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Activity-Based Protein Profiling—Enabling Multimodal Functional Studies of Microbial Communities

Christopher Whidbey and

Chemical Biology and Exposure Sciences Group, Pacific Northwest National Laboratory, Richland, WA 99352, USA; Department of Chemistry, Seattle University, Seattle, WA 98122, USA

Aaron T. Wright

Chemical Biology and Exposure Sciences Group, Pacific Northwest National Laboratory, Richland, WA 99352, USA; The Gene and Linda Voiland School of Chemical Engineering and Bioengineering, Washington State University, Pullman, WA 99163, USA

Abstract

Microorganisms living in community are critical to life on Earth, playing numerous and profound roles in the environment and human and animal health. Though their essentiality to life is clear, the mechanistic underpinnings of community structure, interactions, and functions are largely unexplored and in need of function-dependent technologies to unravel the mysteries. Activity-based protein profiling offers unprecedented molecular-level characterization of functions within microbial communities and provides an avenue to determine how external exposures result in functional alterations to microbiomes. Herein, we illuminate the current state and prospective contributions of ABPP as it relates to microbial communities. We provide details on the design, development, and validation of probes, challenges associated with probing in complex microbial communities, provide some specific examples of the biological applications of ABPP in microbes and microbial communities, and highlight potential areas for development. The future of ABPP holds real promise for understanding and considerable impact in microbiome studies associated with personalized medicine, precision agriculture, veterinary health, environmental studies, and beyond.

Keywords

Activity-based protein profiling; Microbiomes; Microbial communities; Activity-based probes

1 Introduction

Microorganisms impact nearly every facet of life on Earth. Human health, biogeochemical cycles, and the maintenance of a food supply all involve microbial processes. While microbiology is a well-established field, the majority of its history has been focused on the study of single (i.e., axenic) strains. Though the study of axenic cultures is important and can be informative, it misses the fact that the overwhelming majority of microbial life is

spent in complex communities with other microbes. These complex microbial communities can dramatically differ from axenic cultures in regard to gene expression, protein production, and the metabolism they conduct. Understanding the mechanistic underpinnings of community structure, interactions, and functions will be critical for understanding more complex biological systems ranging from wetland and soil ecosystems to the human gut and resulting physiology (Gilbert et al. 2014; Bitten et al. 2016; Fierer 2017). Additionally, microbiomes are not static, and it will also be necessary to understand how microbiomes respond to various perturbations.

While the importance of determining the mechanisms that drive microbial communities is clear, the study of such communities is in its infancy due to its challenging nature. Ideally, one would have the ability to grow communities in a controlled fashion and use traditional genetic and biochemical approaches to test a specific hypothesis. In practice, difficulties isolating microbes from complex matrices, low biomass recovery, and the inability to grow many microbes in the laboratory have been major barriers to working with microbial communities. Furthermore, interindividual and site-specific variance in microbial community structure and function introduce more layers of complication.

In order to address these difficulties and study microbial communities, a new set of molecular tools is emerging (Arnold et al. 2016). Recent technological developments, such as high-throughput (previously described as ‘next-generation’) nucleic acid sequencing, have been a major step forward in this regard. High-throughput sequencing (HTS) has enabled culture-independent detection and identification of microbes, single-cell sequencing, and the ability to know the sequence of all the genes present in the complex community (the metagenome). The utility of HTS approaches can be extended to RNA as well, allowing detection of all of the actively transcribed genes within a sample (the metatranscriptome). The ability to study all of the proteins present within a sample (the metaproteome) has been slower to develop, for reasons discussed in Sect. 3.

The role of HTS techniques in opening up the study of microbial communities cannot be overstated. At the same time, nucleic acid sequencing has some important technical and epistemological caveats (Zarraonaindia et al. 2013). Amplification bias, contamination, undersampling, and a lack of an agreed-upon universal analysis framework have had an appreciable impact on the ability to draw conclusions from different studies (Pollock et al. 2018; Weiss et al. 2014; Kim et al. 2017). More fundamentally, the presence of a gene or transcript does not mean that a functionally active protein is produced. The limitations of gene annotation and presence of closely related organisms mean that metagenomic and metatranscriptomic studies can identify potential function but cannot, in themselves, draw a causal inference between function and gene or organism. Metaproteomics is likely closer to representing true function, but many enzymes require posttranslational modifications or cofactors to be active. Thus, there exists a clear need for tools capable of bridging the gap between the two big questions about any microbial community system—‘who is there?’ and ‘what are they doing?’.

At present, activity-based protein profiling (ABPP) for microbial community applications is in its early stages, but there are unprecedented opportunities for function-dependent studies

to reveal the mechanisms of microbial communities impacting our health and the world around us. ABPP also provides an excellent avenue to determine how external exposures, such as chemical insults, changing climate, or altered diets or physiology result in functional alterations to microbiomes. Herein, we illuminate the current state and prospective contributions of ABPP as it relates to microbial communities. We will discuss the design, development, and validation of probes, challenges associated with ABPP in complex microbial communities, provide some specific examples of the biological applications of ABPP in microbes and microbial communities, and highlight potential areas for development.

2 Probe Design

2.1 Activity- and Affinity-Based Probes for Multimodal Characterization of Microbiome Function

As described throughout this book and in numerous reviews, chemical probes for ABPP consist of three moieties: a reactive group that forms an irreversible covalent bond with a target protein (extracellular or intracellular), a binding group (e.g., protein substrate or metabolite) that biases the probes toward a target protein or protein family and may also impart cell permeability, and a reporter tag for rapid and sensitive measurement of labeled enzymes. In microbiomes, where so little is known about organism content or overall functional capacity, the potential power of probes to reveal the functional landscape is enormous and varying the reactive groups can yield probing of diverse functions.

The reactive group of the probe can be developed in two ways. First, probes can incorporate electrophilic moieties that enable selective targeting of intracellular catalytically active enzymes by a direct mechanism-based reaction between the enzyme and the probe (Sadler and Wright 2015; Cravatt et al. 2008; Sadaghiani et al. 2007). Probes of this nature can target hydrolases, kinases, oxidation and reduction catalyzing enzymes, and others in a direct activity-dependent manner. The second type of probe mimics a small molecule and preserves the physiochemical properties of the small molecule but does not directly react with target proteins (Dubinsky et al. 2012); therefore, a photoaffinity moiety such as a diazirine or benzophenone must be incorporated to enable covalent probe-protein binding. These probes can enable characterization of microbial transporters and intracellular proteins involved in the metabolism of the small molecule or enzymes utilizing that small molecule as a cofactor (Romine et al. 2017; Anderson et al. 2016; Nair et al. 2017).

An advantage of ABPP is the ability to characterize the functional capacity of a microbiome at both cell and protein scales. Multimodal profiling of probe targets by altering the reactive group can enable sorting and imaging at the cell scale, and SDS-PAGE and chemoproteomics at the protein scale (Fig. 1). Once proteins are probe labeled, options exist for directly incorporating a reporter tag (e.g., fluorophore or biotin) in the probe or exploiting click chemistry (CC) reactions to attach a reporter to the probe after it has bound its target. The latter option maintains a smaller probe size which can minimize undesirable impacts on reactivity with the target protein or cell permeability. Furthermore, it permits the user to readily exchange the type of reporter that is applied based on the desired application and outcome of the study, and properties of the sample being assayed.

For microbiome characterization, probe-assisted fluorescence-activated cell sorting (FACS) is a powerful tool for characterizing the functional capacity of the microbiome at the cell scale. Choosing a fluorophore to enable FACS is dictated by the background fluorescence of microbiome samples, the fluorescent signal strength required to detect the desired functional cell type, permeability of the cells to the fluorophore, and compatibility with other fluorescent reagents. Activity-based probe-enabled cell sorting enables the isolation of functional guilds of microbial cells from complex microbiomes. Additionally, probe-enabled sorting can provide reduced sample complexity to assist with proteomics analyses and partitioning of cells displaying an active function for subsequent DNA sequencing. Due to microbiome complexity, as well as redundancy when looking at a specific enzyme type, cell sorting can help yield a reduced organism search space for complementing proteomic studies (see Sect. 3). Finally, in the future probes may be used to isolate functional groups of living organisms from microbiomes for subsequent cultivation and analytical studies providing an unprecedented view of microbiome function (Fig. 1).

2.2 Click Chemistry and Sample Analysis

One of the biggest challenges in microbial community research is the complexity of the sample matrix. For both environmental and host-associated communities, samples from the native environment are more complex than those from laboratory media. As will be discussed in Sect. 3, this complicates recovery of biomaterial for analysis. However, it also complicates the ability to perform otherwise straightforward chemistry in the sample. The application of CC approaches to ABPP has been especially useful where the incorporation of a full reporter tag would impede probe function and/or in cases where multimodal analyses are desired, e.g., chemoproteomics and fluorescence imaging. The most common CC reactions in ABPP are strain promoted azide–alkyne cycloaddition (SPAAC) and copper-catalyzed azide–alkyne cycloaddition (CuAAC) (Kolb et al. 2001; Speers et al. 2003; Agard et al. 2006). Unfortunately, both exhibit a large degree of non-specific labeling in samples of microbial mats, cecal content, fecal material, and soil (unpublished observations). CuAAC, in addition, has dramatically reduced efficacy in microbial cell lysate from fecal material or cecal content. This can be overcome by increasing the concentration of copper, at the expense of increasing Cu(I)-induced biomolecule damage. To address this, it is possible to ‘pre-click’ alkyne and azide probes to their reporter group prior to probing the sample or to utilize probes that were synthesized with reporter groups attached. The introduction of a large and (in the case of fluorophores) charged group to the probe may cause a reduction in probe labeling. The trade-off between background and possible loss of signal is likely specific to the probe, sample, and question being addressed.

The complex sample matrix also impacts the ability to analyze samples after probe labeling. SDS-PAGE of fluorescently labeled sample, the traditional approach to optimize labeling conditions, often fails to resolve proteins from these more complex sample matrices (Snelling and Wallace 2017). Two-dimensional SDS-PAGE gives discrete spots for highly abundant proteins, but still exhibits strikingly poor resolution of these more complex samples. Thus, there is a clear need for a high-throughput way to optimize labeling conditions for such samples; for instance, surface immobilized chemical probes may enable more stringent wash methods to remove contaminating content. It is important to note that

these contaminating substances carry through sample preparation (enrichment, washing, and protein digestion) and are present to some degree in the final sample. They are also prone to precipitating during LC, potentially plugging columns and emitter tips during proteomics analyses. Because of this, liquid chromatography–mass spectrometry (LC-MS) analysis of samples from complex matrices requires additional strategies to protect the instrument and produce quality quantitative data.

3 Challenges of Microbiome Proteomics and How ABPP Can Facilitate

Compared to the exponential growth in studies using HTS for metagenomics and metatranscriptomics, the use of mass spectrometry-based metaproteomic analyses of microbial communities has been substantially more limited. The reasons for this have been discussed in depth elsewhere (Arnold et al. 2016; Heyer et al. 2017; Hettich et al. 2012; Lee et al. 2017). However, there are two major ABPP-related hurdles that are important to highlight here. The first hurdle is sample complexity, which ABPP could help address. While sample complexity is a concern for all mass spectrometry-based proteomics studies, it is especially important to consider in metaproteomes from microbial communities. The wide dynamic range of peptide abundances, co-elution limiting detection of low abundance peptides, and difficulties developing sequence databases are even more pronounced in these highly complex systems where hundreds to thousands of different taxa may be present. To address this problem enrichment strategies, sample fractionation, and two-dimensional chromatography have all been employed (Biteen et al. 2016; Moon et al. 2018; Mayers et al. 2017; Gilbert et al. 2016, 2018; Xiao et al. 2017; Jansson and Hofmockel 2018; Jansson and Baker 2016). However, these steps can dramatically limit the number of samples that can be analyzed. ABPP could serve as an excellent tool to address this problem. Using ABPP, it is possible to reduce sample complexity while retaining information about relevant proteins. It is important to note that the diverse nature of microbial communities and their environments mean that each sample type will require to optimization of labeling and enrichment conditions which may be difficult for low biomass samples. To circumvent this, similar but more easily obtained matrices can be employed for protocol development.

The other major hurdle facing proteomic analysis of microbial communities is the development of a suitable sequence database. Ideally, a matched metagenome is available for that specific sample. This is rarely possible due to cost and limited available biomass. As such, a database will often need to be constructed from available genomes. There are three specific problems that must be considered: sequence availability, database size, and functional prediction.

The first is the lack of complete, closed genome sequences for many environmental organisms. While genomes for phylogenetically similar organisms may be available, it is difficult to know how well matched the available sequence and the actual sequence are without doing metagenomic sequencing. For some frequently studied microbial communities (e.g., human gut microbiota), multiple metagenomes are available to the scientific community. This raises the second problem: for many less studied microbial communities, these resources are unavailable. In these cases, one approach is to use a large database of all available protein sequences. This comes with its own difficulties, including

redundant protein sequences and failure of traditional statistical methods to work with such large databases. Several approaches to address this problem have been developed over the past few years (ComPIL, MetaLab, etc.) (Chatterjee et al. 2016; Zhang et al. 2016; Cheng et al. 2017). Such tools will likely play a key role in successfully drawing conclusions out of massive proteomics datasets.

Regardless of how the sequence database is constructed, the identified proteins need to have some predicted function associated with them in order to use these data to begin to tease out the biological mechanisms. This presents the third challenge: the current state of functional annotation and prediction. While the use of homology-based functional prediction is widespread and can be incredibly powerful, misannotations and lack of annotation for genes in many environmental or unculturable organisms introduce a major caveat to the analysis of proteomics data from complex microbial communities. This is also another challenge that ABPP could help to address. Probes for specific activities such as serine-catalyzed hydrolysis or ATP hydrolysis have been used by our group and others to predict function for previously unannotated proteins in *Mycobacterium tuberculosis* (Ortega et al. 2016). Such a strategy could be readily extended to complex microbial communities to identify proteins of interest for further study. Additionally, as mentioned in Sect. 4.1, probe-assisted cell sorting can provide a way to enhance database quality for particular functional guilds within a microbiome and provide much improved proteomics analyses.

4 Biological Applications for ABPP

The use of ABPP in microbial communities has thus far been very limited, due in part to the technical challenges discussed above. However, there have been some excellent studies employing this strategy or a similar approach to characterize the active members within communities. Here, we will highlight the use of ABPP and other strategies employing activity-based probes to study function within both host-associated and environmental microbial communities.

4.1 Host-Associated Microbial Communities

Host-associated microbiome research has grown exponentially in the past decade, with the human gut microbiome being the major focus. The microorganisms that inhabit the human gut play a role in an ever-expanding list of human biological processes ranging from production of neurotransmitters to the development of the immune system [reviewed in Rooks and Garrett (2016), Mayer et al. (2015)]. If the mechanisms underlying these community interactions could be better understood, this could represent a new point of intervention for a number of diseases. This will require answering the two major questions of microbial communities: ‘who’s there?’ and ‘what are they doing?’. The development of HTS has helped to answer the former. The latter question has been more difficult to answer due to the epistemological limits discussed above; metagenomics and metatranscriptomics can describe potential function but cannot themselves demonstrate that an activity is truly present. Here, we highlight two particular areas of host-associated microbiome research where ABPP could be a powerful tool: xenobiotic metabolism and bile acid metabolism and

signaling. However, the potential utility of ABPP in gut microbiome research is far ranging, and it will be of interest to see its role in this field moving forward.

4.1.1 Xenobiotic Metabolism—Broadly speaking, host metabolism of xenobiotics (compounds foreign to the mammalian body including dietary compounds, environmental pollutants, and drugs) occurs in two steps called phase I and phase II metabolism. Phase I metabolism typically consists of a redox reaction that increases compound polarity. This is most often a cytochrome P450- or flavin-containing monooxygenase-catalyzed oxidation to generate or uncover a reactive moiety on the xenobiotic. In phase II metabolism, a transferase typically conjugates a hydrophilic group such as a sulfate or glucuronate to the previously generated reactive moiety to increase solubility and aid in excretion (Koppel et al. 2017). Importantly, gut microbiota can impact metabolism of xenobiotics through multiple routes (i.e., deconjugation, reduction, or modification of gene expression). Modulation of xenobiotic metabolism by gut microbiota has been shown to impact liver function, immune development, and drug efficacy and may serve as a major system through which host–microbiota interaction occurs (Sousa et al. 2008; Wallace et al. 2010).

While our understanding of the underlying molecular mechanisms is in its infancy, there are an appreciable number of pre-HTS studies focused on the ability of gut microbes to metabolize xenobiotics (Sousa et al. 2008; Scheline 1968; Savage 2001). Much of the early work describing these reactions was done comparing metabolism in colonized and germ-free animals. If a particular transformation was observed in the colonized animals but not in the germ-free animals, the microbiota was presumed to be responsible. Alternatively, microbiota isolated from a host was incubated in vitro with the xenobiotic of interest, and if biotransformation was observed then the microbiota could potentially be responsible for the same reaction in vivo. While such approaches were sufficient to implicate microbiota in xenobiotic metabolism, there is a major limitation to such studies; these approaches cannot identify the (potentially multiple) enzymes or taxa responsible for that biotransformation in vivo. Such information would be useful in the design of probiotics, inhibitors, or screening tools depending on the impact of the biotransformation on the host. ABPP could serve as a powerful tool to aid in these identifications.

As discussed in Sect. 2, one of the principle strategies for probe design in ABPP is to use known mechanism-based, irreversible inhibitors for the enzyme class of interest as the basis for the probe binding and reactive groups of the probe. Unfortunately, very little knowledge is available regarding the protein structure or exact reaction mechanism of many of the enzymes produced by gut microbiota. Chemical transformations that are mediated by the microbiome have been characterized, which may be sufficient in some cases for probe design. These include hydrolytic reactions, lyase reactions, reductive transformations, polysaccharide degradation and utilization, functional group transfers, conjugations, and radical catalyzed reactions (Koppel et al. 2017; Spanogiannopoulos et al. 2016; Koppel and Balskus 2016). For some of these reactions, proteolysis and polysaccharide degradation in particular, probe designs already exist and have been employed eukaryotic and prokaryotic systems. For other reactions such as reductions and conjugations novel probes will need to be developed and validated.

Reduction of alkenes, azo-groups, nitro-groups, and sulfoxide groups by gut microbiota has been well documented (Sousa et al. 2008). In fact, one of the best examples of connecting a metabolic reaction to a particular organism is the reduction of digoxin to dihydrodigoxin by the gut microbe *Eggerthella lenta* (Haiser et al. 2014; Haiser et al. 2013). While general reductase reaction mechanisms are understood, they often lack a covalent substrate–enzyme complex that a probe can be designed to capture. A reductase probe strategy could be inspired by probes for their enzymatic opposites, oxidases. Probes for cytochrome P450 enzymes and monoamine oxidases involve the generation of a reactive intermediate (a ketene or iminium-containing Michael acceptor, respectively), which can react with nearby residues in the enzyme (Wright et al. 2009; Krysiak et al. 2012). Depending on the reactivity of this intermediate, however, the probe can diffuse and label nearby enzymes other than the target. An alternative would be photoreactive reversible inhibitors, although these can result in off-target labeling as well.

One of the important functions of gut microbiota is the degradation of polysaccharides via glycoside hydrolases (GHs) and polysaccharide lyases. Importantly, microbiota possesses not only enzymes capable of degrading dietary polysaccharides, but also GHs capable of removing glucuronic acids from xenobiotics. One of the major reactions in phase II metabolism is the conjugation of a glucuronic acid to xenobiotics to form a glucuronide. Glucuronides can then be transported to the gut where microbial GHs (specifically β -glucuronidases) can deconjugate the glucuronic acid from the xenobiotic, regenerating the metabolite. This can lead to altered pharmacodynamics and potentially severe side effects. The chemotherapeutic irinotecan can interact with microbial glucuronidases in this manner, leading to dose-limiting severe diarrhea. In seminal work, Redinbo and colleagues showed that this process could be inhibited by co-administration of bacterial-specific β -glucuronidases inhibitors (Wallace et al. 2010; Wallace et al. 2015). Nonetheless, the specific taxa responsible for this activity remain unidentified. Recently, our group has developed a FACS-based platform to isolate and identify cells responsible for deglucuronidation in vivo (manuscript pending). Additionally, Overkleeft and colleagues recently described synthesis and application of irreversible inhibitors for retaining β -glucuronidases (Wu et al. 2017). While initially demonstrated in human systems, such probes can be applied to label and identify microbial β -glucuronidases as well (unpublished observations).

4.1.2 Bile Acid Metabolism and Signaling—In addition to xenobiotics, gut microbiota can also act on endogenous metabolites. Some of the most abundant metabolites present in the gut are bile acids and salts. Primary bile acids are amphipathic, cholesterol-derived steroids that are produced in the liver and aid in the emulsification of lipids during digestion (Joyce and Gahan 2016; Li and Chiang 2014). When a bile acid is conjugated to an amino acid (taurine and glycine in humans), the more water-soluble bile salt is produced. Eventually, the bile salt enters the gut where it can be altered by bacterial enzymes. There are two subsequent modifications that can occur. The first is deconjugation of the amino acid by bile salt hydrolases (BSHs), regenerating the bile acid. The newly regenerated bile acid can then undergo the second modification, which is an alteration of the sterol core via redox reaction or isomerization. The resulting compound is called a secondary bile acid.

In addition to their role in emulsification bile acids are also potent signaling molecules, activating host nuclear receptors such as the farnesoid X receptor (FXR) and G-protein coupled receptors such as TGR5. These signaling events influence energy metabolism and dysregulation that may play a role in the development of liver disease and metabolic disorders such as diabetes and obesity (Li and Chiang 2014). Secondary bile acids, which result from gut microbiota transformation of primary bile acids, are structurally distinct from their parent compounds. This can lead to altered receptor binding and thus signaling activity (Joyce and Gahan 2016).

Recently, Lei and colleagues developed bile acid-derived photoreactive probes (Zhuang et al. 2017). Using a competition-based approach, they identified known and novel bile acid-interacting proteins in HeLa cells including carnitine palmitoyl transferase 1A (CPT1A) and ADP-dependent glucokinase (ADPGK). Such probes could be useful in microbial systems as well and could help identify the enzymes and microbes that are responsible for primary bile acid transformation. Furthermore, bile acids are known to trigger germination of some microbial spores, indicating that there are likely other important bile acid binding proteins to be identified (Francis et al. 2013).

The opportunity to utilize ABPP for characterization and quantification bile acid regulation and signaling in the gut microbiota and host tissues is significant. The development of function-dependent approaches in this research realm has been hindered by the lack of technologies capable of identifying mechanistic relationships between bile salt hydrolase activity, the specific enzymes, and taxa within the microbiome that are directly responsible for metabolism, and the host pathways that respond to bile acids. ABPP will have a role in identifying the levels of functionally active bile salt hydrolases in the gut microbiome in healthy and diseased individuals, determining the specific microbes capable of transporting and metabolizing specific bile acids, and revealing interactions between specific bile acids and host nuclear receptors. ABPP can be used to dissect bile acid metabolism and signaling to yield an improved understanding of the mechanisms at play and the role of bile acids in metabolic disease and response to various exposures.

4.2 Environmental Microbial Communities

Like host-associated microbial communities, interest in environmental microbial communities (such as those found in soil or in marine environments) has increased recently, in large part due to the ability to use HTS as an approach to study communities in a culture-free manner. Also, like host-associated communities, environmental communities have seen limited application of ABPP. ABPP has been used to study axenic cultures of environmental microbes such as nitrifying bacteria, extremophilic archaea, and cellulolytic bacteria (Chauvigne-Hines et al. 2012; Bennett et al. 2016; Zweerink et al. 2017; Sadler et al. 2014; Ansong et al. 2014). Because ABPP does not require the ability to genetically manipulate organisms or the use of antibodies, it may prove especially useful in the study of microbial communities where those tools are often not available.

4.2.1 Metabolic Activity and Translation—Heterogeneity between microbial cells has is increasingly being understood to play a key role in community function and survival.

This is especially relevant when considering structurally complex communities such as biofilms, where concentration gradients of nutrients and terminal electron acceptors impact cell physiology (Jansson and Hofmockel 2018; Deveau et al. 2018). Where HTS-based analysis of community composition cannot easily distinguish between active and inactive cells, ABPP can. The capacity to switch the probes' reporter groups from affinity reagents (e.g., biotin) to fluorophores enables imaging as well. This provides structural information about where the active cells and proteins are localized that would otherwise require genetic manipulation—often not possible in environmental isolates—or the use of immunoreagents such as antibodies, which are not often available commercially and must be generated and validated.

While not targeted at a specific class of enzymes such as proteases or glycoside hydrolases, bioorthogonal noncanonical amino acid targeting (BONCAT) has been employed to detect newly translated proteins and translationally active cells within complex microbial communities. BONCAT has been successfully utilized in the study of planktonic marine and slow-growing archaeal communities to identify actively translating microbes (Leizeaga et al. 2017; Hatzepichler et al. 2016). Unlike axenic systems, however, BONCAT in microbial communities has not yet been extended to identification of the newly translated proteins themselves. In part, this is due to the complications mentioned above.

General measures of metabolic activity, such as active respiration, DNA/RNA synthesis, and membrane integrity have all been used to identify active members within microbial communities (Mou et al. 2008; Maurice et al. 2013). As with BONCAT, these tools have also been coupled to FACS to allow isolation of the active population as well. While these approaches may help to identify live/active cells, they are not designed to directly identify which organisms and enzymes are responsible for a given activity, which is often the more interesting question. Tools capable of labeling and isolating microbes and enzymes based on a more specific function, such as ABPs, will be needed to fill these knowledge gaps.

4.2.2 Vitamin Metabolism—Vitamins and other micronutrients can influence microbial communities in multiple ways primarily by acting as cofactors or precursors to cofactors. As these compounds are often energetically 'expensive' to produce, microbial communities often consist of both organisms capable of producing the vitamin themselves (auxotrophs) and those that must uptake the vitamin from the environment (heterotrophs). Thus, the exchange of vitamins between cells within an environment may play a key role in community dynamics (Konopka et al. 2015). The dynamic microbial interactions involving vitamins also means that they are critical to the formation and fitness of microbiomes in general (Degnan et al. 2014).

As vitamins are involved in a variety of biochemical reactions, a single reaction mechanism-based strategy would likely be insufficient to capture vitamin utilizing enzymes. Additionally, vitamins often affect biological change via non-covalent binding events and characterizing the transporters involved in their cellular incorporation is also important. In order to capture vitamin-interacting proteins, ABPP focused on vitamins has utilized photoreactive tags. By incorporating diazirine and alkyne-containing amino acids into the parent molecules, our group has applied B₁ (thiamine), B₂ (riboflavin), and B₇ (biotin)-

based ABPs to the photosynthetic thermophile *Chloroflexus aurantiacus*, which can be found in microbial mats (Anderson et al. 2016). This work successfully identified a number of proteins including transporters, kinases, and fatty acid biosynthesis machinery. Importantly, we were also able to enrich a number of proteins that were undetectable using a global proteomics approach, demonstrating that ABPP can be used to enrich otherwise undetectable targets in microbial systems.

One B vitamin group, B₁₂ (cobalamin), has attracted particular attention in regard to microbial communities. B₁₂ is exclusively synthesized by bacteria and archaea, and its de novo synthesis is energetically and genetically costly, requiring nearly 30 enzymes (Martens et al. 2002; Roth et al. 1993). As such, most organisms will uptake B₁₂ from the environment rather than produce it for themselves (auxotrophy). B₁₂ availability has been postulated to influence microbial communities in the gut and marine ecosystems, but exact mechanisms remain poorly understood. Our group has also synthesized a B₁₂ mimic that contains a diazirine for photocrosslinking to nearby proteins and an alkyne to enable CuAAC (Romine et al. 2017). Using this probe, we were able to identify proteins involved in folate, methionine, and ubiquinone metabolism as well as a light-sensing transcription factor in the environmental microbe *Halomonas* sp. HL-48 (Romine et al. 2017). We determined that vitamin B₁₂ acts as a novel regulator of various microbial functions and intercellular interactions. Most recently, we have shown that our B₁₂ probe mimics the natural vitamin almost perfectly, such that microbes can be cultured with the probe as the sole source of B₁₂ (Rosnow et al. 2018). This allows for the direct identification of macromolecule-B₁₂ interactions including proteins, DNA, and RNA throughout the growth phase of a microbe or microbiome. We anticipate that future studies will incorporate vitamin or other metabolite-based affinity probes to understand their roles and interactions in complex communities.

4.2.3 Community Signaling—The ability of individual cells to interact with each other via signaling systems is a key feature of microbial communities. One of the best examples of such signaling is quorum sensing (QS), in which a microbe is able to sense the presence and abundance of nearby organisms. While this phenomenon is best understood in proteobacteria, analogous systems in microbes form all domains of life (Atkinson and Williams 2009). Fundamentally, QS systems consist of two components: a small, diffusible signal and a response regulator that controls the physiological response. The diffusible signals are chemically diverse, ranging from short peptides to isoprenoids to *N*-acyl homoserine lactones. As QS systems play a critical role in community-level behaviors such as biofilm formation, the ability to identify organisms and enzymes involved in QS signal transduction will likely be key to understanding how microbial communities develop and function. Photoaffinity probes based on QS molecules have been designed and applied to axenic *Pseudomonas aeruginosa* and mammalian cells (Dubinsky et al. 2009; Garner et al. 2011; Baker et al. 2017). Application of such probes could be extended to microbial communities as well and may help to better establish the role QS systems play in interspecies interaction.

In addition to QS systems, certain metabolites such as peptidoglycan, vitamin metabolites, and indoles can act as signaling molecules. Peptidoglycan makes up the cell wall of most

bacteria, and peptidoglycan synthesis is required for cell growth and division. Thus, the availability of free peptidoglycan may serve as an indicator of favorable growth conditions to nearby bacterial cells (Dworkin 2014). Indeed, metabolic activity, cell growth, and cell division are regulated by peptidoglycan-sensing serine/threonine kinases in taxa such as *Bacillus subtilis*, *Mycobacterium tuberculosis*, and *Streptococcus pneumoniae* (Shah et al. 2008; Mir et al. 2011; Beilharz et al. 2012). Peptidoglycan metabolites can also influence eukaryotic microbes as well. In *Candida albicans*, a gut-associated fungal taxa, N-acetylglucosamine signaling was shown to influence physiological changes necessary for mating (Huang et al. 2010). Peptidoglycan is also an important pathogen-/microbial-associated molecular pattern (PAMP or MAMP respectively) sensed by the mammalian immune system and plays a key role in the induction and management of the inflammatory response (Dworkin 2014). This is also true of folate, tryptophan, and retinoid metabolites, which are known to influence immune development and activity at mucosal sites (van de Pavert et al. 2014; Kjer-Nielsen et al. 2012; Schiering et al. 2017).

Probes based on these metabolites would be unique and powerful tools to define these interactions in actual community systems. However, the design of such probes is challenging. The binding modes between these signals and their receptors are non-covalent, necessitating the use of a photoaffinity tag. Depending on the size of the metabolite, this tag may introduce a relatively small change in size (such as vitamin B₁₂) or a large change (such as indole derivatives). The placement of such a tag is critical to whether or not the probe will exhibit the same binding behavior as the native metabolite. If available, protein crystal structures can be helpful to identify potential modification sites that may have limited impact on binding. Given the limited number of protein structures for many environmental microbes, structure prediction tools such as Phyre2 are particularly useful in this area (Kelley et al. 2015). For smaller metabolites probe design can be especially challenging, as most of the synthetically accessible sites (amines, carboxylates, alcohols, etc.) are often essential for protein binding. As such, it may be necessary to employ a subtractive approach where a general reactive group is competed with the native substrate in order to successfully profiling small metabolite–protein interactions.

5 Current Challenges and Outlook

The application of ABPP to study microbial communities remains promising, though critical challenges in sample preparation, probe design, and database availability will need to be addressed (Fig. 2). The presence of a complex, often undefined sample matrix complicates peptide analysis, click chemistry, and potentially even probe labeling. All ABPP strategies fundamentally rely on a chemical ‘warhead,’ a group that is prone to making covalent bonds with an enzyme when that enzyme participates in a reaction. The presence of both biotic and abiotic molecules such as carbohydrates, salts, and surfactants can all interfere with this reaction by quenching the reactive group or inhibiting the enzyme. While this can be somewhat controlled for in laboratory culture, ‘real-world’ samples consist of such chemically diverse substituents that removing them all from the cells is often impossible. Strategies that incorporate a non-denaturing purification step, such as size-exclusion chromatography prior to assaying for activity, may be useful to clean up samples before labeling, at the cost of destroying the native cellular environment (Beller et al. 2018).

Alternatively, strategies such as an enzymatic digest to break up a defined matrix can be employed. Post-probe labeling, SDS-PAGE or 2D-DIGE to cut out fluorescence protein bands can also be effective in some sample types.

Probe development for microbial communities also remains a challenge. Most activity-based probes take inspiration from known irreversible inhibitors of an enzyme or enzyme class. Historically, the development and characterization of these compounds have mainly been to study or act as a therapeutic for a mammalian system. The same enzyme class or metabolic pathway may not be present in the phylogenetically distant fungi and prokaryotes. While there are some irreversible inhibitors of microbial systems (penicillin, artemisinin, difluoromethylornithine, etc.), they are mostly antimicrobial and cannot be used studies where the organisms need to be viable. Alternatively, probes can be designed with some knowledge of the reaction mechanism. For enzymes where homologs are present in commonly studied microbes, such as *E. coli* or *C. albicans*, mechanisms and structures may already be described. Given that such probes often need to be optimized and validated, assumptions based on homology may not be optimal for preliminary studies. In the near term, it will likely be more beneficial to utilize either a subtractive approach or photoaffinity probes based on the substrate of interest.

Despite these challenges, activity-based approaches are powerful tools for the study of complex microbial communities. At present, the functional capacity of microbiomes is largely inferred from genomic and transcriptomic data. Determining the contribution of individual microbial species and enzymes for a particular function is largely outside the capability of existing technologies. ABPP has a real potential to make specific measurements to report upon the functional ‘health’ of a microbiome. Within medicine, ABPP provides a platform to evaluate individual variability and susceptibility to diseases, to understand consequences associated with exposures in adults and developing children, and track microbe functions longitudinally throughout the development of an individual. ABPP may also be used to characterize functions associated with nutrient acquisition in soil/aquatic microbiomes. If successful, one could imagine agricultural fields, marginal lands, or other environmental sites (e.g., those undergoing bioremediation) being temporally tested and the data used for ‘prescriptive’ amendments to alter plant productivity, test for other features of soil health, or to improve ecosystem health. Such insights offered by ABPP constitute a major advancement in our understanding of complex microbial communities and will lay a foundation for new strategies to improve agricultural productivity, bioremediation efforts, and human health.

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Fig. 1.

Multimodal analyses of microbial communities by ABPP. Probes can be used for enrichment and proteomics identification and quantification of target proteins in complex microbiomes or via attachment of a fluorophore spatial resolution of functional cells and proteins can be determined and/or cells can be isolated by cell sorting and sequenced. The future may also see function-dependent live cell sorting for subsequent cultivation and enhanced characterization of active cells from microbiomes

Fig. 2.

Potential applications and challenges for ABPs in microbial communities. Once designed, probes can be used coupled to an enrichable moiety or surface. The probes can then be used for ABPP to identify and quantify active enzymes (top left) or used to functionally annotate proteins of unknown function (top right). Alternatively, the probe can be coupled to a fluorophore and then applied to the sample. This allows the use of FACS to isolate microbes possessing that function for cultivation or sequencing (bottom left) as well as imaging to study the spatial distribution of activity (bottom right)