The ever increasing necessity of mass spectrometry in dissecting protein post-translational modifications catalyzed by bacterial effectors

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Abstract

Protein post-translational modifications (PTMs), such as ADP-ribosylation and phosphorylation, regulate multiple fundamental biological processes in cells. During bacterial infection, effector proteins are delivered into host cells through dedicated bacterial secretion systems and can modulate important cellular pathways by covalently modifying their host targets. These strategies enable intruding bacteria to subvert various host processes, thereby promoting their own survival and proliferation. Despite rapid expansion of our understanding of effector-mediated PTMs in host cells, analytical measurements of these molecular events still pose significant challenges in the study of host-pathogen interactions. Nevertheless, with major technical breakthroughs in the last two decades, mass spectrometry (MS) has evolved to be a valuable tool for detecting protein PTMs and mapping modification sites. Additionally, large-scale PTM profiling, facilitated by different enrichment strategies prior to MS analysis, allows high-throughput screening of host enzymatic substrates of bacterial effectors. In this review, we summarize the advances in the studies of two representative PTMs (i.e., ADP-ribosylation and phosphorylation) catalyzed by bacterial effectors during infection. Importantly, we will discuss the ever increasing role of MS in understanding these molecular events and how the latest MS-based tools can aid in future studies of this booming area of pathogenic bacteria-host interactions.

The ever increasing necessity of mass spectrometry in dissecting protein post-translational modifications catalyzed by bacterial effectors

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Abstract

Protein post-translational modifications (PTMs), such as ADP-ribosylation and phosphorylation, regulate multiple fundamental biological processes in cells. During bacterial infection, effector proteins are delivered into host cells through dedicated bacterial secretion systems and can modulate important cellular pathways by covalently modifying their host targets. These strategies enable intruding bacteria to subvert various host processes, thereby promoting their own survival and proliferation. Despite rapid expansion of our understanding of effector-mediated PTMs in host cells, analytical measurements of these molecular events still pose significant challenges in the study of host-pathogen interactions. Nevertheless, with major technical breakthroughs in the last two decades, mass spectrometry (MS) has evolved to be a valuable tool for detecting protein PTMs and mapping modification sites. Additionally, large-scale PTM profiling, facilitated by different enrichment strategies prior to MS analysis, allows high-throughput screening of host enzymatic substrates of bacterial effectors. In this review, we summarize the advances in the studies of two representative PTMs (i.e., ADP-ribosylation and phosphorylation) catalyzed by bacterial effectors during infection. Importantly, we will discuss the ever increasing role of MS in understanding these molecular events and how the latest MS-based tools can aid in future studies of this booming area of pathogenic bacteria-host interactions.

INTRODUCTION

Gram-negative bacterial pathogens such as *Shigella flexneri* ,*Salmonella enterica* , *Legionella pneumophila* , etc., cause a broad range of communicable human diseases (Jennison & Verma, 2004; Knodler & Elfenbein, 2019; Gonçalves et al., 2021). Their virulence is often mediated by dedicated protein secretion systems (i.e., type III for *S. flexneri* and *S. enterica* or type IV for *L. pneumophila*), which can deliver effector proteins directly into host cells to modulate diverse cellular pathways (Muthuramalingam et al., 2021; Jennings et al., 2017; Hubber & Roy, 2010; Isberg et al., 2009). In the last decade, a growing body of studies has shown that many secreted bacterial effectors harbor enzymatic activities that often lead to post-translational modifications (PTMs) of their host targets (Jennings et al., 2017; Mattock & Blocker, 2017; Qiu & Luo, 2017; Macek et al., 2019; Chambers & Scheck, 2020). From the perspective of bacterial pathogens, covalently modifying key host proteins represents a more economic and rapid means (relative to mechanisms altering protein abundance) to regulate host biological processes including anti-bacterial defense system, thereby promoting bacterial survival and/or proliferation (Ashida et al., 2014). Indeed, accumulating evidence suggests that bacteria-mediated modifications of host targets are much more prevalent than we initially thought.

Rapid growth of this emerging field (i.e., pathogen-mediated host modifications) is, at least in part, spurred by technical innovations that facilitate accurate and precise measurements of these molecular events such as protein PTMs. Among those tools probing covalent modifications, mass spectrometry (MS) is arguably the most powerful and versatile analytical approach thanks to major technological breakthroughs (e.g., in terms of instrument sensitivity, mass resolution and throughput) that were witnessed in the last two decades. Nevertheless, the application of this analytical tool in dissecting effector-mediated PTMs is, at least in our opinion, still far below where it is supposed to be. This review will focus on the application of MS-based approaches in the study of host protein modifications catalyzed by bacterial effectors. Specifically, we will highlight some recent work on protein ADP-ribosylation (and related ADP-riboxanation) and classical phosphorylation as examples to illustrate our current understanding of this fascinating area in host-pathogen interactions (Figure 1). Furthermore, we will critically discuss the increasing role and capability of MS in dissolving some looming challenges for further advancements in the field.

ADP-ribosylation and ADP-riboxanation

ADP-ribosylation involves the attachment of ADP-ribose (ADPr) groups from nicotinamide adenine dinucleotide (NAD^+) to substrates by enzymes called ADP-ribosyltransferases (ARTs), together with the release of nicotinamide (NA) (Palazzo et al., 2017). Given bacterial toxins (i.e., Cholera and Diphtheria toxins) as the first reported enzymes of this group (Honjo et al., 1968; Cassel & Pfeuffer, 1978), the other ARTs identi-

fied afterwards are often classified as either Cholera toxin-like ART (ARTC) or Diphtheria toxin-like ART (ARTD) superfamily (Hottiger et al., 2010). Recently, there seems to be a spike in the number of reports on bacterial virulence factors possessing ART activities. For instance, *Salmonella* Typhimurium effector SopF targets Gln124 of ATP6V0C in host V-ATPase for ADP-ribosylation, thereby blocking antibacterial autophagy (xenophagy) via disrupting V-APTase-ATG16L1 association (Xu et al., 2019). In a follow-up study, the authors found SopF stably associates with ADP-ribosylation factor (ARF) GTPases and requires these host factors for its activation (Xu et al., 2022). Another type III effector CetC from *Chromobacterium violaceum* catalyzes ADP-ribosylation of ubiquitin to disrupt host ubiquitin signaling, representing the first of its kind on the threonine residue (Yan et al., 2020). In the above studies, tandem mass spectrometry (MS/MS) involving dissociation of modified protein fragments/peptides plays an indispensable role in ascertaining the modification in the first place as well as pinpointing the exact modified sites.

Recently, a novel PTM dubbed as ADP-riboxanation, a derivative of ADP-ribosylation, was uncovered and catalyzed by bacterial effectors OspC3 and CopC from *S. flexneri* and *C. violaceum* , respectively (Li et al., 2021; Peng et al., 2022). Distinct from ADP-ribosylation, ADP-riboxanation involves an additional deamination upon the classical modification. In other words, this novel modification couples two enzymatic reactions through the activity of a single effector. This unique reaction mechanism was supported by the effector variants ($OspC3^{D177A}$ and $CopC^{D172E}$) capable of catalyzing conventional ADP-ribosylation without the subsequent deamination. The initial assignment/characterization of this unconventional modification was greatly facilitated by a suite of MS tools including multi-stage MS (MS/MS and MS/MS/MS), high-resolution measurements and stable isotope labeling MS. Unlike canonical arginineN^{ω} -ADP-ribosylation (ANT1 by Ceg3 or Rab4a and Ras by ExoS) (Fu et al., 2022; Bette-Bobillo et al., 1998; Vareechon et al., 2017), OspC3-catalyzed ADP-riboxanation proceeds withN^{δ} -ADP-ribosylation and subsequent deamination with the ribosyl-2'-OH of ADPR to remove oneN^{ω} , resulting in an oxazolidine ring. Thus far, both *S. flexneri* OspC3 and *C. violaceum* CopC were found to modify host caspases via ADP-riboxanation. While OspC3 targets inflammatory caspases (-4 and -11) to block host pyroptosis (Li et al., 2021), CopC inactivates a broader spectrum of caspases (-3, -7, -8, and -9) to interfere with host cell death pathways including apoptosis, necroptosis and pyroptosis (Peng et al., 2022).

Of note, upon elucidation of enzymatic activities of bacterial effectors, a rate-limiting step of most studies can often be associated with the discovery of their modified targets. As discussed above, enzymatic substrates are mostly identified by inference from perturbed cellular pathways or through conventional genetic and biochemical assays. Nevertheless, in many cases one could encounter a scenario where there are no observable phenotypes associated with the effector of interest. Then we would argue that cellular ADP-ribosylome profiling (i.e., global analysis of ADP-ribosylated proteome) can be a generic and unbiased strategy for high-throughput screening of ART substrates. Such work is facilitated by the development of an engineered macro domain-containing protein (eAf1521) from *Archaeoglobus fulgidus* . This variant of Af1521 exhibits high affinity towards ADP-ribosylated proteins, thereby allowing efficient enrichment of cellular ADP-ribosylome prior to MS analyses (Hendriks et al., 2019; Nowak et al., 2020). Indeed, such a strategy has been applied in the study of*S.* Typhimurium SopF (Xu et al., 2019) and *L. pneumophila*effector Ceg3 (Fu et al., 2022; Kubori et al., 2022), though in a more targeted manner as only those Af1521-enriched proteins with the size of interest were analyzed by LC-MS. Notably, Ceg3 and Larg1 were found to target ADP/ATP translocases by reversible ADP-ribosylation in host mitochondria to temporally regulate their ADP/ATP exchange activity during *L. pneumophila* infection. Alternatively, the use of an NAD derivative, biotin-17-NAD, in *in vitro* ADP-ribosylation assays permits pull-down of modified ART substrates prior to MS identification. By using this strategy, *L. pneumophila* effector Lart1 was found to ADP-ribosylate yeast glutamate dehydrogenase 2 (Black et al., 2021).

Phosphorylation

Phosphorylation often occurs on serine, threonine, and tyrosine residues of target proteins with the addition of phosphate by kinases and its removal by phosphatases. As a highly efficient modification, phosphorylation modulates protein functions by altering conformation, promoting ubiquitination and degradation, influenc-

ing protein-protein interactions, or regulating enzyme activities (Huttlin et al., 2010). Due to long-standing co-evolution with mammalian host cells, many bacterial pathogens have evolved effector proteins harboring kinase or phosphatase activities to directly engage with host phosphorylation signaling (Grishin et al., 2015). *Yersinia pseudotuberculosis*YpkA is arguably the first reported prokaryotic virulence factor possessing Ser/Thr protein kinase activity (Galyov et al., 1993) and its activation is dependent on a host factor, actin (Barz et al., 2000, Dukuzumuremyi et al., 2000, Juris et al., 2000). YpkA phosphorylates the heterotrimeric G protein, Gαq, on a critical serine residue, thereby inhibiting its GTP binding (Navarro et al., 2007). In addition to its kinase activity, YpkA also possesses the guanidine nucleotide dissociation inhibitor (GDI) domain, and together they work synergistically to disrupt host actin cytoskeleton (Prehna et al., 2006). Later, vasodilator-stimulated phosphoprotein (VASP) was reported to be another enzymatic substrate of YpkA and its modification led to the disruption of host actin dynamics (Ke et al., 2015). *S.*Typhimurium effector SteC harbors kinase activity that is required for the formation of the F-actin meshwork associated with*Salmonella* -containing vacuoles (SCVs). Several enzymatic substrates of SteC have been identified by various approaches, including Hsp27 (Imaimi et al., 2013), formin family FMNL proteins (Walch et al., 2021; Poh et al., 2008) and the MAP kinase MEK (Odendall et al., 2012). Together, SteC-mediated phosphorylation of these target proteins coordinately modulates the host F-actin cytoskeleton (Heggie et al., 2021).

L. pneumophila exploits phosphorylation signaling pathways by delivering an array of kinase effectors into host cells including LegK1-K4, LegK7 and Lem28/Lpg2603 (Hervet et al., 2011; Lee et al., 2018; Sreelatha et al., 2020). LegK1 was first identified in a screen of*L. pneumophila* effectors that activate the NF-κB pathway (Ge et al., 2009). Mechanistically, LegK1 directly phosphorylates the IκB family of inhibitors. LegK2 targets the ARP2/3 complex whose phosphorylation triggers actin cytoskeleton remodeling in host cells (Michard et al., 2015). LegK7 hijacks the host Hippo pathway by phosphorylating the scaffolding protein MOB1, leading to the degradation of the transcriptional regulators TAZ and YAP1 (Lee et al., 2018). The host targets of LegK4 were identified to be the Hsp70 chaperone family by using a chemical genetic screen (Moss et al., 2019). LegK4-dependent phosphorylation of cytosolic Hsp70 inhibits its ATPase activity and hence protein folding capacity, resulting in global translation arrest in host cells. Thus far, host targets of other *L. pneumophila*kinase effectors are still elusive and their functions in bacterial infection remain to be investigated.

Other than kinases, bacterial pathogens also encode and secret effector proteins harboring phosphatase activities. *Y. pseudotuberculosis*YopH is perhaps the first reported bacterial effector possessing potent tyrosine phosphatase activity (Bliska et al., 1991). Later, two independent reports found that YopH targets focal adhesions in host cells by dephosphorylating p130Cas and FAK, thereby disrupting peripheral focal complexes and inhibiting bacterial uptake into HeLa cells (Persson et al., 1997; Black et al., 1997). In T cells, YopH targets the tyrosine kinases Lck and ZAP-70 together with the adaptors SLP-76 and LAT, and their dephosphorylation alters T cell-mediated immune responses (Alonso et al., 2004; Gerke et al., 2005). Overlapping with the previously reported targets in different host cells, the PRAM-1/SKAP-HOM and SLP-76 signaling pathways are also dephosphorylated in a YopH-dependent manner to impair neutrophil responses in an animal infection model (Rolán et al., 2013). In many of these studies, a catalytic mutant of YopH was often employed to precipitate the interacting proteins for identifying potential phosphatase substrates. Furthermore, *Salmonella* effector SptP has a C-terminal protein tyrosine phosphatase domain fused to its N-terminal GTPase activating protein (GAP) domain (Stebbins & Galán, 2000). It dephosphorylates the host AAA+ ATPase VCP to regulate the biogenesis of*Salmonella* -containing vacuoles (SCVs) (Humphreys et al., 2009). Again, the substrate VCP was identified from the pull-down samples by using a phosphatase inactive mutant of SptP. Furthermore, SptP targets a tyrosine kinase Syk and a vesicle fusion protein, N-ethylmalemide-sensitive factor to suppress immune responses in mast cells through its phosphatase activity (Choi et al., 2013). Additional phosphatase effectors include WipA, WipB, Ceg4 and Lem4 from *L. pneumophila* (Pinotsis & Waksman, 2017; Prevost et al., 2017; Quaile et al., 2018; Beyrakhova et al., 2018) and *Coxiella burnetii*effector CinF that dephosphorylates IκBα to inhibit host NF-κB signaling (Zhang et al., 2022).

Nevertheless, our understanding of bacterial manipulation of host phosphorylation signaling pathways remains rather limited, largely because of technical challenges in sensitively and reliably measuring phosphorylation events of proteins (especially on a global level). Therefore, many studies on bacterial kinases or phosphatases, in particular some of the early work as we discussed above, were carried out without the assistance of the latest MS tools on more definitive measurements of protein modifications as well as precise determination of phosphorylation sites. In addition, the potential impact(s) of a bacterial kinase/phosphatase was not probed on systems-level during bacterial infection. Such holistic views are particularly desired when a single effector (e.g., YpkA, SteC, YopH and SptP) may target multiple host pathways. Fortunately, the arrival and maturation of MS-based phosphoproteomics in the last two decades renders large-scale analyses of cellular phosphorylation network possible.

Indeed, phosphopeptides can be efficiently enriched (e.g., by titanium dioxide (TiO_2)) from digested total cell lysates at relatively low cost, allowing high-throughput phosphoproteome profiling by LC-MS (Olsen et al., 2010; Villén et al., 2007). Global phosphoproteomic analyses of host cells infected by bacterial pathogens were also reported (Rogers et al., 2011; Imami et al., 2013; Schmutz et al., 2013). Such phosphoproteome measurements, if designed with proper controls, can be exploited as a universal and powerful approach to identify host enzymatic substrates of a given effector protein with either kinase or phosphatase activities. For example, enteropathogenic*E. coli* (EPEC) effectors NleH1/2 are Ser/Thr protein kinases and they have been shown by a number of studies to attenuate NF-κB activation (Gao et al., 2009; Royan et al., 2010; Wan et al., 2011) and inhibit host apoptotic pathway during infection (Hemrajani et al., 2010). Nevertheless, no phosphorylated substrates of NleH1/2 were reported until a recent study in which a large-scale phosphoproteomic screen successfully identified host microvillus protein Eps8 as the kinase target (Pollock et al., 2022). Phosphorylation of Eps8 on Ser775 inhibits its bundling activity, leading to its dispersal from the attaching and effacing (AE) lesion during EPEC infection. This work exemplifies the utility of phosphoproteomics in identifying enzymatic substrates of bacterial kinases.

CONCLUSION

In general, host target discovery is a demanding task in the study of bacterial effector proteins. In particular, it often becomes more challenging when the effector of interest is an enzyme that may catalyze a potential PTM of host targets. In many cases, one would rely on biochemical isolation of protein complexes comprising effectors and host targets. The success of such strategies often requires the stable interaction/association of host substrates with bacterial factors. There are at least two scenarios where these approaches (e.g., immunoprecipitation of protein complexes) would be less effective in identifying target proteins. First, the transient or weak enzyme-substrate affinities prevent successful isolation of protein complexes unless additional techniques such as crosslinking methods are employed to preserve such labile interactions before immunoprecipitation. For instance, in addition to its N-terminal ADP-riboxanase domain *S. flexneri* effector OspC3 has a C-terminal ARD domain that is thought to recognize specific caspases for ADP-riboxanation. That being said, the binding constant (K_D) between OspC3 and caspases were measured at ˜5 μM (Hou et al., 2023). In contrast, efficient immunoprecipitation often requires binding affinities in the range of nM at least. Additionally, another complication may arise from the scenario where effectors do have stably interacting partners and yet such interactors are not enzymatic substrates. Rather, stably associated factors can be host factors required for activation of bacterial effectors. For example, both *S. flexneri* OspC3 and *C. violaceum* CopC rely on the interaction with an abundant host factor, calmodulin, for their activation (Hou et al., 2023; Peng et al., 2022; Liu et al., 2022). The stable association of ARF GTPases with*Salmonella* effector SopF also falls into this category (Xu et al., 2022). Therefore, alternative approaches without the necessity of stable protein-protein interactions (i.e., effector-substrate association) would be highly desired for identification of enzymatic targets.

As we discussed above, recent advancements in MS-based proteomic approaches make it possible to comprehensively identify host proteins modified by bacterial effectors. Successful application of such strategies has been seen at least in ADP-ribosylation and phosphorylation mediated by bacterial effectors. As the macro domain-containing protein Af1521 is able to recognize the newly discovered ADP-riboxanation as well, we

would anticipate similar utilization of PTM profiling to identify modified substrates for the class of effectors possessing ADP-riboxanase activity. Furthermore, as effectors may have multiple substrates by targeting different host processes, such PTM profiling strategies can also be utilized to uncover any additional host targets of some characterized effectors (e.g., potential non-caspase substrates of OspC3 and CopC). Last but not the least, large-scale analyses of additional PTMs (e.g., ubiquitination and acetylation) are also feasible with the development of specific antibodies recognizing these modifications (Xu et al., 2010; Choudhary et al., 2009).

In summary, we believe that mass spectrometry will play a more prevalent role in studying host protein modifications catalyzed by bacterial effectors. It will not only aid in the characterization of novel modifications (e.g., ADP-riboxanation), but also greatly facilitate the global, unbiased discovery of enzymatic substrates of bacterial effectors. We certainly envisage a flourishing field of bacteria-mediated PTMs and further expansion of our knowledge on host-pathogen interactions in the near future.

CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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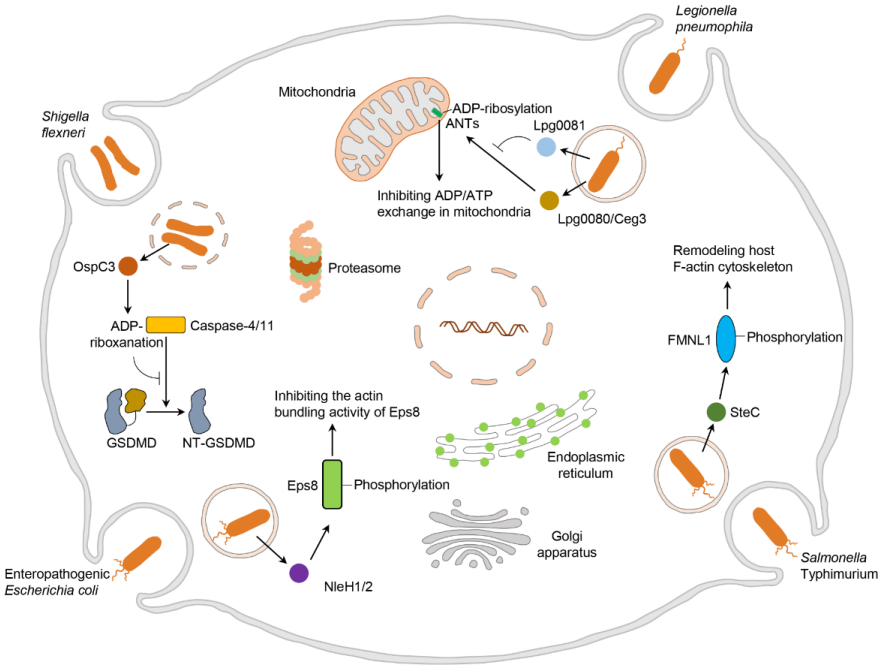

FIGURE 1 Schematic representation of exploitation of host cell signaling by bacterial pathogens via effector-mediated post-translational modifications. Effector proteins secreted by invading pathogenic bacteria have been shown to harbor diverse enzymatic activities, subverting host cellular pathways. For instance, *Shigella*effector OspC3 targets host caspase-4/11 for ADP-riboxanation, which blocks the cleavage of GSDMD by caspase-4/11 (Li et al., 2021).*Salmonella* kinase SteC remodels the host F-actin cytoskeleton by phosphorylating FMNL1 (Poh et al., 2008), whereas enteropathogenic*Escherichia coli* effector kinases NleH1 and NleH2 target Ser775 of host microvillus protein Eps8 for phosphorylation, thus inhibiting its bundling activity and driving its dispersal from the attaching and effacing (AE) lesion during infection (Pollock et al., 2022). In addition, Lpg0080 (also named as Ceg3) and Lpg0081 from *Legionella pneumophila* coordinately modulate host mitochondrial ADP/ATP exchange via reversible ADP-ribosylation of ANTs (Kubori et al., 2022; Fu et al., 2022). Due to space limitation, only a few representative effectors and their associated pathways are shown in the figure.