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Article:
Wright, MH orcid.org/0000-0003-2731-4707 (2018) Chemical proteomics of host-microbe interactions. Proteomics, 18 (18). ARTN1700333. ISSN 1615-9853

https://doi.org/10.1002/pmic.201700333

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Chemical proteomics of host-microbe interactions

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Abbreviations:

ABPP	activity-based protein profiling
ADP	adenosine diphosphate
AfBPP	affinity-based protein profiling
Aha	azidohomoalanine
AMP(ylation)	(attachment of) adenosine monophosphate
Anl	azidonorleucine
Aoa	2-aminooctynoic acid
ARF	ADP-ribosylation factor
ATP	adenosine triphosphate
BONCAT	bio-orthogonal non-canonical amino acid tagging
CuAAC	copper-catalysed azide-alkyne cycloaddition
GPI	glycosylphosphatidylinositol
HCV	Hepatitis C Virus
HIV	Human immunodeficiency virus
HSV	Herpes Simplex Virus
IBD	inflammatory bowel disease
isoTOP-ABPP	isotopic tandem orthogonal proteolysis
MetRS	methionine-tRNA ligase

MOE metabolic oligosaccharide engineering

Keywords: activity-based protein profiling, chemical probe, chemical proteomics, metabolic tagging, photoaffinity labelling

Total number of words: 7526

Abstract

The dynamic proteome plays numerous roles in the interactions of microbes - whether they are invading pathogens or symbiotic organisms - and their hosts. Host and microbe sense, respond and manipulate each other's biology via a multitude of mechanisms, resulting in alterations in protein expression or post-translational modification that influence protein localisation, activity or binding partners. The intrinsic, temporal and spatial complexity of multi-species systems makes identifying the molecular players challenging. Chemical proteomic approaches apply small molecule chemical tools to interrogate protein function, interactions or modifications. Here I highlight recent advances in the application of these methods at the host-microbe interface.

1 Introduction

Microbes - encompassing bacteria, viruses, fungi, archae and protists - are ubiquitous at the interface of multicellular organisms and their environments. Worldwide, infectious diseases such as tuberculosis, HIV and malaria remain significant health problems, and rising antibacterial resistance is widely recognised as an increasingly urgent and severe threat to human and animal health. Microbes range from pathogens causing diseases of varied severity and acuteness, to pathobionts (commensals with pathogenic potential but that do not always cause disease[1]), to non-harmful symbionts that co-exist with their hosts. The microbiota - the community of microbes living on or in a multicellular host - has been linked to susceptibility and progression of diseases such as diabetes, obesity, inflammatory bowel disease and cancer in humans.[2, 3] However, >50% of human gut microbe genes are unannotated[2, 4] and only a tiny portion of microbial small molecule metabolites and metabolic capacities are characterised.[5]

'Omics methodologies are powerful, unbiased, discovery tools for deriving connections between proteins/genes and effects at the cellular or organismal level. Proteomics provides molecule-level insight into the machinery of the cell and lends itself well to comparative studies e.g. of infected vs. uninfected cells; diseased vs. non-diseased tissue. The huge advances in mass spectrometry (MS) sensitivity in the last decade,[6] mean that deep proteome analyses of complex systems are now possible, although there are ongoing challenges for multi-species systems, including the high complexity of samples, how to deal with sequence similarity across proteomes, and analytical challenges associated with unsequenced organisms.[7] Moreover, there is a bottle neck in moving from statements of correlation (this protein/gene is present and is linked to a specific phenomenon) to causation (this protein influences biology via this molecular mechanism). Pressing questions in the field of host-microbe interactions include: What are the microbial small molecules that influence the host (e.g. the immune system), and what are the mechanisms mediating this at the molecular level? How can we annotate proteins with specific enzymatic activities and how do activity levels change in different environments or over time? How do endogenous microbiomes influence the effects of small molecules on the host through metabolism of drugs and other environmental chemicals in humans?[8] In contrast to 'omics analyses, which generate huge datasets, the experimental characterisation of protein function or interactions is painstaking and low-throughput. Furthermore, certain protein post-translational modifications (PTMs) are still largely undetectable via global proteomics approaches. This review discusses small molecule-based methods, termed chemical proteomics, that can help bridge the gap between large descriptive datasets and functional studies, or can aid in detecting low abundance proteoforms in complex samples.

Chemical proteomics is the application of small molecule chemical tools to label and detect specific proteins or subsets of the proteome. This encompasses methods that label proteins based on their enzyme activity, employ metabolic incorporation of functionality for cell-specific labelling or PTM detection, or label proteins with chemical tools to determine the direct interactors of a small molecule. Modern tools often exploit bio-orthogonal ligation chemistry - chemical reactions occurring between two functional groups that are individually unreactive in the cellular environment but that react together rapidly and specifically under biocompatible conditions (Fig. 1). Such ligations enable tools to be functionalised with small tags that ideally do not interfere with interactions in cells. One of the most commonly used tag is a terminal alkyne, which can be ligated to an azide-containing label via a copper-catalysed reaction (CuAAC, a click reaction).[9]

Here I review recent examples of chemical proteomics approaches applied to host-microbe interactions, with an emphasis on bacteria and on small molecule chemical probes - especially those that are cell permeable and that therefore enable in situ interrogation of biological systems.

2 Activity-based protein profiling

Activity-based protein profiling (ABPP) is a powerful approach for functional proteomics, enabling the annotation of uncharacterised protein function, the identification of small molecule enzyme inhibitors, and allowing changes in protein activity to be tracked in a time- and context-dependent manner.[10]

2.1 ABPP methodology

In ABPP a small molecule chemical probe is applied to specifically label catalytically active proteins. Probes include those that react covalently with nucleophilic enzyme active-site residues, or form reactive intermediates in situ that label the enzyme in a mechanism-dependent manner. The activity-based probe (ABP) is functionalised with a tag such as a fluorophore, affinity label (usually biotin) or with a bio-orthogonal handle for two-step labelling, allowing the application of these molecules in live cells or even organisms (Fig. 2A). Thus, the classical ABP contains three features: the warhead that reacts with protein residues, an element that provides specificity for a protein or class of proteins, and the tag. Upon incubation of a two-step ABP with the biological sample, labelled proteins are ligated to an affinity tag (typically biotin) via bio-orthogonal chemistry, tagged proteins are pulled-down, digested into peptides and subject to standard LC-MS/MS proteomic workflows. ABPP can also be powerful when applied in competitive mode: here a broad-spectrum probe is typically used to label a class of enzymes and compounds are screened to search for

inhibitors that block labelling.[11] This can lead to the generation of new chemical tools to block the activity of specific proteins in biological settings.

ABPs have been developed to target multiple enzyme classes, including serine hydrolases, cysteine proteases, glycosidases, ATPases/kinases, fatty acid synthases and bacterial cell wall antibiotic targets, some of which are discussed below.

2.2 ABPP in bacterial-host interactions

ABPs, many based on natural products,[9] have been applied to characterise bacterial enzymes in a variety of Gram-negative and positive organisms. Several recent reviews have covered chemical proteomic approaches to reveal mechanisms of bacterial pathogenesis [12]. Therefore, I highlight here four recent examples where ABPP has contributed to our mechanistic understanding of host response to bacteria or been applied to analyse protein function in the context of interactions. For a discussion of ABPP applied to host-virus interactions, the reader is referred to a recent review by Pezacki et al.,[13] who have developed and applied multiple ABPs in the context of viral infection.

Characterising host and pathogen enzymes. In an example of simultaneous interrogation of host and pathogen enzymes in an animal model, Hatzios et al. employed ABPP in samples from rabbits or human infected with Vibrio cholerae to search for novel secreted enzymes involved in cholera.[14] They used the well-characterised and widely-used probe **1** (Fig. 2B), which has a fluorophosphonate warhead and promiscuously labels serine hydrolases, to label proteins in cell-free supernatants. The majority of the proteins identified after biotin enrichment were host-derived proteases, but four predicted serine hydrolases from V. cholerae were also isolated. The authors went on to characterise one of these, applying ABPP to analyse the different active processed forms of the protease under different growth conditions.

ABPP for deep interrogation of the proteome. ’Omics approaches are increasingly revealing correlations between specific gut microbes and human diseases.[1] However, a major challenge in metaproteomics studies is coverage. Mayers et al. recently applied a quantitative isotope labelling approach to analyse the proteome of inflammatory bowel disease (IBD) mice in comparison to healthy animals.[15] They noted an overabundance of host anti-proteolytic proteins in the IBD animal model, but could not detect gut bacterial proteases despite extensive fractionation prior to LC-MS/MS. The authors synthesised a biotinylated chloromethyl ketone probe (**2**, Fig. 2B) and applied this to fecal samples to enrich reactive cysteine-containing proteins. Indeed the probe enriched for proteins with cysteine-type peptidase activity, as well as dehydrogenases. IBD mice showed increased

levels of peptidase/hydrolase activity. This study highlights the utility of ABPP for homing in on specific protein classes in highly complex samples.

Characterising the mode of action of microbiota-derived natural products. Promiscuous ABPs have also been applied to characterise the activity of small molecules originating from the microbiota. Fischbach and co-workers identified a family of nonribosomal peptide synthetase gene clusters in gut bacteria, and determined that the natural products produced were likely peptide aldehydes and derivatives thereof.[16] They then performed competitive ABPP with a cysteine-reactive probe iodoacetamide-alkyne (**3**, Fig. 2C)[17] to search for block of cysteine labelling by the natural products. For site-specific quantification, they applied isotopic tandem orthogonal proteolysis (isoTOP)-ABPP, a method in which the cysteine-containing peptide-probe adduct is selectively released via an orthogonal proteolysis step after enrichment.[17] The authors found that dipeptide aldehyde **4** specifically inhibited the activity of host cathepsins. These aldehydes may constitute a mechanism for gut bacterial manipulation of the immune system.

Selective probes to analyse the proteasome in plant-microbe interactions. The examples above mostly employ promiscuous probes to broadly label enzyme classes, but chemical biologists have also been remarkably successful at tuning ABPs for selectivity towards specific enzymes. For example, peptide substrate sequence selectivity has been widely exploited for development of probes for proteases.[18] The proteasome is a multi-subunit machine exerting control over many cellular processes, and several subunit-selective probes have been developed. The van der Hoorn group, who have established the use of many ABPs in plant science, employed two probes (**5** and **6**, Fig. 2D) developed by Li et al.[19] to analyse activity of the β1 and β5 subunits in Nicotiana benthamiana plants infected with the pathogen Pseudomonas syringae.[20] Interestingly, they observed a variable response, with upregulation or suppression of the β5 subunit in response to infection, and uncoupling of β1 and β5 activities. Based on this the authors hypothesise that proteasome complexes composed of different subunits may form in plants under different conditions.

2.3 ABPP: strengths and limitations

A clear strength of ABPP is its ability to report on protein enzymatic activities, not just levels, thereby providing direct functional information. Furthermore, this information is obtained in a whole proteome, and often live cell, context. Depending on the promiscuity of the probe, ABPP combined with modern quantitative proteomics can provide data on the activities of 10s to 1000s of proteins simultaneously and enable screening of small molecule libraries to identify inhibitors and assess their selectivity across the proteome in a native environment. This generates novel small molecule tools as well as annotating uncharacterised proteins.

ABPP is limited to detection of enzyme classes for which probes are available. Similarly, ABPP is subject to some of the inherent limitations of MS-based proteomics, such as difficulties detecting or quantifying across the dynamic range present in biological samples, and a lack of spatial resolution in the sample. Like every small molecule-based approach, the exogenous application of a chemical probe has the potential to perturb the biological system under study, although in the case of ABPP the timescale of probe application is often short and so this is of less concern than with some methods. However, this limitation also extends to genetic methods: any manipulation of the sample has the potential to alter function.

There is clearly still significant mileage in applying well-established probes (such as **1** and **3**) in complex host-microbe settings, and the commercial availability of these reagents facilitates their application. Meanwhile, research efforts into new probes targeting thus far un-addressed proteome space should continue to contribute to the expanding toolbox of ABPs.

3 Metabolic labelling

The development of bio-orthogonal ligation chemistry has led to a whole suite of approaches that exploit metabolic incorporation to introduce functionality into cells. Small tags such as azides and alkynes can be introduced into synthetic metabolite analogues that are taken up and incorporated in place of natural building blocks into proteins. Such metabolic tagging strategies fall into two camps: tagging with amino acid analogues to incorporate unnatural functionality into the protein sequence, and tagging with metabolite precursors to profile PTMs.

3.1 Metabolic tagging with unnatural amino acids

Distinguishing proteins originating from different populations of cells is challenging, even with the increasing power of metaproteomic analysis tools.[7] Amongst the various genetic and chemical tools available for cell-selective labelling,[21] BONCAT (bio-orthogonal non-canonical amino acid tagging[22]) is a chemical proteomic method where functionalised amino acid analogues are incorporated into proteins by the native or engineered translation machinery (Fig. 3). Site-specific incorporation is possible via genetic code expansion (reviewed in [23]), but here I focus on global incorporation of amino acid analogues into the proteome. BONCAT enables labelling and enrichment of newly synthesised proteins and is well-suited for pulse-chase analyses due to the high level of temporal control achievable.[21] Incorporation can also be placed under genetic control, to restrict labelling to a specific cell type.

One of the most commonly used non-canonical amino acid analogues is **Aha** (Fig. 3), first applied to monitor protein synthesis in mammalian cells.[22] **Aha**, and its alkynyl analogue **Hpg**,[24] mimics methionine and **Aha** is also tolerated by the native translation machinery in a variety of bacteria.[25, 26] **Aha** labelling can be made cell selective; for example, by feeding a precursor to cells that requires expression of a specific nitrilase for conversion into **Aha**.[27] Other tagged analogues are poor substrates for native MetRS and therefore require expression of an engineered MetRS for incorporation; these include **Anl**[28], **Aoa**[29] and **Pra**[30] (Fig. 3). Combined use of **Pra** and **Anl** in a co-culture system of bacteria expressing the appropriate MetRS enzymes enabled independent labelling of the two strains.[30]

Many bacterial pathogens can invade host cells, exploiting the host cell for nutrients and evading immune detection. Monitoring the proteome of the pathogen during infection is challenging due to the high relative abundance of host proteins and extremely low abundance of many bacterial virulence factors. Recognising this, the Hang lab developed alkynylated amino acid analogue **Aoa** and applied this to label and identify Salmonella typhimurium proteins during host cell invasion.[29] One rational for developing **Aoa** is that having the azide as the labelling reagent has been observed by numerous groups to give lower background labelling than the reverse reaction (e.g.[31]). However, azides can be ligated to labels via either CuAAC or strain-promoted cyclooctyne reactions, giving them the advantage of flexibility, and both azides and alkynes are widely used as tags. Although **Aoa** resulted in some background labelling via the native translation machinery, the signal to noise of CuAAC was indeed better than **Anl.**[29] **Aoa** or **Anl** were incorporated into Salmonella proteins to identify virulence factors expressed during infection.

A study from the Tirrell lab applied amino acid tagging to identify bacterial proteins that are secreted or injected into the mammalian cell by Yersinia enterocolitica.[32] Cell selective labelling of bacteria was accomplished via expression of the MetRS mutant for incorporation of **Anl**. Following infection with Yersinia in the presence of **Anl**, host cells were selectively lysed, leaving the bacteria intact, and biotin enrichment followed by LC-MS/MS used to identify proteins injected into the host. Amongst hits were expected type III-secretion system substrates, including the Yop (Yersinia outer membrane) proteins. The authors also analysed Yersinia proteins secreted into the medium, revealing that only a subset of proteins are secreted by intracellular cells. Finally, this study exploited the high temporal control afforded by pulse-labelling with **Anl** to analyse the order in which Yops were injected into host cells.

In a similar approach, Chande et al. used Mycobacterium tuberculosis expressing the **Anl**-specific MetRS to identify mycobacterial proteins.[33] This study illustrates a caveat of

BONCAT: the mutant MetRS strains were less efficient at infecting macrophages than wild type bacteria. As noted by the authors and others,[21] careful control of labelling conditions and validation of results is important in this approach. A slowing of growth over long labelling times with **Anl** was observed in another study, which applied a similar approach to host cell infection with the protozoan Toxoplasma gondii.[34] Nevertheless, the parasites could still invade cells and pulse labelling with **Anl** showed that many T. gondii proteins are stable in the host for hours. The authors of this study note a possible incompatibility of the mutant MetRS proteins, which are E. coli derived, and the eukaryotic translation machinery: their data suggests that **Anl** is only being incorporated into leader methionines, and not into internal methionines. These limitations may be addressed by engineering improved MetRS variants.

3.2 Metabolic tagging for PTM analysis: Glycosylation, lipidation and AMPylation

Proteins in cells are frequently subject to diverse and dynamic post-translational modifications (PTMs) that modulate their function. Metabolic tagging approaches have been developed to address several PTMs, using the same concept of unnatural but functional analogue incorporation (Fig. 4A).

Glycosylation of proteins is one of the most common and diverse PTMs, and is correspondingly involved in many cellular processes, including cell-cell recognition and bacterial virulence.[35, 36] MS-proteomics is the gold standard method for analysing glycoproteins but assigning glycan structures to specific sites in a high-throughput manner is still challenging.[37] A chemical glycoproteomics termed metabolic oligosaccharide engineering (MOE), pioneered by Bertozzi et al. amongst others, applies bio-orthogonally tagged sugar analogues to label glycans and glycoproteins in live cells.[38] MOE exploits cellular pathways that scavenge and permissively incorporate monosaccharide building blocks (Fig. 4B), and has been widely applied in mammalian cells for both imaging[39] and proteomics.[36] Fewer studies have applied MOE to bacterial glycans, because microbe-specific monosaccharide analogues have received less attention and bacteria can rapidly catabolise some sugars in the medium, necessitating high concentrations of analogues.[38] Despite these challenges, MOE has been applied to label glycoproteins in H. pylori,[40] C. jejuni,[41] Bacteroidales,[42] and recently Dube and coworkers designed analogues of three rare monosaccharides for labelling in H. pylori, C. jejuni, B. thailandensis and the plant pathogen R. solanacearum.[43] Further tool development – and particularly application of microbe-specific sugars – should enable researchers to interrogate bacterial glycoproteins in the context of interaction with hosts.

Attachment of lipids to proteins often directs membrane localisation.[44] In eukaryotes, the best characterised modifications include N-terminal N-myristoylation (C14:0), S-palmitoylation/acylation (often C16:0) on cysteine, prenylation and GPI-anchor modification. Identifying lipidated proteins by MS is challenging because these proteins are often of low abundance, modifications may be of low stoichiometry, and lipid-modified peptides are difficult to detect due to their physicochemical properties. The development of bio-orthogonal ligation chemistry has made significant impact in this field: lipid analogues bearing small azide or alkyne tags are well tolerated by the biosynthetic machinery and incorporated into proteins.[45] Whilst most work has focused on eukaryotes, there are also examples of bacterial lipoprotein labelling, notably by Hang et al., who first reported the use of fatty acyl analogues in bacteria,[46] and by Tate et al. for the analysis of lipoproteins in intestinal pathogen C. difficile.[47] Here I focus on select examples where this approach has provided insight into interactions at host-microbe interfaces.

Given the important signalling functions of protein lipidation in eukaryotes, it is not surprising that lipidated proteins are involved in defence against infection, or that intracellular pathogens can hijack host lipidation machinery for their own ends. Hang et al. used analogue **7** (Fig. 4C) to globally profile S-palmitoylation in dendritic cells, revealing modification of IFITM3, a protein upregulated by the antiviral cytokine interferon-γ.[48] Further experiments demonstrated that the activity of IFITM3 in influenza infection is dependent on S-palmitoylation. Similarly, Charron et al. used alkynyl-farnesol analogue **8** to identify prenylated proteins in macrophages.[49] They applied their previously developed cleavable azido-biotin reagent, which improves identification by enhancing release of enriched proteins from streptavidin beads.[25] Such cleavable linkers can sometimes also enable identification of the site of modification.[17][50] Amongst the hits, Charron et al. identified ZAPL (long isoform of the zinc-finger antiviral protein), a protein not previously annotated as prenylated; they then demonstrated that farnesylation promotes membrane localisation and anti-viral activity of ZAPL against Sindbis virus.

A recent study by Serwa et al. employed myristate and palmitate analogues **9** and **10** to study protein acylation in Herpesvirus (HSV) infection.[51] The authors used SILAC quantification for chemical proteomic enrichment of acylated proteins in HSV-infected and mock-infected cells, and used total proteome analysis to control for changes in global protein abundance. Interestingly, their results revealed that reduced N-myristoylation of host proteins results largely from suppression of host protein synthesis, whereas reduced S-acylation does not. To distinguish S- and N-linked acylation, Serwa et al. employed a specific inhibitor of N-myristoyltransferase to demonstrate that, although viral proteins are labelled by shorter analogue **9**, this is likely via an S-acyl linkage. They also used an

orthogonal S-acylation detection method, resin-assisted capture [52], to confirm S-acylation of several host proteins.

Metabolic labelling approaches could be particularly powerful for facilitating unbiased discovery of new mechanisms. N-Myristoylation was historically thought to be an irreversible modification mediated by one or two related myristoyltransferases. However, Burnaevskiy et al. recently reported the identification of a Shigella cysteine protease (IpaJ) that cleaves N-myristoyl glycine from host ARF1, resulting in Golgi disruption in infected cells.[53] Labelling with **9** revealed that IpaJ removes lipidation from multiple proteins in HEK cells. The authors followed this study with global chemical proteomic profiling of cells infected with Shigella wild type or ipaJ knockout bacteria, showing that despite broad in vitro specificity, in vivo the protease shows high selectivity for host ARF and ARF-like isoforms and an E3 ubiquitin ligase.[54] This highlights the value of performing such functional studies in a biologically relevant context.

Bacteria use injection of effector proteins that modify host proteins to subvert host cell function.[55] AMPylation, the attachment of adenosine monophosphate onto proteins, and ADP-ribosylation are PTMs mediated by bacterial effectors and several chemical probes have been developed to identify the host substrates subject to these modifications.[56] In 2011 Hang et al. reported the first alkyne-tagged probe to monitor AMPylation;[57] **11** (Fig. 4E) was incubated with HeLa cell lysates in the presence of bacterial effectors, biotin attached via CuAAC and protein substrates isolated and identified by proteomics.[57] This identified Cdc42 as a novel target of Vibrio effector VopS. **11** has also been applied to identify new substrates of VopS in conjunction with protein microarrays [58], of a L. pneumophila effector,[59] and of a human AMPylator, HYPE.[60] The latter study employed cleavable pull-down reagents to enable identification of the site of AMPylation via proteomics.[60] The unphosphorylated version of **11** was recently applied to study ADP-ribosylation in response to oxidative stress in whole mammalian cells.[61]

3.3 Metabolic tagging: strengths and limitations

Incorporation of unnatural precursors offers the advantages of high sensitivity and specificity: the cellular machinery imparts high specificity, and covalent incorporation of the tag means that proteins can be pulled-down and enrichment resin washed stringently to remove non-specific binders in a manner not possible with other affinity-based methods (such as antibodies). Some PTMs (such as certain lipidations) are currently undetectable in high throughput via other methods.

Metabolic incorporation affords high temporal control, providing a snapshot of protein synthesis or modification within a specified timeframe. However, this can also be a limitation:

only specific subsets of the proteome are captured by the experiment. Low incorporation can reduce sensitivity, and unnatural metabolites may perturb the system, as noted for some unnatural amino acid analogues.[33, 34] Alternative methods exist to detect some PTMs such as S-acylation, which can be identified by biotin-switch or resin-assisted (acyl-RAC) approaches where thioesters are selectively cleaved and subsequently enriched based on thiol-reactivity. For example, a recent in-depth quantitive study compared metabolic labelling with an alkynyl-palmitoyl analogue and acyl-RAC, concluding that ideally both experiments should be performed to fully characterise the S-acylated proteome of a cell:[62] whilst metabolic tagging is highly sensitive, and highly specific when used in conjunction with thioester hydrolysis control experiments, acyl-RAC captures some proteins that may have slow palmitoyl turnover and thus are not labelled by the fatty acid analogue.

To effectively apply metabolic tagging it is also important to understand the uptake and metabolism of the analogue. This has been shown to be particularly important in chemical glycoproteomics, where analogues can be subject to epimerase activity.[36]

Whilst metabolic labelling cannot be applied in human samples, researchers are beginning to exploit the availability of non-natural analogues of metabolites to label and track specific populations or bacteria in increasingly complex systems and even in vivo. Examples include: using **Aha** to follow bacterial degradation in phagocytes;[63] imaging bacteria in the mouse gut;[64] and applying sugar and amino acid analogues to label surface molecules to track anaerobes in a live mouse.[65]

4 Photoaffinity labelling

ABPP relies on intrinsic protein reactivity to introduce labels that facilitate proteome detection and enrichment, and metabolic labelling exploits the biosynthetic machinery. However, many small molecule-protein and protein-protein interactions regulating host-microbe interactions are non-covalent in nature, posing a challenge for chemical proteomic methods.

Affinity resin approaches, where a molecule of interest is attached to a solid support and a lysate flowed across, have been widely and in some cases very successfully applied to identify protein binders.[66] However, this method suffers from several limitations: weak binders may be washed away; attachment to the resin can perturb binding; and, crucially, the lysate environment is artificial and not well suited to detecting membrane proteins. An alternative is photoaffinity labelling, a strategy that is experiencing a resurgence in popularity due to the increased sensitivity of modern MS techniques and the development of bio-orthogonal ligation chemistry. In photoaffinity labelling, an affinity-based protein profiling (AfBPP) method, a molecule of interest is functionalised with a photoreactive moiety,

creating a probe that, upon UV irradiation, forms a highly reactive intermediate that crosslinks it to adjacent binding partners.[9, 67, 68] By using a small bio-orthogonal tag, a probe that closely resembles its parent compound can be designed; the tag then enables downstream enrichment of the probe-protein complexes for identification (Fig. 5A). Common photoreactive groups include benzophenone **12**, aryl azide **13**, aryl diazirine **14** and alkyl diazirine **15** (Fig. 5B); all possess advantages and disadvantages, as reviewed by others.[67] Although photoaffinity labelling has not yet been widely applied for analysis of host-microbe interactions, I discuss below a few recent examples that illustrate the potential of this technology.

Sherratt et al. reported a chemical probe based on a reversible inhibitor of phosphatidylinositol kinases to monitor these enzymes during infection with HCV.[69] The probe, **16**, incorporated a benzophenone photoreactive group and alkyne tag for isolation of target proteins (Fig. 5C). Although lipid kinases could not be detected by proteomics, likely due to their low abundance relative to nucleotide-binding proteins labelled by the probe (a common challenge with ATP-competitive probes), the authors used pull-down and Western blot to demonstrate increased labelling of PI4K-IIIβ in response to HCV infection. Interestingly, this did not correlate with abundance of the kinase, suggesting that the affinity-based probe may be selectively labelling active kinase.

Benzophenone is commonly used due to its stability and the commercial availability of building blocks. However, choice of photoreactive group is by no means straightforward, and appears to be rather context dependent,[67] although a recent comparative study suggests that diazirines may generate the lowest background.[70] We recently explored several different photoreactive groups to identify the bacterial binding partners of a human hormone, dynorphin, that enhances virulence of the opportunistic pathogen Pseudomonas aeruginosa.[71] After investigating several different probes based on the dynorphin sequence, we found that an alkyne- and diazirine-modified probe (**17**, Fig. 5C) was able to enrich a bacterial sensor kinase from live Pseudomonas cells, as detected by label-free quantitative proteomics.[71] Subsequent global proteome analyses showed that binding of the peptide to this sensor triggers a bacterial defence response. This report is the first demonstration of direct binding of such a peptide to a low abundance sensor in live cells, illustrating the potential of photoaffinity labelling for target identification in host-pathogen interactions.

The bacterial response we observed was similar to that triggered by cationic peptides, which in nature are common host defence and immunomodulatory molecules.[72] Others have applied photoaffinity labelling to study bacterial targets of cationic peptides to define their mode of action. For example, Volke et al. devised benzophenone and alkyne-tagged

versions of proline-rich antimicrobial peptides and found that these labelled ribosomal proteins in E. coli.[73]

Probes for other signals active at the host-microbe interface have also been developed, including photoreactive analogues of the P. aeruginosa quorum sensing molecules acyl homoserine lactones and quinolones (e.g. **18**).[74, 75] These tools have thus far been mostly applied to identify bacterial protein binders but could reveal the mechanism by which these signals interact with the host to induce responses.

An exciting emerging area is the combination of metabolic tagging and photoaffinity labelling, where photoaffinity groups are incorporated via metabolite analogues or unnatural amino acids. Several groups have developed photo-sugar analogues, demonstrated their incorporation into cell glycans in human cells and applied them for glycan-protein interaction mapping (reviewed in [76] and [36]). Thus far such approaches have not been widely applied to profile host-microbe interactions, but are poised to make significant contributions to this field. Incorporation of photo-tagged amino acids into specific proteins also shows promise for protein-protein interaction analysis in complex systems. One study investigated interactions between a bacterial endosymbiont and its filarial nematode host by incorporating photoreactive analogues of methionine and leucine (**19** and **20**, Fig. 5D) into worms.[77] Following labelling and UV irradiation, two bacterial surface proteins were immunoprecipitated and their host interactors identified by MS.

4.1 Photoaffinity labelling: strengths and limitations

The most significant technical challenge with photoaffinity labelling is distinguishing non-specific from functional binders. Quantification and use of controls (e.g. competition experiments or control probes with similar physicochemical properties – and hence similar non-specific interactions – but lacking the biological effect of interest) are absolutely required, as is validation of protein hits via other methods. However, photoaffinity labelling combined with modern proteomics is an exceptional unbiased and system-independent discovery tool for small molecule-protein interactions. As with all small molecule-based methods, the probe must be carefully compared with the parent compound to ensure that alterations to the chemical structure do not change bioactivity significantly. Design of probes has become easier with the development of bio-orthogonal ligation chemistry, which enables use of minimal tags.

5 Conclusions

Exploring the interface between microbes and their hosts at the molecular level enhances our understanding of pathogenicity, virulence, symbiosis and dysbiosis - disease as a result of dysfunction of the normal microflora. Improvements in MS-based proteomics have had

widespread impact on our ability to catalogue and compare biological systems, but there is a long way to go to unravel the complex interplay between organisms at the molecular level. Concurrent advances in the development of chemical probes have furnished tools which, particularly when combined with cutting edge quantitative proteomics methods, can interrogate protein functions and interactions in complex settings. Here I have highlighted recent chemical proteomics studies that have revealed host - microbe interactions in mechanistic detail, with a focus on small molecule probes applied in live cells. A major strength of a chemical probe is that it is applicable even to complex samples, as illustrated in several of the studies described above. Further developments in probe design, innovative approaches to label the proteome, and advances in analytical methods, should aid in further characterising interactions in these complex systems.

6 Acknowledgements

I gratefully acknowledge support from a University Academic Fellowship from the University of Leeds, and a Royal Society Research Grant (RG160914). I apologies to researchers whose work could not be cited and discussed here due to space limitations.

The authors have declared no conflict of interest.

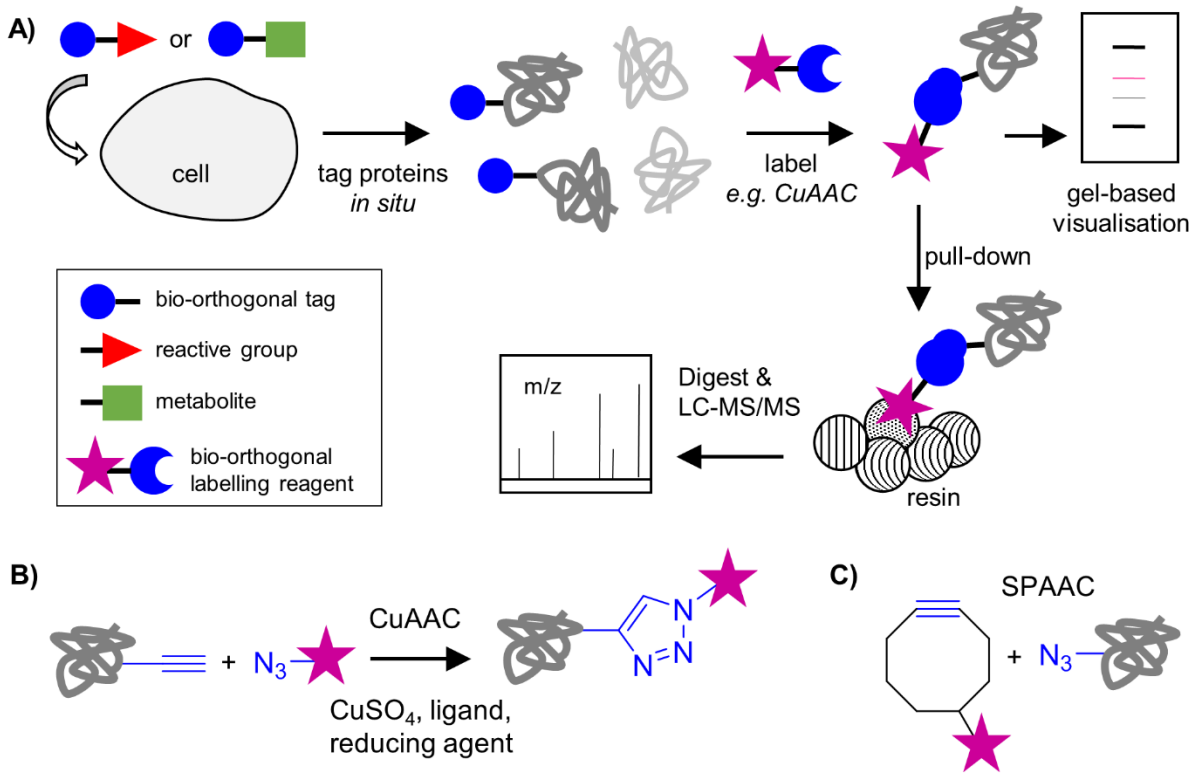
A)
or
cell
tag proteins
in situ
label
e.g. CuAAC
gel-based
visualisation
pull-down
bio-orthogonal tag
reactive group
metabolite
bio-orthogonal
labelling reagent
m/z
Digest &
LC-MS/MS
resin
B)
CuAAC
CuSO4, ligand,
reducing agent
C)
SPAAC

Figure 1

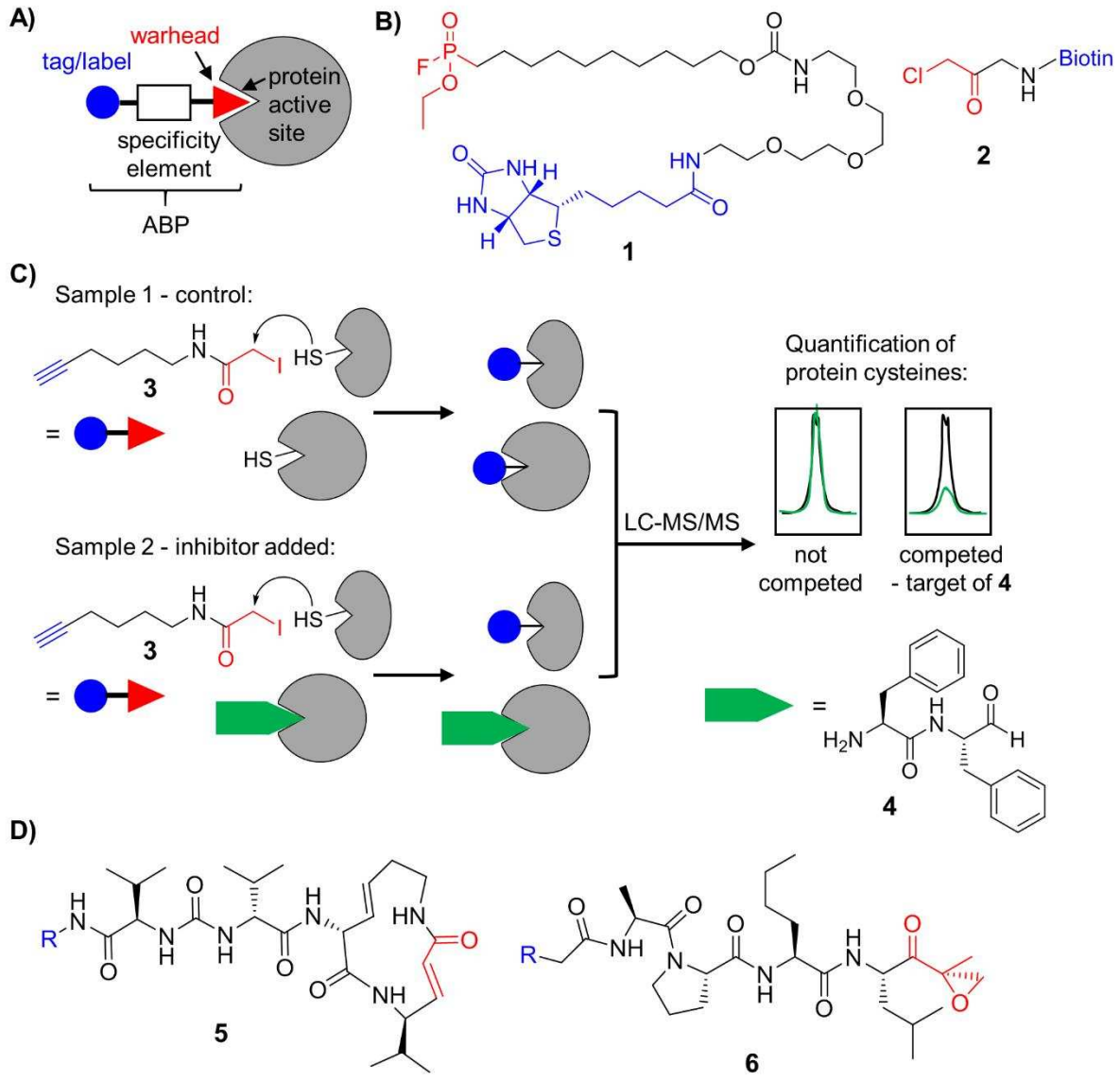
A)
warhead
tag/label
protein active site
specificity element
ABP
B)
1
2
Biotin
C)
Sample 1 - control:
3
HS
Quantification of protein cysteines:
LC-MS/MS
not competed
competed - target of 4
Sample 2 - inhibitor added:
4
D)
5
6

Figure 2

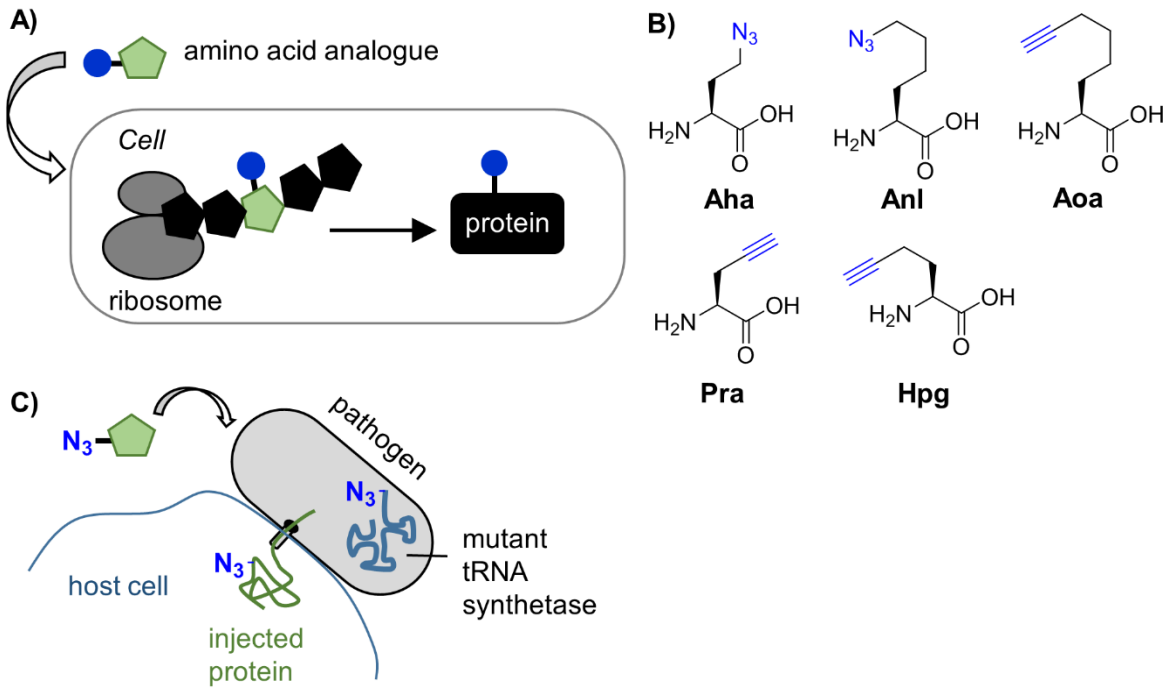

Figure 3

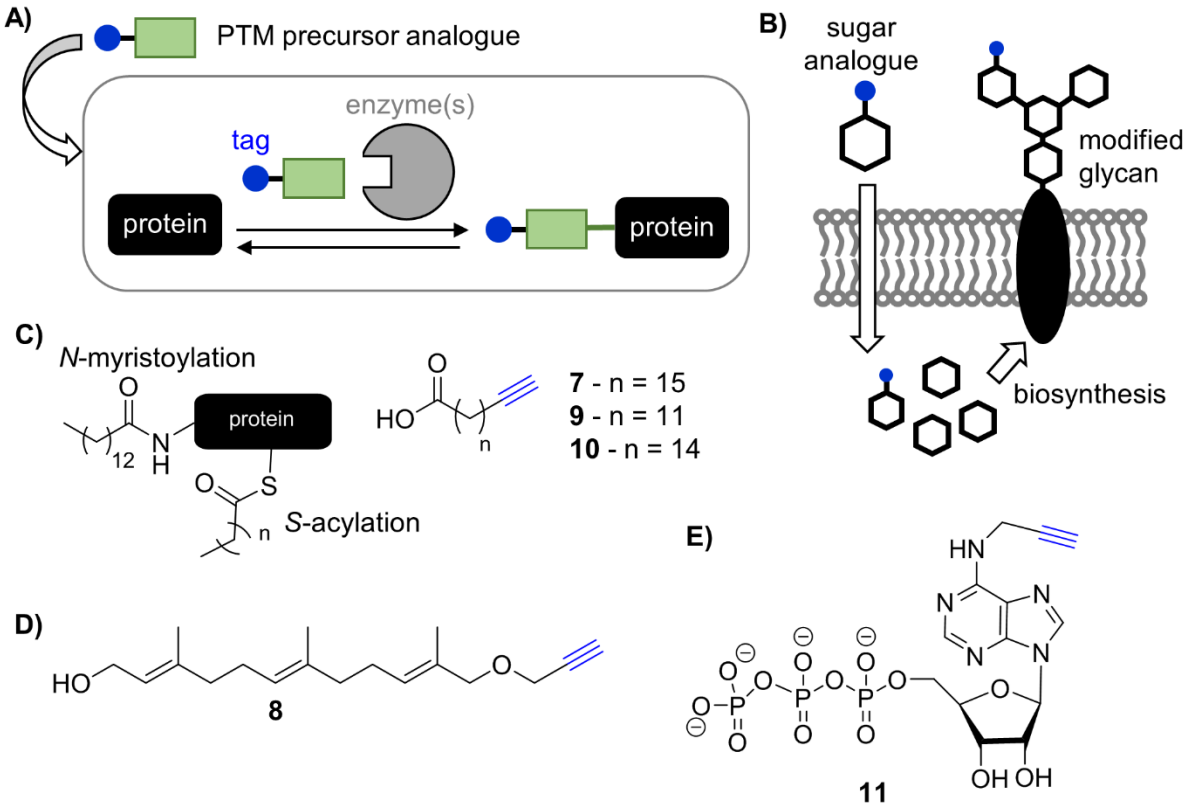

Figure 4

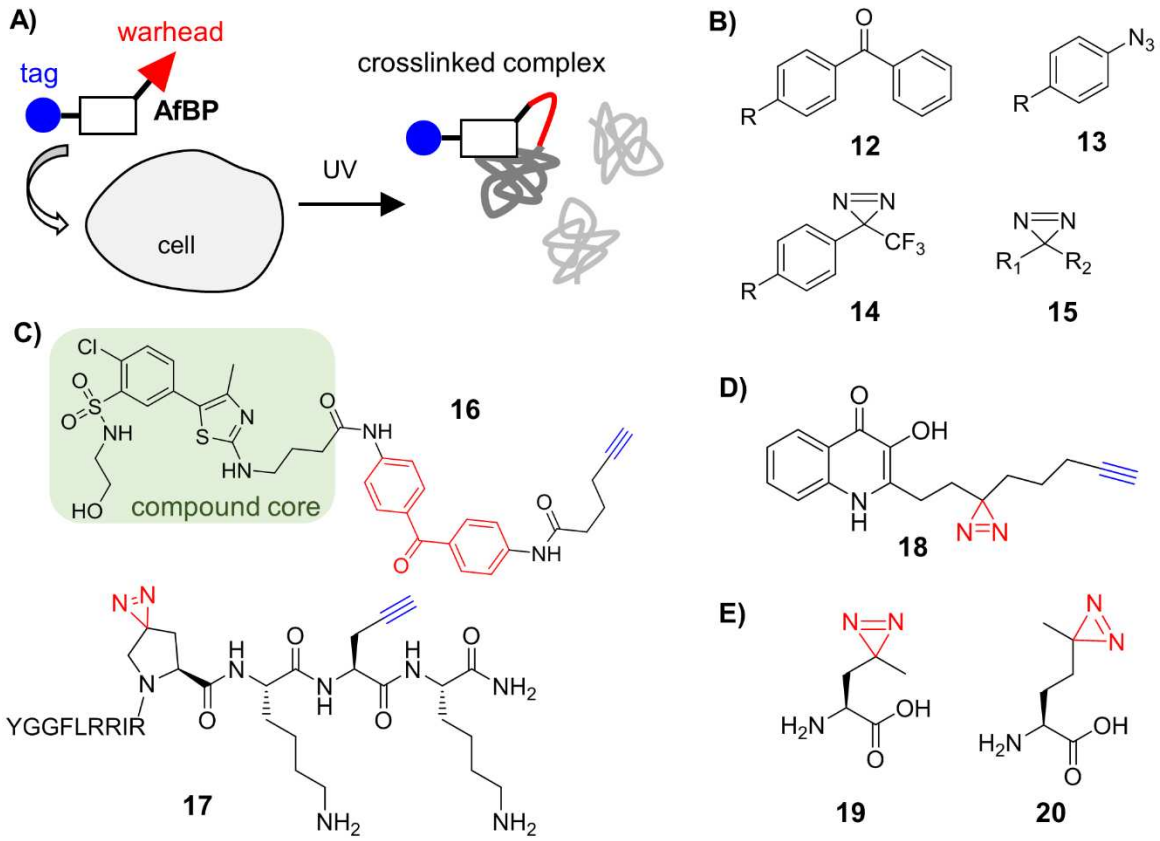
A)
warhead
tag
AfBP
crosslinked complex
UV
cell
B)
12
13
14
15
C)
16
compound core
YGGFLRRIR
17
D)
18
E)
19
20

Figure 5

Figure legends

Figure 1. A) Overview of a general two-step labelling chemical proteomic workflow to analyse subsets of the proteome captured via chemical probes. Probes incorporate a bio-orthogonal tag for labelling and isolation of proteins, and may be analogues of metabolites (e.g. amino acids, PTMs) or contain reactive groups to capture specific enzyme classes (Activity-based protein profiling, ABPP) or binding partners (Affinity-based protein profiling, AfBPP, or photoaffinity labelling). **B)** Copper-catalysed azide-alkyne cycloaddition (CuAAC) ligation to label alkyne/azide tagged proteins. **C)** Strain-promoted ligation chemistry (SPAAC).

Figure 2. Activity-based protein profiling (ABPP) of host-microbe interactions. **A)** Anatomy of an ABP, and interaction of the probe with an enzyme active site**. B)** Examples of ABPs that have been applied in the context of host-bacteria interactions. **C)** Competitive ABPP: a promiscuous probe (e.g. **3**) is used to label the proteome; competition of small molecules for binding at the enzyme active site can be detected as a loss of labelling of that protein. This was applied for detection of **4** as a cysteine protease inhibitor from the microbiome [16]. **D)** Proteasome subunit-specific probes **5** and **6**.

Figure 3. Bio-orthogonal non-canonical amino acid tagging (BONCAT). **A)** Incorporation of unnatural amino acids by the cellular machinery. **B)** Bio-orthogonally tagged amino acid analogues. **C)** Cell selective labelling of proteins: pathogen containing a mutant tRNA synthetase is able to incorporate an unnatural azide-containing amino acid analogue into proteins; this enables subsequent proteome analysis of bacterial proteins injected into the host.

Figure 4. Metabolic tagging of post-translationally modified (PTM) proteins. **A)** PTM tagging approach: relies on the incorporation of small bio-orthogonal tags via metabolite analogues or PTM precursors. **B)** Metabolic Oligosaccharide Engineering (MOE) approach. **C)** Lipid acylation of proteins and acyl-mimetic chemical probes. **D)** Prenyl chemical probe. **E)** AMPylation probe.

Figure 5. Photoaffinity profiling. **A)** Affinity-based probe (AfBP) is added to cells and crosslinked via UV irradiation to target proteins. **B)** Different photoreactive groups. **C)** Examples of two photoaffinity probes that have been used to study proteins involved in host-microbe interactions [69, 71]. **D)** Probe mimicking a quorum sensing quinolone compound; used to identify interactors in P. aeruginosa [75]. **E)** PhotoLeu (**19**) and PhotoMet (**20**).

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