Activity-based protein profiling in bacteria: Applications for identification of therapeutic targets and characterization of microbial communities
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Abstract
Activity-based protein profiling (ABPP) is a robust chemo-proteomic technique that uses activity-based probes to globally measure endogenous enzymatic activity in complex proteomes. It has been utilized extensively to characterize human disease states and identify druggable targets in diverse disease conditions. ABPP has also recently found applications in microbiology. This includes using activity-based probes (ABPs) for functional studies of pathogenic bacteria as well as complex communities within a microbiome. This review will focus on recent advances in the use of ABPs to profile enzyme activity in disease models, screen for selective inhibitors of key enzymes, and develop imaging tools to better understand the host–bacterial interface.

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Introduction
Bacteria have profound impacts on human health, ranging from life-threatening infections by pathogenic bacteria to commensal bacteria living in symbiosis to maintain the healthy state of a host. ‘Omics’-based techniques have been invaluable for mapping interactions between pathogenic bacteria and the host (reviewed in Ref. [1]) as well as dynamic changes in the composition and functional output of bacterial communities in response to diet [2,3] or disease [4,5]. While these studies are able to capture snapshots of complex functional profiles of host–bacteria and bacteria–bacteria interactions, unbiased ‘omics’-based approaches alone do not inherently provide ways to prioritize key regulators of a given state. The application of chemical tools has become invaluable for the study of bacterial systems. Of note, activity-based protein profiling (ABPP) has been applied to overcome some of the limitations of unbiased ‘omics.’ This approach uses chemical probes that provide a direct readout of enzyme activity, thus enabling enrichment of relevant enzymatic targets in complex and dynamically regulated systems. Furthermore, because ABPP identifies enzymes via chemical modification of their active sites, it focuses on inherently druggable targets that have the potential for modulation by small molecule therapeutics [6]. In this review, we will highlight the recent applications of ABPP to studies of both pathogenic and commensal bacteria, including early advances in the use of ABPP to characterize gut microbiome function.

Activity-based protein profiling
ABPP measures enzyme activity using an activity-based probe (ABP) that covalently modifies active-site nucleophiles in an activity-dependent manner. ABPs comprise a warhead for covalent bond formation, a linker that confers specificity, and a tag for the visualization or enrichment of the labeled enzymes (Figure 1a) [7]. An ABP-labeled proteome can either be separated by gel electrophoresis and visualized via a fluorophore tag or be enriched by affinity purification using an affinity tag and digested, so that targets can be identified by LC-MS/MS (Figure 1b) [8,9]. Additionally, some ABPs have an alkyne-based tag such that an azide-containing fluorophore or biotin can be added via copper-catalyzed or copper-free alkyne–azide cycloaddition (CuAAC) [10] after probe labeling.

One of the most significant benefits of ABPP is that it allows the dynamic profiling of multiple enzymes in their endogenous environments [8,11]. Furthermore, it...
selects for enzymes that are amenable to modulation by small molecules and provides a platform to screen for selective inhibitors, even for enzymes with unknown function and unknown substrates, through competitive ABPP (Figure 1c) [7,12]. There are a number of excellent reviews that cover the application of ABPP to bacterial pathogenesis [13,14], host–microbe interactions [15,16], and host-associated and environmental microbial communities [17]. In addition, there have been many studies using affinity-based protein profiling (reviewed in Ref. [18]), which utilizes a photoreactive group to covalently label a target with the probe rather than relying on the reactivity of the catalytic residues of a target. Here, we will discuss recent examples of diverse applications of ABPP, including profiling enzymatic activity in vitro, identifying enzymes involved in the host–bacteria interface, and developing inhibitors and imaging probes for important bacterial enzyme targets (see Table 1).

ABPP as a profiling approach for bacteria in culture

As a first step toward understanding which proteins are important for host–microbe interactions, ABPP can be used to identify and classify enzymes from bacteria grown in vitro. While these studies are limited in that they cannot accurately replicate the complex environment inside a host, certain aspects of the pathogenic state of bacteria can be mimicked in vitro, and the ability to precisely manipulate experimental conditions allows for well-controlled comparative studies of how enzyme activities change.

*Mycobacterium tuberculosis*, the causative agent of tuberculosis, infects almost a quarter of the world’s population in a dormant state [19], which is especially hard to treat because of its reduced metabolism [20]. To understand which enzymes remain active in dormancy, Ortega and coworkers used the serine-reactive fluorophosphonate-based ABP 1 containing a polyethylene glycol-based linker to profile serine hydrolase activity in a hypoxic model of *M. tuberculosis* dormancy [21]. Of the 75 enzymes annotated as hydrolases or hypothetical proteins that were identified under exponential growth conditions, approximately half were detected under non-replicative hypoxic conditions, albeit with reduced activity. The others were not detected at all, consistent with reduced metabolism during dormancy. Furthermore, the authors were able to define the hydrolytic function of several identified unannotated enzymes [21], highlighting the shortcomings of bioinformatics-based annotation of highly divergent bacterial genomes and the power of ABPP to experimentally annotate and confirm biochemical functions of enzymes. Tallman and coworkers performed a similar study with fluorophosphonate-based ABP 2, which has a saturated alkyl chain linker to specifically target lipid-metabolizing esterases [22]. This study identified five esterases active in both normoxic and hypoxic states that were also identified by Ortega and coworkers [21], suggesting that these enzymes are promising targets for drugs to treat both active and latent tuberculosis. In fact, several drugs that have anti-mycobacterial effects, including the anti-diabetes drug tetrahydrolipstatin (compound 3) [23] and analogs of the natural product cyclosporin P (compound 4) [24], have been shown via ABPP to act by covalently inhibiting the activity of multiple lipid-metabolizing enzymes. While neither of the studies by Ortega and coworkers [21] and Tallman and coworkers [22] went beyond profiling the enzymes active in hypoxic *M. tuberculosis*, both demonstrate the value of ABPP in poorly annotated bacterial genomes.

Chemoproteomic approaches have also been developed to profile reactive cysteine [25] and lysine [26] residues in mammalian proteomes, using iodoacetamide alkyne (ABP 5) and sulfotetrafluorophenyl ester alkyne (ABP 6), respectively. However, these probes label active-site residues as well as cysteines and lysines that play other roles in protein function. Cysteine residues, for example, can serve as metal ligands or redox-active disulfides [27]. Deng and coworkers used the general cysteine-reactive ABP 5 to identify oxidation-sensitive cysteines in *Pseudomonas aeruginosa* and *Staphylococcus aureus*, mapping out pathogen response to oxidative stress induced by hydrogen peroxide [28]. The host uses oxidative stress as one tactic to combat infections, so understanding how bacteria respond to this defense mechanism can help predict behavior in an actual infection. As these probes and others [29,30] can target both catalytic and noncatalytic sites, they are also valuable tools for identifying novel druggable targets across the bacterial proteome.

ABPP as a tool for characterizing host–bacteria interactions in vivo

Beyond using ABPs to profile enzyme activity in individual species of pathogenic bacteria, their application is especially powerful in analyzing complex host–bacteria interactions. There have been a number of recent examples of ABPs used to profile enzyme activity in in vivo models of infection and in the gut microbiome.

Using the fluorophosphonate-based ABP 7, Hatzios and coworkers profiled active serine hydrolases in the cecal fluid of *Vibrio cholerae*-infected infant rabbits and in human choleic stool [31]. This ABP-based enrichment identified four *V. cholerae* serine proteases that were not detected in a total proteomic analysis. One of these proteases, IvpP, modulates the activity of both host and *V. cholerae* serine hydrolases, and all four serine proteases together decreased the abundance of several host proteins, including one that enhances macrophage phagocytosis of bacteria [32]. Consequently, the identification of *V. cholerae* enzymes active in infection pinpointed
Activity-based probes and compounds referenced in this review, including the name of the molecule in the original paper, its targets, and the biological sample(s) in which it was tested. For each molecule, the warhead is colored red and the tag is colored blue.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Compound Name</th>
<th>Enzyme Class</th>
<th>Biological Sample</th>
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<td>1</td>
<td>FP-ABP</td>
<td>Serine hydrolases</td>
<td><em>Mycobacterium tuberculosis</em></td>
<td>78 serine hydrolases or hypothetical proteins; Rv2224c (CaeA/Hip1), Rv2284 (LipM), Rv2970c (LipN), Rv0183, Rv1683</td>
<td>Ortega et al., <em>Cell Chem Biol.</em>, 2016</td>
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<td>2</td>
<td>Desthiobiotin-FP</td>
<td>Serine hydrolases</td>
<td><em>Mycobacterium tuberculosis</em></td>
<td>52 serine hydrolases; Rv2224c (CaeA/Hip1), Rv2284 (LipM), Rv2970c (LipN), Rv0183, Rv1683</td>
<td>Tallman et al., <em>ACS Infect Dis.</em>, 2016</td>
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<tr>
<td>5</td>
<td>IA probe</td>
<td>Reactive cysteine residues</td>
<td><em>Pseudomonas aeruginosa, Staphylococcus aureus</em></td>
<td><em>P. aeruginosa</em>: 82 oxidation-sensitive Cys residues, LasR, ExaC, ArcA, GAPDH; <em>S. aureus</em>: 113 oxidation-sensitive Cys residues, GAPDH</td>
<td>Weerapana et al., <em>Nature</em>, 2010; Deng et al., <em>Cell Host Microbe</em>, 2013</td>
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<td>6</td>
<td>STP alkyne 1</td>
<td>Reactive lysine residues</td>
<td>–</td>
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<td>Hacker et al., <em>Nat Chem.</em>, 2017</td>
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<td>8</td>
<td>BioGlyCMK</td>
<td>Reactive cysteine residues</td>
<td>Mouse fecal pellets</td>
<td>Peptidases, alcohol dehydrogenases, acetaldehyde dehydrogenases</td>
<td>Mayers et al., <em>J Proteome Res.</em>, 2017</td>
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<td>Bile salt hydrolases</td>
<td>Mouse fecal pellets</td>
<td>Bacteroides sp., Bacteroidales bacterium, Muribaculaceae bacterium</td>
<td>Parasar et al., <em>ACS Cent Sci.</em>, 2019</td>
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<td>FphB</td>
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<td>Mycobacterium tuberculosis</td>
<td>Ag85, Pks13</td>
<td>Lehmann et al., <em>Angew Chem Int Ed.</em>, 2018</td>
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several bacterial proteases that manipulate the host response and potentially allow for bacterial evasion of the innate immune system [31].

Unlike infections by a single pathogenic bacterial species, in the gut, there exists a rich community of commensal bacteria whose makeup changes in response to many different external stimuli and disease states. With this added complexity, enrichment for low abundance proteins with ABPs is especially advantageous. A recent effort by Mayers and coworkers applied ABPs to the gut microbiome in a mouse model of colitis [33]. A metaproteomic analysis of both host- and microbial-derived proteins from fecal pellets found that mice with colitis had elevated host inflammatory proteins, host serine protease inhibitors, and general microbial metabolic proteins. Interestingly, the authors did not detect bacterial proteases, which are known to play an important role in the pathogenesis of colitis (reviewed in Ref. [34]). By enriching with the cysteine-reactive ABP 8, elevated activity of microbial Cys-type peptidases and dehydrogenases was detected in animals with colitis [33]. While this study was not able to single out a key enzyme from a particular bacterial species with this broadly reactive ABP, the authors demonstrated that ABPs can successfully enrich for relevant yet low-abundant enzymes from complex samples.

More recent work applying ABPs to the microbiome has approached this complexity by focusing on a specific
class of enzymes. Microbial β-glucuronidases, for example, remove glucuronic acid, which host enzymes conjugate to drugs to enhance clearance. As a result, high expression of these microbial enzymes has detrimental effects on drug clearance [35]. To understand which of the 250+ identified bacterial β-glucuronidases [36] are active in vivo, Whidbey and coworkers developed the substrate-based ABP 9 that is processed by β-glucuronidases to produce a reactive intermediate that labels nearby nucleophilic residues [37]. The authors labeled bacteria isolated from the mouse gut with fluorophore-conjugated ABP 9 and used fluorescence-activated cell sorting (FACS) to isolate the bacteria positive for β-glucuronidase activity. This workflow allowed both the quantification of total β-glucuronidase activity and the identification of bacterial species with active β-glucuronidases via 16S rRNA sequencing. For example, perturbation by vancomycin treatment globally decreased β-glucuronidase activity, although the bacterial species that had active β-glucuronidases differed across litters of mice [37]. Despite the small size of their study, the authors showed that the strategy of combining ABPs and FACS in the gut microbiome will be very useful in connecting molecular-level mechanistic results with systems-level bacterial community studies.

An ABP has also been developed for bile salt hydrolases [38], a class of gut bacterial enzymes that catalyze the first step of microbial metabolism of host-secreted conjugated bile acids. The downstream secondary bile acids affect host metabolism [39–41] and are dysregulated in inflammatory bowel diseases [42]. Although a photoreactive probe based on the structure of bile acids was developed and used to identify bile acid-interacting proteins in mammalian cells [43], the bile salt hydrolases in bacteria that are active in vivo had not been well characterized. To address this gap in knowledge, Parasar and coworkers treated bacteria isolated from mouse fecal pellets with the cholic acid-based covalent ABP 10 [38]. In a mouse model of colitis, the global activity of bile salt hydrolases was elevated, although the level of activity of individual bile salt hydrolases across several species in the phylum Bacteroidetes varied from mouse to mouse. Similar to the work by Whidbey and coworkers [37], only a small cohort of mice was used, so the biological relevance of this inter-mouse variability is not well understood. However, future studies with larger sample sizes and additional ABPs will further the understanding of host and bacterial enzyme function in the gut microbiome, the contribution of individual bacteria to this overall function, and the alterations that occur upon disease progression.

**ABPP as a method for developing inhibitors**

Beyond simply identifying enzymes whose activities are relevant and druggable in a pathogenic context, ABPP can also directly enable the development of selective inhibitors for enzymes of interest. By measuring enzyme activity in its endogenous environment, inhibitors can be tested without expressing recombinant protein or even knowing the native substrates of the enzymes. Selective inhibitors can then be used to control enzyme activity temporally, even in currently genetically intractable species, and to visualize bacterial subcellular enzyme activity in complex environments. There are two primary approaches for identifying new classes of inhibitors, either screening a compound library via competitive ABPP (Figure 1c) or developing electrophile-containing molecules inspired by the endogenous substrate of an enzyme, both of which will be explored in this section.

**S. aureus**, a leading cause of nosocomial infections, can form biofilms that are resistant to antibiotic treatment [44]. To identify serine hydrolases that are active in this state, ABPP was performed using the fluorophosphonate-based ABP 11 [45]. This chemoproteomic study resulted in the identification of 12 α,β-hydrolase domain-containing serine hydrolases active in live bacteria during biofilm growth. Within this newly discovered enzyme family named fluorophosphonate-binding hydrolases (Fphs), only two enzymes had been previously studied and the majority lacked even basic annotation as hydrolases in the Uniprot database ([https://www.uniprot.org/](https://www.uniprot.org/)). Because the targets were identified using an ABP, it was possible to immediately screen a focused library of covalent serine-reactive electrophiles to identify selective inhibitors of individual members of this family of hydrolases. Chloroisocoumarin 12, for example, showed potent and selective inhibition of FphB. Pretreatment of bacteria with compound 12 decreased the colonization efficiency of wild-type *S. aureus* in the liver during a systemic infection mouse model, suggesting this enzyme may be an important virulence factor that is required for the early colonization of host tissues [45]. In a follow-up study, competitive ABPP was used to screen a library of serine-reactive 1,2,3-triazole urea-containing compounds to identify additional specific inhibitors of Fph enzymes [46]. One of the most promising hits from the library screen was converted to the fluorescent ABP 13, and its use as a selective imaging probe highlighted the heterogeneity of FphE enzyme activity across a population of isogenic bacteria and identified compensatory functions within this network of Fph enzymes [46].

Inspired by natural products, the Sieber lab and others have used β-lactone-containing molecules to design a number of inhibitors [47,48]. One such compound, compound 14, inhibited *M. tuberculosis* growth at low doses [49]. Using an alkyl handle-containing analog of compound 14, Lehmann and coworkers identified two targets of this molecule, antigen 85 and polyketide synthase 13, both of which are serine hydrolases.
instrumental in mycolic acid biosynthesis. They confirmed these ABPP results in vitro and demonstrated that compound 14 was capable of decreasing the pool of newly synthesized mycolate α-chains. Furthermore, compound 14 had synergistic killing effects with other approved antibiotics such as rifampicin and vancomycin, showing that compounds that mimic crucial bacterial metabolites, in this case, the β-hydroxy motif of mycolic acid, can be used as novel antibiotics [49].

Conclusions and perspectives
To truly understand how bacteria, both good and bad, can affect human health, understanding of the context of host—bacteria and bacteria—bacteria interactions is essential. As demonstrated in this review, small molecule ABPs are valuable tools for capturing enzyme activity in the native environment of bacteria. They have applications ranging from profiling enzymes active in disease to developing small molecule inhibitors against identified enzymes. The ability of ABPs to enrich for active enzymes is especially powerful in complex systems such as the gut microbiome.

Moving forward, simpler models of gut microbial communities, ranging from gnotobiotic mice with defined microbial communities to mono- or co-culturing commensal bacterial in vitro, can provide insight into the activity of relevant enzymes in a well-controlled manner. In particular, we think these models can help narrow the focus specifically onto pathobiont, or contextually pathogenic, bacteria. Many diseases, such as inflammatory bowel diseases or colon cancer, are characterized by a shift in gut microbial communities to establish a state of dysbiosis that favors the growth and

Figure 2

Future applications for ABPP for treating diseases mediated by microbiome dysbiosis. (a) Schematic diagram of the gut microbial communities of healthy mice and diseased mice with a bloom of a pathobiont bacteria. Representative gel-based ABPP profiles are shown for the healthy and disease-state microbiomes, which enables identification of enzymes that are specific to the diseased microbiome. Competitive ABPP can then be used to screen for inhibitors that selectively target the disease-specific microbial enzyme leading to decreased viability of the pathobiont bacteria in the community. (b) Treating the diseased mouse with a pathobiont-specific inhibitor selectively kills the pathobiont bacteria and allows the microbial community to return to a healthy state.
dominance of pathobiont bacteria [50–53]. We believe that, by using approaches such as ABPP to identify relevant bacterial enzymes that are essential for maintaining a disease state and then developing selective inhibitors for those enzymes (Figure 2), it will be possible to shift the composition of a microbiome back to a healthy state to treat diverse human diseases.

Conflict of interest statement
Nothing declared.

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References
Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest
** of outstanding interest


38. Coupling activity-based probes and fluorescence-activated cell sorting enabled the enrichment of bacteria with specific enzymatic activity from a complex environment and the identification of the bacterial species.


40. Use of a substrate-based activity-based probe for the identification of specific bacterial bile salt hydrolases from the fecal pellets of conventionally-raised mice. Activity of individual enzymes varied from mouse to mouse but global activity across the entire microbiome shifted upon disease.


48. Activity-based protein profiling identified a new family of S. aureus bacterial serine hydrolases including a context-dependent virulence factor whose activity could be selectively inhibited by a small molecule resulting in reduced infection of specific tissues in a mouse model of infection.


50. Converting a selective inhibitor to a fluorescent imaging tool enabled the measurement of the subcellular localization of enzyme activity and overall heterogeneity within a population.


54. A small molecule mimicking a vital biosynthetic pathway can serve as a potent enzymatic inhibitor and its effects can be confirmed by metabolomic analyses of its products.


