSP: a curated database for protein phosphorylation sites in prokaryotes. *Database (Oxford)* **2015**, bav031.
 - 154 Pagano GJ and Arsenault RJ (2019) Advances, challenges and tools in characterizing bacterial serine, threonine and tyrosine kinases and phosphorylation target sites. *Expert Rev Proteomics* **16**, 431–441.
 - 155 Nita-Lazar A, Saito-Benz H and White FM (2008) Quantitative phosphoproteomics by mass spectrometry: past, present, and future. *Proteomics* **8**, 4433–4443.
 - 156 Mann M (2006) Functional and quantitative proteomics using SILAC. *Nat Rev Mol Cell Biol* **7**, 952–958.
 - 157 Misra SK, Moussan Désirée Aké F, Wu Z, Milohanic E, Cao TN, Cossart P, Deutscher J, Monnet V, Archambaud C and Henry C (2014) Quantitative proteome analyses identify PrfA-responsive proteins and phosphoproteins in *Listeria monocytogenes*. *J Proteome Res* **13**, 6046–6057.
 - 158 Ziveri J, Chhuon C, Jamet A, Rytter H, Prigent G, Tros F, Barel M, Coureuil M, Lays C, Henry T *et al.* (2019) Critical role of a sheath phosphorylation site on the assembly and function of an atypical type VI secretion system. *Mol Cell Proteomics* **18**, 2418–2432.
 - 159 Parandhaman DK, Sharma P, Bisht D and Narayanan S (2014) Proteome and phosphoproteome analysis of the serine/threonine protein kinase E mutant of *Mycobacterium tuberculosis*. *Life Sci* **109**, 116–126.
 - 160 Silvestroni A, Jewell KA, Lin WJ, Connelly JE, Ivancic MM, Tao WA and Rajagopal L (2009) Identification of serine/threonine kinase substrates in the human pathogen group B streptococcus. *J Proteome Res* **8**, 2563–2574.

- 161 Baer CE, Iavarone AT, Alber T and Sassetti CM (2014) Biochemical and spatial coincidence in the provisional Ser/Thr protein kinase interaction network of *Mycobacterium tuberculosis*. *J Biol Chem* **289**, 20422–20433.
- 162 Imami K, Bhavsar AP, Yu H, Brown NF, Rogers LD, Finlay BB and Foster LJ (2013) Global impact of *Salmonella* pathogenicity island 2-secreted effectors on the host phosphoproteome. *Mol Cell Proteomics* **12**, 1632–1643.
- 163 Holland C, Schmid M, Zimny-Arndt U, Rohloff J, Stein R, Jungblut PR and Meyer TF (2011) Quantitative phosphoproteomics reveals link between *Helicobacter pylori* infection and RNA splicing modulation in host cells. *Proteomics* **11**, 2798–2811.
- 164 Glowinski F, Holland C, Thiede B, Jungblut PR and Meyer TF (2014) Analysis of T4SS-induced signaling by *H. pylori* using quantitative phosphoproteomics. *Front Microbiol* **5**, 356.
- 165 Scholz R, Imami K, Scott NE, Trimble WS, Foster LJ and Finlay BB (2015) Novel host proteins and signaling pathways in enteropathogenic *E. coli* pathogenesis identified by global phosphoproteome analysis. *Mol Cell Proteomics* **14**, 1927–1945.
- 166 Fabrik I, Link M, Putzova D, Plzakova L, Lubovska Z, Philimonenko V, Pavkova I, Rehulka P, Krocova Z, Hozak P *et al.* (2018) The early dendritic cell signaling induced by virulent *Francisella tularensis* strain occurs in phases and involves the activation of extracellular signal-regulated kinases (ERKs) and p38 in the later stage. *Mol Cell Proteomics* **17**, 81–94.
- 167 Michalik S, Depke M, Murr A, Gesell Salazar M, Kusebauch U, Sun Z, Meyer TC, Surmann K, Pförtner H, Hildebrandt P *et al.* (2017) A global *Staphylococcus aureus* proteome resource applied to the *in vivo* characterization of host-pathogen interactions. *Sci Rep* **7**, 9718.
- 168 Malherbe G, Humphreys DP and Davé E (2019) A robust fractionation method for protein subcellular localization studies in *Escherichia coli*. *Biotechniques* **66**, 171–178.
- 169 Yang Y, Hu M, Yu K, Zeng X and Liu X (2015) Mass spectrometry-based proteomic approaches to study pathogenic bacteria-host interactions. *Protein Cell* **6**, 265–274.
- 170 Kelstrup CD, Bekker-Jensen DB, Arrey TN, Högrelbe A, Harder A and Olsen JV (2018) Performance evaluation of the Q Exactive HF-X for shotgun proteomics. *J Proteome Res* **17**, 727–738.
- 171 Humphrey SJ, Karayel O, James DE and Mann M (2018) High-throughput and high-sensitivity phosphoproteomics with the EasyPhos platform. *Nat Protoc* **13**, 1897–1916.
- 172 Leutert M, Rodríguez-Mias RA, Fukuda NK and Villén J (2019) R2–P2 rapid-robotic phosphoproteomics enables multidimensional cell signaling studies. *Mol Syst Biol* **15**, e9021.
- 173 Högrelbe A, von Stechow L, Bekker-Jensen DB, Weinert BT, Kelstrup CD and Olsen JV (2018) Benchmarking common quantification strategies for large-scale phosphoproteomics. *Nat Commun* **9**, 1045.
- 174 Wilkes E and Cutillas PR (2017) Label-free phosphoproteomic approach for kinase signaling analysis. *Methods Mol Biol* **1636**, 199–217.
- 175 Cox J, Hein MY, Luber CA, Paron I, Nagaraj N and Mann M (2014) Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. *Mol Cell Proteomics* **13**, 2513–2526.
- 176 Ludwig C, Gillet L, Rosenberger G, Amon S, Collins BC and Aebersold R (2018) Data-independent acquisition-based SWATH-MS for quantitative proteomics: a tutorial. *Mol Syst Biol* **14**, e8126.
- 177 Bekker-Jensen DB, Bernhardt OM, Högrelbe A, Martinez-Val A, Verbeke L, Gandhi T, Kelstrup CD, Reiter L and Olsen JV (2020) Rapid and site-specific deep phosphoproteome profiling by data-independent acquisition without the need for spectral libraries. *Nat Commun* **11**, 787.
- 178 Peckner R, Myers SA, Jacome ASV, Egerton JD, Abelin JG, MacCoss MJ, Carr SA and Jaffe JD (2018) Specter: linear deconvolution for targeted analysis of data-independent acquisition mass spectrometry proteomics. *Nat Methods* **15**, 371–378.
- 179 Tiwary S, Levy R, Gutenbrunner P, Salinas Soto F, Palaniappan KK, Deming L, Berndt M, Brant A, Cimermanic P and Cox J (2019) High-quality MS/MS spectrum prediction for data-dependent and data-independent acquisition data analysis. *Nat Methods* **16**, 519–525.
- 180 Tran NH, Qiao R, Xin L, Chen X, Liu C, Zhang X, Shan B, Ghodsi A and Li M (2019) Deep learning enables de novo peptide sequencing from data-independent-acquisition mass spectrometry. *Nat Methods* **16**, 63–66.
- 181 Banerjee SL, Dionne U, Lambert JP and Bisson N (2018) Targeted proteomics analyses of phosphorylation-dependent signalling networks. *J Proteomics* **189**, 39–47.
- 182 Taumer C, Griesbaum L, Kovacevic A, Soufi B, Nalpas NC and Macek B (2018) Parallel reaction monitoring on a Q Exactive mass spectrometer increases reproducibility of phosphopeptide detection in bacterial phosphoproteomics measurements. *J Proteomics* **189**, 60–66.
- 183 Landry CR, Levy ED and Michnick SW (2009) Weak functional constraints on phosphoproteomes. *Trends Genet* **25**, 193–197.
- 184 Levy ED, Michnick SW and Landry CR (2012) Protein abundance is key to distinguish promiscuous

- from functional phosphorylation based on evolutionary information. *Philos Trans R Soc Lond B Biol Sci* **367**, 2594–2606.
- 185 Beltrao P, Albanese V, Kenner LR, Swaney DL, Burlingame A, Villén J, Lim WA, Fraser JS, Frydman J and Krogan NJ (2012) Systematic functional prioritization of protein posttranslational modifications. *Cell* **150**, 413–425.
 - 186 Strumillo MJ, Oplová M, Viéitez C, Ochoa D, Shahrzad M, Busby BP, Sopko R, Studer RA, Perrimon N, Panse VG *et al.* (2019) Conserved phosphorylation hotspots in eukaryotic protein domain families. *Nat Commun* **10**, 1977.
 - 187 Lienhard GE (2008) Non-functional phosphorylations? *Trends Biochem Sci* **33**, 351–352.
 - 188 Studer RA, Rodriguez-Mias RA, Haas KM, Hsu JJ, Viéitez C, Solé C, Swaney DL, Stanford LB, Liachko I, Böttcher R *et al.* (2016) Evolution of protein phosphorylation across 18 fungal species. *Science* **354**, 229–232.
 - 189 Macek B, Gnad F, Soufi B, Kumar C, Olsen JV, Mijakovic I and Mann M (2008) Phosphoproteome analysis of *E. coli* reveals evolutionary conservation of bacterial Ser/Thr/Tyr phosphorylation. *Mol Cell Proteomics* **7**, 299–307.
 - 190 Holt LJ, Tuch BB, Villén J, Johnson AD, Gygi SP and Morgan DO (2009) Global analysis of Cdk1 substrate phosphorylation sites provides insights into evolution. *Science* **325**, 1682–1686.
 - 191 Iakoucheva LM, Radivojac P, Brown CJ, O'Connor TR, Sikes JG, Obradovic Z and Dunker AK (2004) The importance of intrinsic disorder for protein phosphorylation. *Nucleic Acids Res* **32**, 1037–1049.
 - 192 Craveur P, Rebehmed J and de Brevern AG (2014) PTM-SD: a database of structurally resolved and annotated posttranslational modifications in proteins. *Database (Oxford)* **2014**, <https://doi.org/10.1093/database/bau041>
 - 193 Gao J, Prlic A, Bi C, Bluhm WF, Dimitropoulos D, Xu D, Bourne PE and Rose PW (2017) BioJava-ModFinder: identification of protein modifications in 3D structures from the Protein Data Bank. *Bioinformatics* **33**, 2047–2049.
 - 194 Li F, Fan C, Marquez-Lago TT, Leier A, Revote J, Jia C, Zhu Y, Smith AI, Webb GI, Liu Q *et al.* (2020) PRISMOID: a comprehensive 3D structure database for post-translational modifications and mutations with functional impact. *Brief Bioinform* **3**, 1069–1079. <https://doi.org/10.1093/bib/bbz050>
 - 195 Kuhlman B and Bradley P (2019) Advances in protein structure prediction and design. *Nat Rev Mol Cell Biol* **20**, 681–697.
 - 196 Jones DT (2019) Setting the standards for machine learning in biology. *Nat Rev Mol Cell Biol* **20**, 659–660.
 - 197 Camacho DM, Collins KM, Powers RK, Costello JC and Collins JJ (2018) Next-generation machine learning for biological networks. *Cell* **173**, 1581–1592.
 - 198 Ching T, Himmelstein DS, Beaulieu-Jones BK, Kalinin AA, Do BT, Way GP, Ferrero E, Agapow PM, Zietz M, Hoffman MM *et al.* (2018) Opportunities and obstacles for deep learning in biology and medicine. *J R Soc Interface* **15**, <https://doi.org/10.1098/rsif.2017.0387>
 - 199 He W, Wei L and Zou Q (2018) Research progress in protein posttranslational modification site prediction. *Brief Funct Genomics* **18**, 220–229.
 - 200 Wong YH, Lee TY, Liang HK, Huang CM, Wang TY, Yang YH, Chu CH, Huang HD, Ko MT and Hwang JK (2007) KinasePhos 2.0: a web server for identifying protein kinase-specific phosphorylation sites based on sequences and coupling patterns. *Nucleic Acids Res* **35**, W588–W594.
 - 201 Miller ML, Soufi B, Jers C, Blom N, Macek B and Mijakovic I (2009) NetPhosBac - a predictor for Ser/Thr phosphorylation sites in bacterial proteins. *Proteomics* **9**, 116–125.
 - 202 Li Z, Wu P, Zhao Y, Liu Z and Zhao W (2015) Prediction of serine/threonine phosphorylation sites in bacteria proteins. *Adv Exp Med Biol* **827**, 275–285.
 - 203 Hasan MM, Rashid MM, Khatun MS and Kurata H (2019) Computational identification of microbial phosphorylation sites by the enhanced characteristics of sequence information. *Sci Rep* **9**, 8258.
 - 204 Durek P, Schudoma C, Weckwerth W, Selbig J and Walther D (2009) Detection and characterization of 3D-signature phosphorylation site motifs and their contribution towards improved phosphorylation site prediction in proteins. *BMC Bioinformatics* **10**, 117.
 - 205 Wang D, Zeng S, Xu C, Qiu W, Liang Y, Joshi T and Xu D (2017) MusiteDeep: a deep-learning framework for general and kinase-specific phosphorylation site prediction. *Bioinformatics* **33**, 3909–3916.
 - 206 Luo F, Wang M, Liu Y, Zhao XM and Li A (2019) DeepPhos: prediction of protein phosphorylation sites with deep learning. *Bioinformatics* **35**, 2766–2773.
 - 207 Xiao Q, Miao B, Bi J, Wang Z and Li Y (2016) Prioritizing functional phosphorylation sites based on multiple feature integration. *Sci Rep* **6**, 24735.
 - 208 Ochoa D, Jarnuczak AF, Viéitez C, Gehre M, Soucheray M, Mateus A, Kleefeldt AA, Hill A, Garcia-Alonso L, Stein F *et al.* (2020) The functional landscape of the human phosphoproteome. *Nat Biotechnol* **38**, 365–373.
 - 209 Kanshin E, Giguère S, Jing C, Tyers M and Thibault P (2017) Machine learning of global phosphoproteomic profiles enables discrimination of direct versus indirect kinase substrates. *Mol Cell Proteomics* **16**, 786–798.

- 210 Fondi M and Liò P (2015) Multi-omics and metabolic modelling pipelines: challenges and tools for systems microbiology. *Microbiol Res* **171**, 52–64.
- 211 Jean Beltran PM, Federspiel JD, Sheng X and Cristea IM (2017) Proteomics and integrative omic approaches for understanding host-pathogen interactions and infectious diseases. *Mol Syst Biol* **13**, 922.
- 212 Deatherage Kaiser BL, Li J, Sanford JA, Kim YM, Kronewitter SR, Jones MB, Peterson CT, Peterson SN, Frank BC, Purvine SO *et al.* (2013) A multi-omic view of host-pathogen-commensal interplay in *Salmonella*-mediated intestinal infection. *PLoS ONE* **8**, e67155.
- 213 Ramos PIP, Fernández Do Porto D, Lanzarotti E, Sosa EJ, Burguener G, Pardo AM, Klein CC, Sagot MF, de Vasconcelos ATR, Gales AC *et al.* (2018) An integrative, multi-omics approach towards the prioritization of *Klebsiella pneumoniae* drug targets. *Sci Rep* **8**, 10755.
- 214 Chen WH, van Noort V, Lluch-Senar M, Hennrich ML, Wodke JA, Yus E, Alibés A, Roma G, Mende DR, Pesavento C *et al.* (2016) Integration of multi-omics data of a genome-reduced bacterium: prevalence of post-transcriptional regulation and its correlation with protein abundances. *Nucleic Acids Res* **44**, 1192–1202.
- 215 Khan MM, Ernst O, Manes NP, Oyler BL, Fraser IDC, Goodlett DR and Nita-Lazar A (2019) Multi-omics strategies uncover host-pathogen interactions. *ACS Infect Dis* **5**, 493–505.
- 216 Wani N and Raza K (2019) Integrative approaches to reconstruct regulatory networks from multi-omics data: a review of state-of-the-art methods. *Comput Biol Chem* **83**, 107120.
- 217 Prestinaci F, Pezzotti P and Pantosti A (2015) Antimicrobial resistance: a global multifaceted phenomenon. *Pathog Glob Health* **109**, 309–318.
- 218 Huang YY, Sun YH, Huang N, Liu XX, Yan J and Sun AH (2018) Sublethal β -lactam antibiotics induce PhpP phosphatase expression and StkP kinase phosphorylation in PBP-independent β -lactam antibiotic resistance of *Streptococcus pneumoniae*. *Biochem Biophys Res Commun* **503**, 2000–2008.
- 219 Pensinger DA, Aliota MT, Schaezner AJ, Boldon KM, Ansari IU, Vincent WJ, Knight B, Reniere ML, Striker R and Sauer JD (2014) Selective pharmacologic inhibition of a PASTA kinase increases *Listeria monocytogenes* susceptibility to β -lactam antibiotics. *Antimicrob Agents Chemother* **58**, 4486–4494.
- 220 Labbe BD, Hall CL, Kellogg SL, Chen Y, Koehn O, Pickrum AM, Mirza SP and Kristich CJ (2019) Reciprocal regulation of PASTA kinase signaling by differential modification. *J Bacteriol* **201**, <https://doi.org/10.1128/JB.00016-19>
- 221 Beltramini AM, Mukhopadhyay CD and Pancholi V (2009) Modulation of cell wall structure and antimicrobial susceptibility by a *Staphylococcus aureus* eukaryote-like serine/threonine kinase and phosphatase. *Infect Immun* **77**, 1406–1416.
- 222 Dias R, Félix D, Caniça M and Trombe MC (2009) The highly conserved serine threonine kinase StkP of *Streptococcus pneumoniae* contributes to penicillin susceptibility independently from genes encoding penicillin-binding proteins. *BMC Microbiol* **9**, 121.
- 223 Boudreau MA, Fishovitz J, Llarrull LI, Xiao Q and Mobashery S (2015) Phosphorylation of BlaR1 in manifestation of antibiotic resistance in Methicillin-resistant *Staphylococcus aureus* and its abrogation by small molecules. *ACS Infect Dis* **1**, 454–459.
- 224 Kellogg SL and Kristich CJ (2018) Convergence of PASTA kinase and two-component signaling in response to cell wall stress in *Enterococcus faecalis*. *J Bacteriol* **200**, <https://doi.org/10.1128/JB.00086-18>
- 225 Canova MJ, Baronian G, Brelle S, Cohen-Gonsaud M, Bischoff M and Molle V (2014) A novel mode of regulation of the *Staphylococcus aureus* Vancomycin-resistance-associated response regulator VraR mediated by Stk1 protein phosphorylation. *Biochem Biophys Res Commun* **447**, 165–171.
- 226 Truong-Bolduc QC and Hooper DC (2010) Phosphorylation of MgrA and its effect on expression of the NorA and NorB efflux pumps of *Staphylococcus aureus*. *J Bacteriol* **192**, 2525–2534.
- 227 Wolff KA, Nguyen HT, Cartabuke RH, Singh A, Ogowang S and Nguyen L (2009) Protein kinase G is required for intrinsic antibiotic resistance in mycobacteria. *Antimicrob Agents Chemother* **53**, 3515–3519.
- 228 Leiba J, Carrère-Kremer S, Blondiaux N, Dimala MM, Wohlkönig A, Baulard A, Kremer L and Molle V (2014) The *Mycobacterium tuberculosis* transcriptional repressor EthR is negatively regulated by serine/threonine phosphorylation. *Biochem Biophys Res Commun* **446**, 1132–1138.
- 229 Bem AE, Velikova N, Pellicer MT, Baarlen P, Marina A and Wells JM (2015) Bacterial histidine kinases as novel antibacterial drug targets. *ACS Chem Biol* **10**, 213–224.
- 230 Bhullar KS, Lagarón NO, McGowan EM, Parmar I, Jha A, Hubbard BP and Rupasinghe HPV (2018) Kinase-targeted cancer therapies: progress, challenges and future directions. *Mol Cancer* **17**, 48.
- 231 Fernandez P, Saint-Joanis B, Barilone N, Jackson M, Gicquel B, Cole ST and Alzari PM (2006) The Ser/Thr protein kinase PknB is essential for sustaining mycobacterial growth. *J Bacteriol* **188**, 7778–7784.
- 232 Wehenkel A, Fernandez P, Bellinzoni M, Catherinot V, Barilone N, Labesse G, Jackson M and Alzari PM (2006) The structure of PknB in complex with

- mitoxantrone, an ATP-competitive inhibitor, suggests a mode of protein kinase regulation in mycobacteria. *FEBS Lett* **580**, 3018–3022.
- 233 Loughheed KE, Osborne SA, Saxty B, Whalley D, Chapman T, Boulloc N, Chugh J, Nott TJ, Patel D, Spivey VL *et al.* (2011) Effective inhibitors of the essential kinase PknB and their potential as anti-mycobacterial agents. *Tuberculosis* **91**, 277–286.
 - 234 Chapman TM, Boulloc N, Buxton RS, Chugh J, Loughheed KE, Osborne SA, Saxty B, Smerdon SJ, Taylor DL and Whalley D (2012) Substituted aminopyrimidine protein kinase B (PknB) inhibitors show activity against *Mycobacterium tuberculosis*. *Bioorg Med Chem Lett* **22**, 3349–3353.
 - 235 Xu J, Wang JX, Zhou JM, Xu CL, Huang B, Xing Y, Wang B, Luo R, Wang YC, You XF *et al.* (2017) A novel protein kinase inhibitor IMB-YH-8 with anti-tuberculosis activity. *Sci Rep* **7**, 5093.
 - 236 Wlodarchak N, Teachout N, Beczkiewicz J, Procknow R, Schaezner AJ, Satyshur K, Pavelka M, Zuercher W, Drewry D, Sauer JD *et al.* (2018) *In silico* screen and structural analysis identifies bacterial kinase inhibitors which act with β -lactams to inhibit mycobacterial growth. *Mol Pharm* **15**, 5410–5426.
 - 237 Schaezner AJ, Wlodarchak N, Drewry DH, Zuercher WJ, Rose WE, Striker R and Sauer JD (2017) A screen for kinase inhibitors identifies antimicrobial imidazopyridine aminofurazans as specific inhibitors of the *Listeria monocytogenes* PASTA kinase PrkA. *J Biol Chem* **292**, 17037–17045.
 - 238 Vornhagen J, Burnside K, Whidbey C, Berry J, Qin X and Rajagopal L (2015) Kinase inhibitors that increase the sensitivity of Methicillin resistant *Staphylococcus aureus* to β -lactam antibiotics. *Pathogens* **4**, 708–721.
 - 239 Schaezner AJ, Wlodarchak N, Drewry DH, Zuercher WJ, Rose WE, Ferrer CA, Sauer JD and Striker R (2018) GW779439X and its pyrazolopyridazine derivatives inhibit the serine/threonine kinase Stk1 and act as antibiotic adjuvants against β -lactam-resistant *Staphylococcus aureus*. *ACS Infect Dis* **4**, 1508–1518.
 - 240 Kant S, Asthana S, Missiakas D and Pancholi V (2017) A novel STK1-targeted small-molecule as an "antibiotic resistance breaker" against multidrug-resistant *Staphylococcus aureus*. *Sci Rep* **7**, 5067.
 - 241 Scherr N, Honnappa S, Kunz G, Mueller P, Jayachandran R, Winkler F, Pieters J and Steinmetz MO (2007) Structural basis for the specific inhibition of protein kinase G, a virulence factor of *Mycobacterium tuberculosis*. *Proc Natl Acad Sci USA* **104**, 12151–12156.
 - 242 Kanehiro Y, Tomioka H, Pieters J, Tatano Y, Kim H, Iizasa H and Yoshiyama H (2018) Identification of novel Mycobacterial inhibitors against mycobacterial protein kinase G. *Front Microbiol* **9**, 1517.
 - 243 Khan MZ, Kaur P and Nandicoori VK (2018) Targeting the messengers: Serine/threonine protein kinases as potential targets for antimycobacterial drug development. *IUBMB Life* **70**, 889–904.
 - 244 Mori M, Sammartino JC, Costantino L, Gelain A, Meneghetti F, Villa S and Chiarelli LR (2019) An overview on the potential antimycobacterial agents targeting serine/threonine protein kinases from *Mycobacterium tuberculosis*. *Curr Top Med Chem* **19**, 646–661.
 - 245 Wang T, Bemis G, Hanzelka B, Zuccola H, Wynn M, Moody CS, Green J, Locher C, Liu A, Gao H *et al.* (2017) Mtb PKNA/PKNB dual inhibition provides selectivity advantages for inhibitor design to minimize host kinase interactions. *ACS Med Chem Lett* **8**, 1224–1229.
 - 246 Ortiz-Lombardía M, Pompeo F, Boitel B and Alzari PM (2003) Crystal structure of the catalytic domain of the PknB serine/threonine kinase from *Mycobacterium tuberculosis*. *J Biol Chem* **278**, 13094–13100.
 - 247 Jone G and Dyson P (2006) Evolution of transmembrane protein kinases implicated in coordinating remodeling of gram-positive peptidoglycan: inside versus outside. *J Bacteriol* **188**, 7470–7476.
 - 248 Cozzone AJ (2009) Bacterial tyrosine kinases: novel targets for antibacterial therapy? *Trends Microbiol* **17**, 536–543.
 - 249 Standish AJ, Salim AA, Zhang H, Capon RJ and Morona R (2012) Chemical inhibition of bacterial protein tyrosine phosphatase suppresses capsule production. *PLoS ONE* **7**, e36312.
 - 250 Sens L, de Souza ACA, Pacheco LA, Menegatti ACO, Mori M, Mascarello A, Nunes RJ and Terenzi H (2018) Synthetic thiosemicarbazones as a new class of *Mycobacterium tuberculosis* protein tyrosine phosphatase A inhibitors. *Bioorg Med Chem* **26**, 5742–5750.
 - 251 Young C, Walzl G and Du Plessis N (2019) Therapeutic host-directed strategies to improve outcome in tuberculosis. *Mucosal Immunol* **13**, 190–204.
 - 252 Napier RJ, Norris BA, Swimm A, Giver CR, Harris WA, Laval J, Napier BA, Patel G, Crump R, Peng Z *et al.* (2015) Low doses of imatinib induce myelopoiesis and enhance host anti-microbial immunity. *PLoS Pathog* **11**, e1004770.