

## REVIEW

# Proteomic discovery of host kinase signaling in bacterial infections

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Protein phosphorylation catalyzed by protein kinases acts as a reversible molecular switch in signal transduction, providing a mechanism for the control of protein function in cellular processes. During microbial infection, cellular signaling essentially contributes to immune control to restrict the dissemination of invading pathogens within the host organism. However, pathogenic microbes compete for the control of host signaling to create a beneficial environment for successful invasion and infection. Although efforts to achieve a better understanding of the host–pathogen interaction and its molecular consequences have been made, there is urgent need for a comprehensive characterization of infection-related host signaling processes. System-wide and hypothesis-free analysis of phosphorylation-mediated host signaling during host–microbe interactions by mass spectrometry (MS)-based methods is not only promising in view of a greater understanding of the pathogenesis of the infection but also may result in the identification of novel host targets for preventive or therapeutic intervention. Here, we review state-of-the-art MS-based techniques for the system-wide identification and quantitation of protein phosphorylation and compare them to array-based phosphoprotein analyses. We also provide an overview of how phosphoproteomics and kinomics have contributed to our understanding of protein kinase-driven phosphorylation networks that operate during host–microbe interactions.

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## 1 Introduction

Protein phosphorylation, which acts as a reversible molecular switch, provides a mechanism for the control of protein function during virtually all cellular processes and is essential for the rapid responses of cells to internal and external cues. The covalent attachment of phosphate groups from adenosine triphosphate (ATP) to proteins at serine, threonine and tyrosine residues is achieved by protein kinases, and the

phosphate groups attached at these residues are removed by protein phosphatases. The human kinome consists of approximately 520 protein kinases [1], but only up to 70–80% of these are likely expressed in eukaryotic cells at a given time [2–4]. On the other hand, recent studies have identified tens of thousands of phosphorylation events in up to 8000 proteins and shown that individual proteins are often phosphorylated at multiple sites, leading to estimations that at least two-thirds of the proteins in most cells are phosphorylated [5, 6].

Protein function is regulated by phosphorylation through conformational changes, which may either directly modulate enzymatic activity or provide a docking site for intra- or intermolecular protein interactions. These changes influence the subcellular localization and turnover of proteins as well as the interplay of phosphorylation with other posttranslational protein modifications (PTMs). Kinases and their substrates thereby form dynamic complexes and temporal information-processing networks that facilitate cell–cell communication and cellular responses to changing environmental conditions.

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**Abbreviations:** ATP, adenosine triphosphate; HILIC, hydrophilic interaction chromatography; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; PRR, pattern recognition receptor; RPPA, reverse-phase protein microarray; TLR, Toll-like receptor; TMT, tandem mass tag

Phosphorylation-based signaling contributes in an essential manner to innate and adaptive immunity, e.g., in the defense of host cells against pathogenic bacteria. Key processes of the immune system, such as differentiation, cytokine/chemokine production, inflammation and bacterial killing, are controlled to a large extent by protein phosphorylation and consequently by the corresponding protein kinases. In the innate immune system, microbe-associated molecular patterns are recognized by specific pattern recognition receptors (PRR) that upon engagement trigger proinflammatory and antimicrobial responses. These receptors also represent an important link to the adaptive immune system that ultimately results in resolution of infection and immunological memory. For example, the Toll-like receptor (TLR) family of PRRs has been extensively studied. Stimulation of TLR proteins results in the consecutive activation of many kinases, including interleukin-1 receptor-associated kinases (IRAK1, 2 and 4), mitogen-activated protein kinases (MAPKs; e.g., MAP3K7/TAK1, p38-alpha and JNK) and I $\kappa$ B kinase (IKK) and the subsequent phosphorylation of downstream targets, such as activator protein 1 (AP-1) and nuclear factor  $\kappa$ B (Nf- $\kappa$ b), which function as master transcriptional regulators during the induction of proinflammatory and anti-apoptotic mediators (reviewed in [7, 8]). Deregulation of these signaling processes is associated with inflammatory diseases, autoimmunity and the pathogenesis of infections.

On the other hand, it has recently become clear that during an infection bacterial pathogens exploit host signaling pathways associated with major processes, such as membrane and cytoskeleton dynamics, autophagy, vesicle trafficking, cell death, inflammation and immunity. These bacterial pathogens have evolved mechanisms, such as the production of specific toxins, effector molecules or virulence factors, by means of which they subvert and control these signaling pathways for their own benefit by supporting bacterial adherence, survival, replication or dissemination. Interference with kinase-mediated phosphorylation in host signaling is a beneficial strategy used by many pathogens. Examples include the uptake of cells of *Yersinia*, *Salmonella* and *Staphylococcus* species, which has been demonstrated to be dependent on the formation of focal adhesion-like complexes and the activities of associated protein kinases, including the cytoplasmic tyrosine kinases FAK and SRC [9–14]. Several Gram-negative bacterial pathogens (e.g., enteropathogenic and enterohemorrhagic *Escherichia coli* and *Pseudomonas aeruginosa*) modulate critical host regulators of actin dynamics, such as small Rho GTPases [15, 16] and the Src-family and Abl tyrosine kinases [17–19]. Furthermore, after successful internalization, *S. Typhimurium* activates the host protein kinases A (PKA) and PKB/AKT1 to promote its intracellular lifestyle [20–22]. Eventually, *Yersinia* spp. disrupt the innate immune response by irreversible inactivation of MAPKs and IKK, resulting in attenuation of the immune response and cell death [23, 24].

Although the examples provided above demonstrate the significance of host protein kinases and phosphorylation in

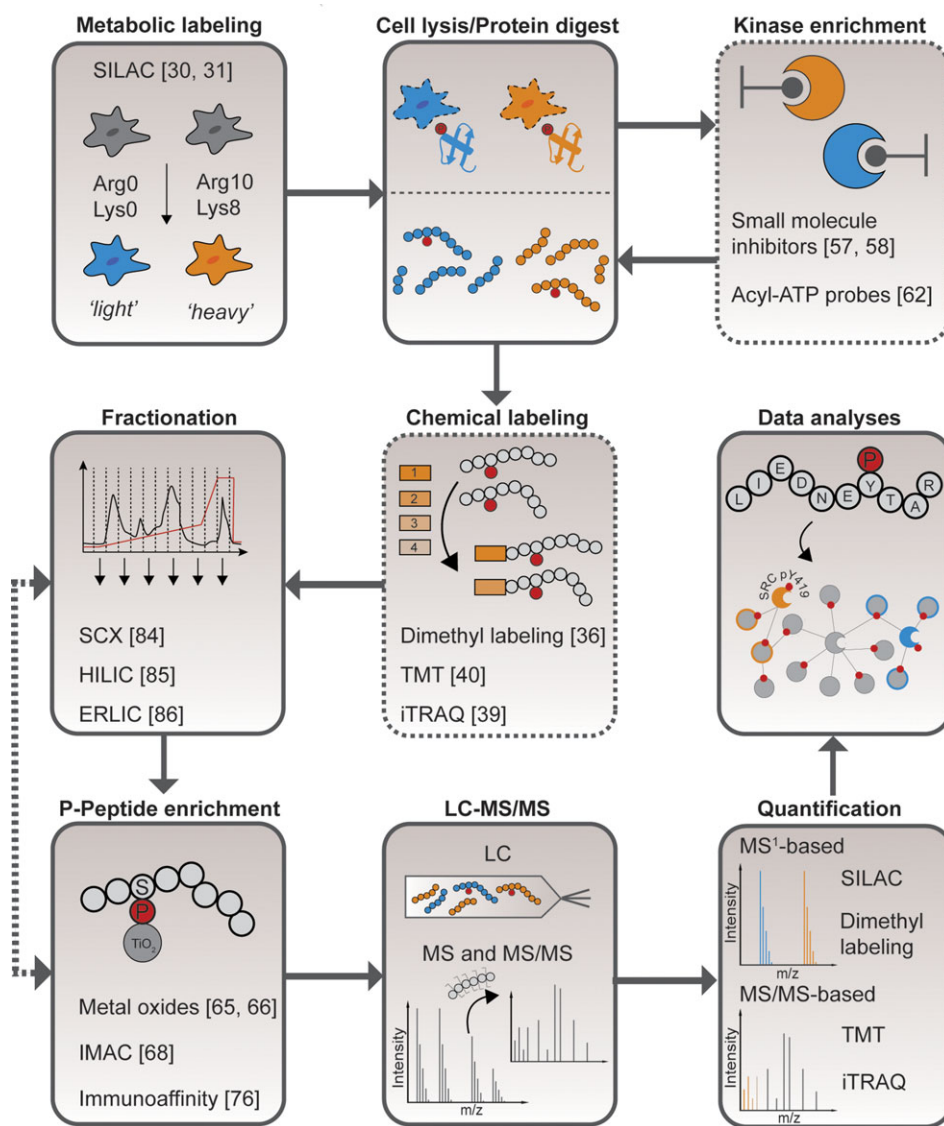
the pathogenesis of bacterial infections, cellular signaling during infection remains to be elucidated in full detail. Widely used classical single-observation experiments are hypothesis driven and do not provide a global view of the biological system. In contrast, proteomics of PTMs has been limited to research groups with specialized knowledge and equipment. However, the system-wide and hypothesis-free characterization of phosphorylation-mediated host signaling during host–microbe interactions is not only promising in view of its potential to provide a deeper and more rapid understanding of the pathogenesis of microbial and viral infections, but it may also result in the system-wide identification of novel host targets that are suitable for preventive or therapeutic intervention.

In this review, we will describe current phosphoproteomic and kinomic workflows, describe state-of-the-art mass spectrometry (MS)-based methodology for the global identification and quantitation of protein phosphorylation and compare this methodology with array-based technologies. In addition, we will exemplify how unbiased system-wide phosphoproteomic and kinomic screening has contributed to our understanding of protein kinase-driven phosphorylation networks in host–microbe interactions.

## 2 MS-based phosphoproteomic and kinomic technologies

MS-based proteomics usually involves the proteolytic digestion of proteomes into peptide mixtures that are separated by nano-liquid chromatography (LC) using reversed-phase (C18) material. The mass-to-charge ratio ( $m/z$ ) and intensity of the eluted ionized molecules are subsequently measured in a survey spectrum or precursor scan ( $MS^1$  spectrum) in a mass spectrometer. At the same time, peptides are fragmented and the resulting fragment ions are detected in an MS/MS spectrum. The acquired spectra provide the basis for the identification of the proteins in the sample and mapping of their PTMs.

Technological development in recent years has yielded super-fast and highly accurate mass spectrometers. Consequently, MS became the method of choice for the study not only of proteins in complex mixtures but also of their PTMs on a global scale. However, protein phosphorylations are generally of low abundance due to the substoichiometric nature of the modification. Recent advances in protein analysis, including the development of enrichment and purification strategies for phosphopeptides and improvements in sample processing and computational data analysis, have accelerated the application of MS-based workflows to the analysis of phosphorylation and enabled scientists to simultaneously monitor the phosphorylation patterns of thousands of proteins with site-specific accuracy. An overview of the steps included in a phosphoproteomic workflow is depicted in Fig. 1.



**Figure 1.** General workflow for MS-based phosphoproteomics. The initial step in a standard phosphoproteomic experiment is the metabolic labeling of cells using SILAC. Sample processing includes cell lysis under denaturing conditions, mixing of corresponding differentially labeled samples and subsequent proteolytic digestion. For kinase analysis, cells are lysed under non-denaturing conditions, and kinase affinity enrichment using small molecules or Acyl-ATP probes is performed prior to tryptic digestion. When SILAC is not applicable, tryptic peptides can be labeled chemically. The samples are then fractionated by chromatography, and phosphopeptides are enriched using TiO<sub>2</sub>, IMAC or pTyr-based immunoaffinity methods. Fractionation and phosphopeptide enrichment can be performed also in the reverse order. LC-MS/MS is then conducted, and the mass spectra of the peptides are recorded, including quantification by precursor ion intensities (MS<sup>1</sup>) or by reporter ions at the MS/MS level. The data are analyzed and examined with respect to differential regulation and the site specificity of protein phosphorylation. Biological pathways and networks are then generated for functional association of the detected signaling activities.

## 2.1 Sample preparation

Characterization of protein phosphorylation requires special considerations regarding the workflow to maintain and successfully measure phosphate groups in proteins by MS. In general, analysis of protein phosphorylation is hampered by the activity of protein phosphatases, which rapidly catalyze the dephosphorylation of rather unspecific target sequences. Therefore, cell disruption is generally performed under denaturing conditions and/or in the presence of phosphatase inhibitors to preserve the native state of protein phosphorylation. As a classical bottom-up proteomics approach, protein phosphorylation is analyzed by proteolytic digestion of proteins prior to MS. The serine protease trypsin is most commonly used for the degradation of proteins to peptides; it cleaves peptide bonds carboxyl-terminal to arginine and lysine residues. Given the distribution of these amino acids in

the proteome, this approach generates a large population of peptides that are suitable for MS-based mass detection and efficient fragmentation, resulting in confident identification of peptides [25]. However, two major drawbacks of using exclusively trypsin for digestion should be noted. First, when the negative charge introduced by phosphorylation is near potential trypsin cleavage sites, it dramatically decreases the cleavage efficiency [26]. Second, although trypsin is superior to other proteases with regard to the theoretical number of observable peptides it can produce, the average length of tryptic peptides is often too short for MS analysis, thus impairing the comprehensive analysis of phosphorylation throughout the proteome. The use of multiple proteases has been suggested as a means of improving the overall sequence coverage that directly yields increased information density regarding the phosphorylation pattern within the proteome [27, 28].

## 2.2 Metabolic and chemical labeling

The systematic analysis of protein phosphorylation requires extensive sample processing by serial application of different methods. Although a high level of standardization of operational procedures can be expected, sample processing may lead to experimental variation because of discrepancies in the working process. Additionally, deviations in peptide elution during LC because of differences in retention times and the continuous change of MS performance contribute to data variation. To minimize this large source of error, multiple proteomic samples can be processed simultaneously in a multiplex format when the biological material is metabolically or chemically labeled with stable isotopes prior to extensive sample processing (comprehensively reviewed in [29]). This approach leads to higher precision and accuracy in quantification.

Metabolic labeling of proteins in cells can be achieved using SILAC, which stands for ‘stable isotope labeling with amino acids in cell culture’ [30, 31]. For SILAC, cells are grown in medium supplemented with stable isotope variants of essential amino acids, usually arginine and lysine, allowing the direct comparison of up to three different samples in a single LC-MS run. The metabolic conversion of arginine to lysine, which is observed in some cell types and may affect accurate quantification, can be largely avoided by using a cell type-specific minimal concentration of arginine [32] and/or by supplementation of the cell culture medium with light proline [33, 34]. Application of a ‘Super-SILAC’ approach using a spiked-in isotope-labeled reference mix generated from SILAC cells can be considered for projects involving cells for which efficient metabolic labeling is impossible, such as for tissue samples [35]. Alternatively, dimethyl labeling has found its way into quantitative phosphoproteomics [36]. In this post-digest isotopic labeling method, peptides are labeled with formaldehyde via the primary amines of lysine and the amino terminus through reductive amination. As in SILAC, the introduction of stable isotopes allows triplex labeling [37]. Quantification in SILAC and dimethyl labeling is performed by comparing the intensities of the peptide ions in a survey spectrum (MS1 scan), allowing determination of changes in the relative abundance of specific (phospho)peptides under various conditions. Dimethyl labeling is similar to SILAC in terms of quantification accuracy but shows poorer reproducibility because sample mixing follows the initial sample processing steps, including protein digestion [38].

In contrast to isotopic labeling strategies in which quantification is based on precursor ion intensities obtained by MS survey scans, isobaric mass tags allow the concurrent identification and multiplexed quantitation of proteins and their PTMs in different samples based on reporter ion intensities in MS/MS scans. Isobaric tags for relative and absolute quantification (iTRAQ) [39] and tandem mass tags (TMT) [40] are two commercially available isobaric label sets with multiplex capacity that are routinely used for quantitative phosphoproteome analyses in up to 8-plex and 10-plex formats,

respectively [39–44]. The overall molecular architecture of the two labels is similar; each includes a reactive group for peptide labeling [N-hydroxysuccinimide (NHS) chemistry targeting primary amines], a region for mass normalization and a reporter group that is quantified via dissociation from peptides and detection in MS/MS scans.

The accuracy of quantification of all the recently introduced labeling approaches is affected by ratio compression and covers a limited dynamic range [31, 38, 45–49]. This results in underestimation of peptide ratios, a problem that becomes more severe as the ratios increase. Various strategies have been introduced to increase accuracy, including decreasing sample complexity by orthogonal fractionation prior to MS analysis [38, 50] and, specifically for metabolic labeling, the use of advanced MS methods [49, 51].

Label-free quantification based on either feature intensities or spectral counting has been successfully used to study protein interactions and changes in protein expression across biological samples [52–56]. However, label-free quantification requires complex statistical analysis, and, from our point of view, mixing of samples that have undergone prior differential labeling at an early stage of the experimental workflow is preferred when extensive sample processing is undertaken, such as in the study of phosphoproteomes.

## 2.3 Kinase enrichment

Information about the temporal abundance and activity of protein kinases can be invaluable when signal transduction networks are studied. For example, active networks may be deduced by bioinformatic approaches when comparative information about kinase abundance and activity has been collected. More importantly, information about these master regulators of cell function can be used to regulate cell activities by kinase-targeting pharmacological intervention strategies. To study the kinomes of cells and tissues in detail, chemoproteomic tools utilizing immobilized small-molecule kinase inhibitors as selective capture reagents for protein kinases have been described [57, 58]. Several suitable reagents with broad target profiles have been employed, including VI16832 [59] and CTx-0294885 [60]. Moreover, to maximize the kinome coverage, different immobilized kinase inhibitors with distinct kinase-binding properties have been combined [58, 59, 61]. An advantage of this chemoproteomic approach is its versatility; it can be combined with SILAC and with phosphopeptide enrichment strategies [59, 61]. A related method of protein kinase enrichment uses modified ATP or ADP probes [62]. These probes consist of a scaffold of ATP or ADP, a biotin tag for enrichment and an acyl group directly attached to the gamma phosphate of ATP for reaction with primary amines. The conserved lysine residue in the ATP-binding loop of protein kinases is the preferred site for nucleophilic attack by these probes, allowing kinase-selective attachment and affinity purification.

## 2.4 Phosphopeptide enrichment

The fact that phosphorylated peptides are found in substoichiometric amounts compared to their unphosphorylated counterparts impedes their rigorous analysis. Accordingly, enrichment of phosphorylated peptides is necessary, and a number of techniques for such enrichment have been developed (these are comprehensively reviewed in [63, 64]). Titanium dioxide (TiO<sub>2</sub>) and immobilized metal affinity chromatography (IMAC) employing Ga<sup>3+</sup>, Zr<sup>4+</sup>, Ti<sup>4+</sup> and especially Fe<sup>3+</sup> have achieved high popularity [65–71]. Selectivity for phosphorylated peptides is highly dependent on the conditions used in the enrichment step, and a major issue is the co-enrichment of peptides rich in glutamic and aspartic acid. Several strategies have been established to prevent the co-purification of acidic peptides, including methyl esterification of carboxyl groups [72], acidification of loading buffers [65, 66, 73] and, for TiO<sub>2</sub> enrichment, addition of direct competitors, such as 2,5-dihydroxybenzoic acid [66], and aliphatic hydroxyl acids (e.g., lactic acid or glycolic acid) [74, 75].

Tyrosine phosphorylation plays an important role in receptor-mediated signal transduction, but the low relative amount of phosphorylated tyrosines ( $\approx 2\%$ ) compared to serine ( $\approx 86\%$ ) and threonine ( $\approx 12\%$ ) requires specific enrichment strategies for their comprehensive characterization. The use of phosphotyrosine-specific antibodies permits the enrichment of phosphotyrosine peptides at the peptide and protein levels via immunoprecipitation. Eluates from phosphotyrosine pull-downs are generally low in complexity and can be analyzed directly via LC-MS without further sample fractionation [76]. However, TiO<sub>2</sub>-based phosphopeptide enrichment prior to or following immunoprecipitation of peptides containing phosphorylated tyrosine residues has been described and has been shown to lead to a reduction in non-phosphorylated peptides [77, 78]. Combinations of antibodies targeting tyrosine phosphorylation may be beneficial for in-depth analysis because different commercial anti-phosphotyrosine antibodies show unique binding preferences with respect to the sequences flanking the phosphorylated tyrosine residues [79]. Considering the low abundance of tyrosine phosphorylation, the analysis of tyrosine-phosphorylated peptides is further complicated by the large amounts of sample material needed, which usually falls within the milligram range [76, 80–82].

## 2.5 Fractionation

Analyzing complex systems, such as (phospho)proteomes, using MS in a comprehensive fashion is a very challenging task. One major issue concerns the extremely large dynamic range of protein concentrations in cellular systems, a range that is usually larger than the dynamic range covered even by modern mass spectrometers. Consequently, proteins of low abundance are not detected when a certain number of highly abundant proteins are present in the same sample. A

strategy that can be used to overcome this issue is to decrease the sample complexity by fractionation prior to MS analysis. Reversed-phase liquid chromatography (RPLC) is routinely used for online fractionation of peptide samples according to their hydrophobicity. In addition, a number of fractionation techniques based on the physicochemical properties of peptides have been applied; these can be used either online or offline to further decrease sample complexity and assist in the enrichment of phosphopeptides (comprehensively reviewed in [29, 83]).

Strong cation-exchange chromatography (SCX) is a very popular technique for fractionation of complex peptide samples [84]. This technique is based on electrostatic interactions between peptides and the ion-exchange resin. SCX is usually performed at a pH of 2.6–2.7, yielding a charge state of +2 resulting from protonation of amino-terminal amino groups for regular tryptic peptides with carboxy-terminal arginine or lysine residues. These peptides are efficiently retained by the anionic resin. In contrast, phosphorylation decreases the net charge according to the number of phosphorylated sites in a given peptide, leading to decreased interaction with the ion-exchange matrix. As a result, phosphorylated peptides either are not retained at all or elute earlier, allowing crude separation of phosphorylated and unphosphorylated peptides.

Separation of peptides using hydrophilic interaction liquid chromatography (HILIC) and electrostatic repulsion hydrophilic interaction chromatography (ERLIC) has been applied as a promising alternative to SCX [85, 86]. The major principle underlying HILIC is the separation of peptides based on their hydrophilicity; HILIC can therefore be regarded as the orthogonal counterpart of RPLC. Addition of phosphate groups increases the hydrophilicity of peptides, allowing semi-selective enrichment of phosphopeptides. ERLIC is a modification of HILIC that employs ionic column surface chemistry; in this method, improved separation of phosphopeptides is achieved by combining the hydrophilic interaction of phosphate groups with their electrostatic attraction to anion- or cation-exchange material [87]. SCX, HILIC and ERLIC have been successfully implemented in phosphoproteomic workflows involving IMAC or TiO<sub>2</sub> for phosphopeptide enrichment as either up- or downstream sample fractionation steps [88–99].

## 2.6 MS and data interpretation

The in-depth identification and quantification of proteins and their PTMs has been greatly advanced by the progress that has been made in the development of high-resolution and high-mass-accuracy mass spectrometers with sensitivities in the ppm or even sub-ppm range. However, the appropriate dissociation of peptide ions and the accurate determination of fragment ions are essential prerequisites for the accurate localization of phosphorylation sites within the peptide sequence, particularly when several potentially modifiable amino acid residues exist, as is often the case with phosphorylated

peptides. Due to the ever-growing number of large-scale studies that are being performed, the number of misidentified phosphorylation sites is increasing, and systematic validation is hampered because of the lack of suitable alternative approaches; e.g., commercial antibodies currently cover less than 1% of the reported sites. This bottleneck emphasizes the requirement for high-quality MS data and well-performing post-acquisition informatics tools for confident assignment of phosphorylation sites.

Several mass spectrometric acquisition strategies for phosphopeptides that take advantage of their specific behaviors during MS have been developed. In MS/MS, serine- or threonine-phosphorylated peptides can show a diagnostic neutral loss of phosphoric acid (–98 Da), whereas tyrosine-phosphorylated peptides generate immonium ions ( $m/z$  216.043). Neutral loss-dependent MS<sup>3</sup> and precursor ion scan methods have, for example, been designed to consider these features for more accurate identification (e.g., reviewed in [100]). In addition, peptide dissociation methods and fragment analyzers exist that differ in the types of ions produced as well as in mass accuracy and speed of detection [101, 102]. Because all of these methods have advantages and disadvantages, the selection of the right mass spectrometer and acquisition method must be planned in advance; the choice is highly dependent on sample complexity and on the selected quantification strategy.

Measurement of samples by MS usually yields numerous large files containing the acquired spectra. Software programs with associated search engines, including MaxQuant ([www.coxdocs.org](http://www.coxdocs.org) [103]), Proteome Discoverer ([www.thermoscientific.de](http://www.thermoscientific.de)) and MASCOT ([www.matrixscience.com](http://www.matrixscience.com) [104]), execute spectra extraction, peak list generation and database searching for the identification and quantitation of peptides and the corresponding proteins. For phosphorylations, an additional algorithm is required that permits automated and confident localization of phosphorylation sites within validated peptide sequences [90, 105–107]. Importantly, for large-scale phosphoproteomics as well as for proteome data sets in general, bioinformatics tools are used to assist in interpreting the ‘omic’ data and to place the results in a biologically relevant context. Publically available protein modification databases comprising large sets of experimentally verified phosphorylation sites of diverse species include PhosphoSitePlus ([www.phosphosite.org](http://www.phosphosite.org) [108]), PHOSIDA ([www.phosida.com](http://www.phosida.com) [109] and Phospho.ELM (<http://phospho.elm.eu.org> [110]). PhosphoSitePlus currently contains approximately 265 000 reported phosphosites for which associated information, including implication in protein function and correlation with specific diseases, is provided if available. The association of proteins with specific molecular functions, biological processes, or cellular components can be used in functional annotation enrichment analysis. PhosphoSitePlus also allows the user to search for experimentally verified upstream kinases for specific substrate phosphosites; these sites can also be predicted by tools, such as NetPhorest (<http://netphorest.info> [111]),

KinasePhos (<http://kinasephos.mbc.nctu.edu.tw> [112]) and GPS (<http://gps.biocuckoo.org> [113]). This information facilitates the generation of signaling networks and the highlighting of key hubs with the aim of gaining a deeper understanding of molecular (patho)mechanisms and targets for pharmacological intervention.

### 3 Application of MS-based screening in infection research

MS-based phosphoproteomics has greatly contributed to a better understanding of the signaling events that are triggered following pathogen recognition. Weintz et al. analyzed the response of primary bone marrow-derived macrophages to the major outer cell wall component of Gram-negative bacteria and TLR4 ligand lipopolysaccharide (LPS) [114]. By using SILAC for quantification and combining SCX with TiO<sub>2</sub> for enrichment of phosphorylated peptides, the authors were able to identify 6956 phosphorylation sites originating from 1850 proteins via LC-MS. TLR4-mediated signaling in macrophages resulted in major changes in the phosphoproteome; 24% of all quantified phosphorylation sites were up-regulated, and 9% were down-regulated. Based on motif analyses of the regulated phosphorylation sites, a number of protein kinases, including MAPK, PKD, AKT, AURORA and CAMK2, were suggested to function as important signaling hubs in immune signaling triggered by LPS. In addition, enrichment analysis of regulated phosphorylation sites and phosphoproteins demonstrated involvement of the mTOR- and GTPase-mediated signaling pathway. Further insights into TLR-induced signaling were obtained in a study by Sjoelund et al., who compared the signaling response of macrophages upon stimulation with LPS, the triacetylated lipopeptide Pam3C (TLR2 ligand) and the imidazoquinoline compound R848 (TLR7 ligand) using SILAC and LC-MS [115]. Enrichment of phosphopeptides with TiO<sub>2</sub> and upstream SCX fractionation enabled reproducible quantification of approximately 500 phosphorylation sites under each of the experimental conditions. The authors observed overlapping signaling patterns triggered by different TLR ligands that were attributed to common signaling through the myeloid differentiation primary response gene MyD88. In addition to the partially overlapping responses, clearly stimulus-specific phosphorylation patterns were reported, including differences in the kinetics of phosphorylation associated with phagocytosis or endocytosis.

A more general view of the host signaling triggered by bacterial components was attempted by Chen et al., who not only studied the response of murine macrophages to LPS but also included heat-inactivated *S. aureus* cells in their analysis [116]. The use of a combination of SILAC, LC-MS, IMAC and HILIC enabled the quantification of 2657 and 1990 phosphopeptides following treatment with LPS and heat-inactivated *S. aureus*, respectively. Of the quantified phosphopeptides, 11.1% changed at least 1.5-fold in their

abundance following LPS stimulation, whereas the abundance of 18.2% of the quantified phosphopeptides was altered by treatment with heat-inactivated *S. aureus*. Stimulation with these agents resulted in a great overlap of phosphorylated peptides that changed in abundance. However, the authors eventually demonstrated that phosphoproteins implicated in the regulation of cytoskeletal dynamics and vesicle transport were more affected by exposure to heat-inactivated *S. aureus* than by LPS stimulation, most likely as a result of phagocytosis of the bacterial cells.

The immediate signaling responses of host cells during an infection may not necessarily be triggered by the physical interaction of macromolecules of the bacterial cell wall with receptors present in the host cell membrane. A wide range of pathogenic bacteria secrete virulence factors that promote infection and disease, for instance through disintegration of the membranes of immune cells or epithelial cells by pore-forming toxins. This led us to investigate the interaction of bronchial epithelial cells with alpha-toxin, a major virulence factor of *S. aureus*, by means of phosphoproteomics [117, 118]. We utilized two bronchial epithelial cell lines, one of which showed a resistant phenotype and one of which was susceptible to alpha-toxin, and compared their responses at the level of signaling to identify differentially regulated pathways that might be implicated in the observed phenotypic outcomes. Indeed, we identified induction of the EGFR-MAPK signaling axis as a key response associated with the tolerance of bronchial epithelial cells to alpha-toxin. Moreover, we were able to demonstrate by pharmacological intervention that inhibition of the EGFR sensitizes initially resistant bronchial epithelial cells to alpha-toxin.

The studies mentioned so far focused on initial host signaling induced by recognition of bacterial constituents. However, bacterial infection is a dynamic process that involves invasion, persistence, phagosomal escape, replication and dissemination of bacteria by exploitation of host cell functions. This exploitation is most likely linked to highly complex alterations in host signaling that may be at least partially critical for the infection process and thus urgently require a deeper understanding. In contrast, only a few studies to date have addressed the host signaling response during bacterial infection, e.g., in cultured cells.

MS-based phosphoproteomics was used by Rogers et al. to analyze host signaling events during the initial stages of *Salmonella* infection of HeLa human epithelial cells [119]. A combination of SILAC and TiO<sub>2</sub>-based phosphopeptide enrichment was applied to cells infected with either a wild-type *S. Typhimurium* strain or a strain lacking the type III secretion system (T3SS) 1 effector protein SopB/SigD, an inositol phosphate phosphatase. The analysis enabled the temporal modeling of infection-induced changes in more than 5800 phosphopeptides. The kinases AKT, PKC and PIM were predicted to target sites with altered phosphorylation, and up to half of the host phosphorylation events required the effector protein SopB, indicating extensive manipulation of host signaling by this effector. The data provide mechanistic

insight into processes associated with *Salmonella* invasion, such as prevention of apoptosis and induction of inflammation, which are crucial host responses that enable *Salmonella* to proliferate in the intestine. In a further phosphoproteomic study conducted in the Foster lab, the impact of *Salmonella* type III secretion effectors encoded by its pathogenicity island 2 (SPI2) on host cell phosphorylation during late stages of infection was analyzed in murine RAW264.7 macrophages and HeLa cells [120]. SPI2 effectors were observed to differentially modulate the host phosphoproteomes in the professional and non-professional phagocytic models of infection. Consistent with the described role of SPI2 T3SS as the main modulator in phagocytic cells, the macrophages were found to be more broadly impacted than the epithelial cells, with altered phosphorylation patterns found in proteins involved in vesicular trafficking, the cytoskeleton, and immune signaling. In addition, several proteins with critical roles in the maturation of the *Salmonella*-containing vacuole and manipulation of host phagosomal functions appeared to be targeted by the SPI2 system, as well as previously unrecognized proteins including E3 ligases, histones and kinases. In additional assays, the authors demonstrated that the only type III secreted kinase, SteC, induces actin rearrangements through direct phosphorylation of HSP27 at multiple sites, thereby providing a novel mechanism for pathogen-induced manipulation of the actin cytoskeleton.

SILAC and TiO<sub>2</sub>-based quantitative phosphoproteomics were also used to assess the impact of infection with extracellular, diarrheagenic *E. coli* (enteropathogenic *E. coli*, EPEC) on the host cell phosphoproteome over time [121]. By the use of wild-type EPEC and an EPEC mutant deficient in T3S, 72 T3SS-independent and 89 T3SS-dependent changes in phosphorylation were identified in HeLa cells. The results emphasize the central role of the established T3SS-target MAPK in phosphorylation during EPEC infection. In addition, T3SS-dependent phosphorylation was found to affect various cellular processes that are known EPEC targets, including immune signaling, vesicle trafficking and cytoskeletal reorganization; in the latter, especially, the role of phosphorylation is not well understood. In this regard, the data provide additional mechanistic insights, such as the phosphorylation of Ser3 in cofilin and of Ser82 in HSP27. Septin-9 (SEPT9) was identified as a novel target in EPEC pathogenesis, and phosphorylation of Ser30 was shown to impact bacterial adherence.

Schmutz et al. analyzed the dynamics of the phosphoproteome of HeLa epithelial cells infected with *Shigella flexneri* in a label-free LC-MS approach [122]. Enrichment of phosphorylation by TiO<sub>2</sub> resulted in the identification of 3234 phosphopeptides from 1183 proteins. During the course of the infection with *S. flexneri*, 14.3% of the detected phosphopeptides changed at least two-fold in abundance, illustrating the substantial impact of infection on host signaling. This included activation of the ribosomal protein S6 kinase and AKT via mTOR complexes 1 and 2, induction of the DNA damage stress response by activation of ATM and extensive

changes in phosphorylation patterns of proteins implicated in cytoskeleton dynamics.

Holland et al. studied the infection of a gastric epithelial cell line with the Gram-negative human pathogen *Helicobacter pylori*, combining SILAC, IMAC, two-dimensional gel electrophoresis and MS to identify protein phosphorylations that are modulated in response to a preceding infection [123]. The experimental setup used by these investigators allowed the differentiation of ‘protein species’, i.e., proteins that show a multitude of post-translational modifications, information that is lost in a typical bottom-up phosphoproteomic approach. The authors were able to quantify 322 protein species originating from 127 proteins; 51 protein species (20 phosphoproteins) changed in abundance upon infection with *H. pylori*. The vast majority of regulated phosphoproteins identified were involved in the splicing of mRNA, suggesting that regulation of splicing occurs upon infection. Kinase motif predictions for the proteins implicated in splicing suggested the involvement of p38 MAPK and PKB in the observed changes in phosphorylation.

A key determinant associated with virulence in *H. pylori* is CagA, a protein that is injected into the cytoplasm of gastric epithelial cells via the T4SS secretion machinery. The structural components of the T4SS secretion machinery, together with CagA, are encoded on a *cag* pathogenicity island [124, 125]. Based on observations that CagA profoundly alters host phosphotyrosine signaling and that components of the T4SS secretion system, including CagL, induce activation of the focal adhesion kinase FAK [126–129], Glowinski et al. performed system-wide phosphotyrosine profiling using immunoaffinity enrichment combined with SILAC and LC-MS analysis of a gastric epithelial cell line infected either with a wild-type *H. pylori* strain, an isogenic CagA deletion mutant or a mutant lacking the entire *cag* pathogenicity island ( $\Delta$ PAI) to resolve component-specific phosphorylation events associated with *H. pylori* infection [130]. CagA deletion resulted in a marked decrease in phosphorylation of the SRC substrates pTyr128, pTyr249 and pTyr387 in BCAR1 and completely abolished activation of MAPK3/ERK1, indicated by reduced phosphorylation at pTyr204. In addition to phosphorylation events modulated by CagA, the authors also observed phosphorylation events that were dependent on the interaction of the T4SS secretion system with the host cell. The increased phosphorylation of several MAPKs, including MAPK13 (p38-delta; pTyr182), MAPK9 (JNK2; pTyr185), MAPK1 (Erk2; pTyr187) and MAPK10 (JNK3; pTyr185) was largely diminished upon infection with *H. pylori*  $\Delta$ PAI, indicating specific signaling in response to components of the T4SS system.

In a comparative phosphoproteomic study, Nakayasu et al. studied the signaling of macrophages infected with wild-type *Francisella novicida* or with a mutant lacking the *lpcC* gene, which encodes a group 1 glycosyl transferase involved in LPS synthesis. Infection with this bacterium generates a phenotype that is associated with increased cytotoxicity and increased rates of phagocytosis by macrophages [131, 132].

By combining iTRAQ, IMAC, and LC-MS, the authors were able to identify 3008 phosphopeptides in total; the highest degree of regulation of phosphorylation occurred at a very early stage of the infection, with 200 phosphopeptides showing profound changes in abundance at this stage. Kinase motif analysis indicated the potential activation of AKT, PKA, CaMKII, CDKs and MAPKs in response to *Francisella* infection. In addition, the authors observed much higher induction of the phosphorylation of Ser178 in tristetraprolin (TTP), a protein implicated in the degradation of mRNA, in cells infected with the *lpcC* knockout than in cells undergoing wild-type infection. Phosphorylation of Ser178 in TTP correlated with an increased abundance of TTP substrate mRNAs and increased expression of pro-inflammatory cytokines, which was attributed to the observed increase in apoptosis.

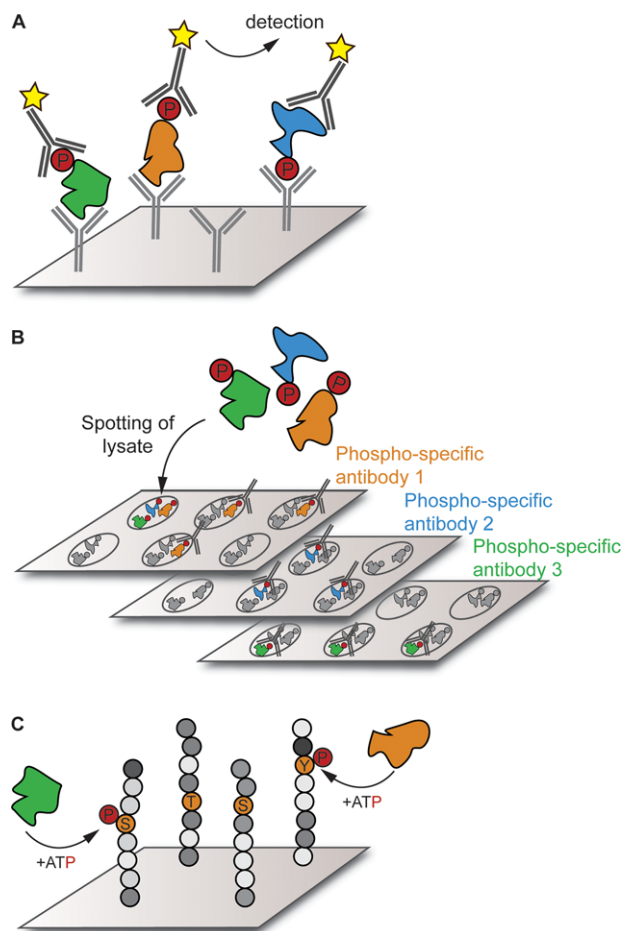
## 4 Array-based phosphoproteomic and kinomic technologies

Alternative approaches to MS-based methods suitable for the profiling of protein phosphorylation or protein kinases in host–microbe interaction experiments include array-based technologies, such as antibody arrays, peptide microarrays and reverse-phase protein microarrays (RPPA). Compared to classical single-observation experiments, these techniques allow investigators to conduct highly parallel surveys (see Fig. 2). Array-based technologies do not involve MS and are thus not dependent on the ionization, transmission or fragmentation efficiencies of analytes and do not suffer from inconsistent peptide selection across sample sets during proteomic discovery-mode mass spectrometry, which can have an impact on repeatable detection, identification and quantification. Moreover, most of these techniques avoid extensive sample processing and require only small amounts of sample. However, in these approaches, a priori knowledge of the (phospho)proteins or kinases to be studied is needed, and the techniques therefore stand in sharp contrast to the system-wide coverage and hypothesis-free MS-based study of protein phosphorylation. In addition, antibody-based techniques are dependent on the availability of high-quality antibodies, and the number of commercially available phosphorylation site-specific antibodies with satisfactory performance is limited.

### 4.1 Antibody arrays

Antibody arrays utilize multiple antibodies that either target proteins or are directed against specific phosphorylation sites (see Fig. 2A). For kinase profiling, antibodies are exclusively directed against protein kinases. Fixed-content panels to identify individual signaling pathways or receptor families of interest are available. One approach to monitoring the phosphorylation status of proteins includes the capture of proteins by antibodies on the array and subsequent detection of phosphorylation using either a cocktail of phosphosite-specific





**Figure 2.** Array-based approaches for phosphoproteomics. (A) Proteins of interest from a cell or tissue lysate are immunoprecipitated using antibody arrays containing immobilized ‘capture’ antibodies. Alternatively, phosphosite-specific antibodies on the array are used to capture exclusively the phosphorylated forms of proteins. Detection of phosphorylation is achieved by chemiluminescence or fluorescence associated with an antibody that targets the protein or a specific phosphorylated site on the protein. (B) Proteins from cell or tissue lysates are spotted on reverse-phase protein arrays. In each array, a single phosphosite-specific antibody is used, making it possible to monitor the phosphorylation of the protein in all lysates. (C) To monitor kinase activities in cell or tissue lysates, kinase peptide arrays utilizing known kinase target sequences are used. Upon incubation with cell or tissue lysates, active kinases transfer phosphate groups from ATP to serine, threonine or tyrosine residues of their target peptides. The incubation can be performed in the presence of  $[\gamma^{33}\text{P}]\text{-ATP}$  or unlabeled ATP; detection of the phosphorylation pattern is achieved by autoradiography, immunodetection or a phosphate-group-specific stain.

antibodies or, specifically for tyrosine phosphorylation, an antibody that binds to phosphotyrosine-containing sequences. Alternatively, phosphosite-specific antibodies can be directly used to capture exclusively the phosphorylated forms of the proteins on the array. The final step of detection is achieved

with chemiluminescence or fluorescence associated with the primary or secondary antibody or with streptavidin if proteins were biotinylated.

## 4.2 Reverse-phase protein arrays

RPPA offer another approach to determining the phosphorylation status of signaling proteins (see Fig. 2B). In this method, parallel dot blots are performed with lysates from differentially treated or healthy and diseased cells or tissues arrayed on a solid support. Immunodetection is performed with a single antibody that recognizes phosphorylated sites or with a phosphosite-specific antibody. Without compromising detection accuracy compared to techniques involving the separation of proteins by PAGE, RPPA increase sample throughput enormously compared to classical western blotting, and the technique is automation ready. However, phosphosite detection requires highly specific antibodies, and this is the major bottleneck for application of this technique in multiplex panels.

## 4.3 Kinase peptide arrays

To profile kinase activities in an array-based format, microarrays with immobilized kinase substrate peptides can be used (see Fig. 2C). Upon incubation with cell or tissue lysates, active kinases transfer phosphate groups from ATP to serine, threonine or tyrosine residues of their target peptides. Incubation can be performed in the presence of  $[\gamma^{33}\text{P}]\text{-ATP}$  or unlabeled ATP; detection of the phosphorylation pattern is achieved by autoradiography or by using a phosphate-group-specific stain, a phosphotyrosine peptide-specific antibody or a phosphoserine/threonine site-specific antibody mix. Use of  $[\gamma^{33}\text{P}]\text{-ATP}$  avoids the need for immunoaffinity reagents and thus overcomes one of the shortcomings of antibody-based assays. On the other hand, target sequences are often not unique to one specific kinase, and the design of peptides with high specificity for individual kinases is a major bottleneck of this method. Only a limited subset of the kinome is accounted for in currently available peptide arrays.

Currently available assays utilizing RPPA or antibody arrays cover a maximum of approximately 100 site-specific phosphorylation events, whereas peptide arrays permit monitoring of the activities of one to two hundred different human kinases. The commercially available PepChip Kinomics Array (PepScan), e.g., contains peptide sequences that are specifically phosphorylated by one or more of approximately 200 different human kinases, of which approximately 160 have at least one unique target on the array. An overview of the potential applications of array-based profiling approaches in infection research is provided in the next chapter.

## 5 Application of array-based screening in infection research

Recently, the RPPA-based strategy has been used to characterize host signaling events during infection of human epithelial cells as well as during infection of murine macrophages and lymph nodes. To address the effects of the *S. Typhimurium* type III secretion system effector proteins SopE/E2 and SopB/SigD on epithelial cells, Molero et al. infected cultured HeLa cells with *S. Typhimurium* depleted of SopE/E2 activity alone or in combination with different mutations in *sigD* [133]. A total of 32 differently treated cell lysates were spotted on chips and profiled using 15 phosphosite-specific antibodies and 10 antibodies specific to the non-phosphorylated forms of the proteins. The phosphoinositide phosphatase SigD was thereby confirmed to be implicated in the phosphorylation of PKB and its targets FoxO and GSK-3 $\beta$ , an involvement that may contribute to its reported anti-apoptotic effect. Furthermore, SopE/E2 promotes *S. Typhimurium* internalization mainly due to activation of CDC42 and Rac1 GTPases, as well as by activation of multiple MAPK (ERK, JNK, p38) pathways. The Hakami laboratory recently employed the RPPA technology to study the host response of 16HBE14o- human bronchial epithelial cells to *Y. pestis*, the cause of plague [134]. Using 111 host-specific antibodies, most of which were phosphosite-specific, host phosphorylation patterns were compared at different times after infection with live or heat-inactivated virulent bacteria and after infection with a non-virulent mutant strain, as well as after treatment with LPS. The analysis showed that 25 proteins, 12 of which were novel for *Y. pestis* infections, underwent significant alteration in the amount of site-specific phosphorylation or in the amount of total protein. The results indicate that modulation of cell growth and survival pathways and negative regulation of autophagy occurs during infection and that the negative regulation of autophagy involves altered phosphorylation-mediated activity of key kinases, including activation of AKT and P53 and inactivation of the AMP-activated protein kinase AMPK. A similar set of 114 antibodies was utilized with RPPA to monitor changes in the expression of host signaling proteins as well as alterations in phosphorylation-mediated host signaling caused by infection of murine RAW264.7 macrophages with *Burkholderia pseudomallei* and *B. mallei* species [135]. The comparison revealed that *B. spp.* trigger signaling through common host pathways and identified 20 and 5 candidate proteins whose phosphorylation states or level of expression, respectively, were altered during infection. In addition to the identification of previously known alterations in the phosphorylation of glycogen synthase kinase 3 beta (GSK3 $\beta$ ) and components of the NF- $\kappa$ B and MAPK pathways, suppression of AMPK- $\alpha$ 1 activation via Ser485 phosphorylation and Tyr416-mediated activation of the tyrosine kinase SRC were shown to be novel host biomarkers of *Burkholderia* infection. Subsequently, Popova et al. demonstrated the application of RPPA

using *Bacillus anthracis* spore-challenged mice [136]. Combining RPPA with laser capture microdissection, the authors studied phosphoprotein signaling in lymph node tissue after challenging mice with toxinogenic *B. anthracis*. Among the tested 31 phosphorylated protein species and 34 total proteins, major alterations were associated with reduced MAPK signaling, upregulation of STAT transcriptional factors and altered levels of many pro- and anti-apoptotic proteins. Through an accompanying immunohistochemical analysis, downregulation of ERK1/2 was shown to be associated with the response of CD11b+ macrophages/dendritic cells, whereas upregulation of the pro-apoptotic protein Puma indicated targeting of CD3+ T-cells.

Several recent studies have utilized multiple immunoblots or antibody arrays to characterize host signaling during interaction with pathogenic bacteria. To study the effect of mycobacterial infection on signal transduction in human macrophages, Hestvik et al. infected human macrophage-like THP-1 cells with live or heat-killed *Mycobacterium bovis* BCG or incubated cells with the mycobacterial cell wall component lipoarabinomannan (LAM) and performed a multiplex screening assay [137]. Lysates from cells obtained 24 hours post-infection were separated in a 20-lane format and probed against an array of 31 phospho-specific antibodies with up to three antibodies per lane. Using this strategy, the authors identified changes in host proteins involved in the regulation of apoptotic pathways (SAPK, c-Jun, and GSK3 $\beta$ ), cytoskeletal rearrangement ( $\alpha$ -adducin), calcium signaling (NR1), and macrophage activation (PKC $\epsilon$ ), and some of these changes were shown to be triggered by LAM. To analyze the receptor tyrosine kinases involved in meningococcal infection, Slanina et al. infected human brain microvascular endothelial cells with *Neisseria meningitidis* and profiled tyrosine phosphorylation events using an array comprising 39 antibodies against the phosphorylated forms of receptor tyrosine kinases and key signaling nodes [138]. Infection resulted in the activation of several kinases including the ErbB-family receptor tyrosine kinases EGFR, ErbB2, and ErbB4. Pharmacological inhibition or genetic ablation of these kinases by RNA interference resulted in decreased bacterial uptake, indicating that activation of EGFR signaling mediates meningococcal infection. Recently, phosphorylation arrays specific for 46 protein kinases and substrates were used to examine signaling downstream of ceramide/TLR4, a pathway triggered by P-fimbriated *E. coli*, which use ceramide-anchored glycosphingolipid receptors [139]. The phosphorylation of 12 proteins was shown to be stimulated following ceramide release; these proteins included antibacterial effectors (eNOS, Hck) and many proteins involved in the activation of the transcription factors AP-1 (Jun, ERK1/2) and IRF3 (PLC $\gamma$ 1, CREB, Fyn, ERK1/2). Based on the results of their study of the ceramide/TLR4-triggered IRF3-dependent transcription of innate immune response genes, the authors propose a model of how TLR4 distinguishes pathogens from commensals at the mucosal level.

The first application of commercially available peptide microarrays (consisting of 192 kinase target sequences) to describe the activities of a large group of kinases in whole cell lysates was published in 2004 [140]. The platform was used to characterize the kinetics of phosphorylation events in human peripheral blood mononuclear cells after induction by LPS. The analysis indicated the activation of p21Ras by LPS; this was confirmed by direct measurement of p21Ras GTP levels in LPS-stimulated human peripheral blood mononuclear cells, thereby providing the first direct demonstration of p21Ras activation by stimulation of a TLR family member. To identify the substrate specificity of the eukaryotic-like serine/threonine protein kinase PknB of *S. aureus*, Miller et al. utilized peptide microarrays; they found that efficient incorporation of radioactive phosphate into a particular subset of 976 human target sequences occurred only if purified PknB was present [141]. The authors identified 68 potential host substrates, approximately half of which were involved in signal transduction and cell communication. Together with a sequence logo analysis, the results imply that staphylococcal PknB is the first prokaryotic representative of the proline-directed kinases. Kinase activity profiling with peptide microarrays was also used to explore signal transduction events in a mouse model of *Klebsiella pneumoniae*-mediated pneumonia [142]. Of 1024 consensus sequences of protein kinase substrates, 179 were significantly differentially phosphorylated 3, 6, 24, or 48 h after infection. In this study, Hoogendijk et al. confirmed the key roles of MAPK and TGF $\beta$  signaling in classical inflammation pathways and demonstrated the involvement of the glycogen synthase kinase 3 $\beta$  (GSK-3 $\beta$ ), AKT and SRC signaling networks in the mouse model.

A chicken-specific peptide array comprising 300 target peptides designed to address cellular metabolic signaling was used by Arsenault et al. for kinomic profiling of changes in skeletal muscle metabolism following *S. Typhimurium* infection [143]. The results point to alterations in AMPK phosphorylation and activity as well as to significant disruptions in the insulin/mTOR signaling pathway over time. This potentially directly affects fatty acid and glucose metabolism and thereby links *S. Typhimurium* infection of the chicken cecum to potential systemic effects on animal health. The Napper lab utilized bovine-specific peptide kinome arrays containing 300 target sequences to analyze the pathological mechanisms of infection with *Mycobacterium avium* subsp. *paratuberculosis*, the causative agent of Johne's disease in cattle. To define the signaling events responsible for the suppression of activation of infected macrophages by gamma interferon (IFN- $\gamma$ ), a key event in subversion of host immune responses and establishment of chronic infection, IFN- $\gamma$ -stimulated infected and uninfected bovine monocytes were subjected to kinome analysis [144]. The peptide array data indicate activation of the IFN- $\gamma$ -associated JAK-STAT pathway in uninfected monocytes but not in *M. avium* subsp. *paratuberculosis*-infected monocytes. This limited responsiveness to IFN- $\gamma$  of infected cells is accompanied by decreased expression of the IFN- $\gamma$  receptor chains and increased expression of the negative IFN- $\gamma$

receptor regulators SOCS1 and SOCS3, providing specific insight into the pathogenic mechanisms of *M. avium* subsp. *paratuberculosis*. Modulation of TLR function during infection of monocytes with *M. avium* subsp. *paratuberculosis* has also been investigated [145]. Using array-based kinome analysis, blocking of classical TLR9 signaling for activation of NF- $\kappa$ B by *M. avium* subsp. *paratuberculosis* infection could be verified. During infection, signaling occurs through PYK2, and inhibition of PYK2 significantly reduced the number of intracellular bacteria, suggesting that PYK2 might be an appropriate therapeutic target. Another bovine-specific peptide-microarray-based study of *M. avium* subsp. *paratuberculosis* infection analyzed tissue samples from the intestinal compartments of calves after surgically isolated infection [146]. Significant differences in the overall intestinal kinome profiles of the infected compartments from four animals could be correlated with the distinct immune responses of cells from these animals to *M. avium* subsp. *paratuberculosis* lysates. Metadata analysis by pathway and gene ontology enrichment indicated that the differences in the kinomes were associated with differences in innate immunity and interleukin signaling and with alterations in the Wnt/-catenin pathway.

## 6 Conclusion and outlook

Eukaryotic protein kinases control virtually all cellular functions, ranging from simple protein translocation to highly complex biological reprogramming of cell function. During microbial infection, these master regulators contribute to innate and adaptive immune control by regulating pathogen recognition, phagocytosis, antigen presentation and many other processes that help restrict the dissemination of invading pathogens within the host organism. Consequently, accurate reception of stimuli and subsequent signal transduction by protein kinases through their corresponding phosphorylation networks must be regarded as essential for eukaryotic cells in general and for cells with barrier functions, such as epithelial cells, in particular. It has recently become clear, however, that pathogenic species employ mechanisms to subvert the host immune system. Hence, not only is host signaling during infection under the control of host cell kinases to stop pathogen invasion but infective agents also compete for the control of host signaling to create a beneficial environment for successful invasion and infection.

Due to their critical role in cellular signaling, members of the kinase superfamily have emerged as the most intensively pursued targets for therapeutic interference in human pathologies. To date, 27 small-molecule protein kinase inhibitors have been approved by the US FDA, predominantly for the treatment of patients suffering from various types of cancer. In contrast, no single kinase inhibitor is currently clinically approved and in use for the treatment of infections.

The dramatically increasing resistance of pathogens to available drugs and antibiotics requires immediate action to increase our understanding of the interactions between

pathogens and their hosts so that innovative tools can be developed to block the dissemination of pathogens as well as to control inflammation and restore immune homeostasis, e.g., in patients with severe sepsis. Although efforts to achieve a comprehensive understanding of the host-pathogen interaction and its molecular consequences have been made, there is an urgent need to better understand infection processes and to develop host-directed therapies that complement clinical research and the production of new antimicrobial agents and vaccines. The introduced techniques for sample preparation and phosphopeptide enrichment as well as MS measurement and quantification provide an integral workflow for the global study of protein phosphorylation and protein kinases. The use of these techniques in research on host-pathogen interactions will beyond doubt accelerate and amplify our understanding of infection processes during microbial and viral attacks. Moreover, it will rapidly provide potential targets for the development of new drugs or the repositioning of clinically approved drugs for host-directed therapy.

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