Metabolic labeling probes for interrogation of the host–pathogen interaction

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Bacterial infections are still one of the leading causes of death worldwide; despite the near-ubiquitous availability of antibiotics. With antibiotic resistance on the rise, there is an urgent need for novel classes of antibiotic drugs. One particularly troublesome class of bacteria are those that have evolved highly efficacious mechanisms for surviving inside the host. These contribute to their virulence by immune evasion, and make them harder to treat with antibiotics due to their residence inside intracellular membrane-limited compartments. This has sparked the development of new chemical reporter molecules and bioorthogonal probes that can be metabolically incorporated into bacteria to provide insights into their activity status. In this review, we provide an overview of several classes of metabolic labeling probes capable of targeting either the peptidoglycan cell wall, the mycomembrane of mycobacteria and coryne-bacteria, or specific bacterial proteins. In addition, we highlight several important insights that have been made using these metabolic labeling probes.

Introduction

Despite the near-ubiquity of antibiotics in modern times, bacterial infections are still among the leading causes of death globally.4 Overuse of antibiotics in high-income countries has contributed to an evolutionary selection pressure that is driving antibiotic resistance.2 New antibiotic resistance mechanisms are rapidly emerging,3 resulting in a staggering increase in multi-drug resistant (MDR), extensive drug resistant (XDR) and pan-drug resistant (PDR) strains.4 At the same time, the development of novel classes of antibiotics has been extremely slow, with only two new classes of broad-spectrum antibiotics introduced to the clinic since 1962.5,6 Development of antibiotics for a certain class of pathogenic bacteria has been particularly difficult: the intracellular bacteria. Intracellular bacteria reside inside host cells, where they can evade immune detection and persist for months or even years in a dormant state.7,8 In order for an antibiotic to be active against infections caused by intracellular bacteria, the drug not only has to cross the bacterial cell envelope but also the host cell membrane. The study of (obligate) intracellular pathogens is further complicated by the fact that they are often difficult to culture in vitro.9,10 In light of these challenges, novel antibiotics against intracellular bacteria are much needed, as the disease burden from these species is very high.

The deadliest bacterial species is arguably Mycobacterium tuberculosis (Mtb), a facultative intracellular bacterium that is the major cause of tuberculosis (TB). This pathogen is responsible for approximately 2 million deaths annually11 and antibiotic-resistant strains are continually emerging. Treating infections due to Mtb is made difficult by the heterogeneity in its intracellular life cycle: after infecting macrophages it diverges into fast and very slow-growing forms.12,13 The slow growing form is generally hard to treat with conventional antibiotics such as those that target the nascent cell wall of the dividing bacteria.14–20 As a result, TB is typically treated with a complex cocktails of antibiotics that need to be taken for long periods of time. Addressing infections due to MDR Mtb is even more challenging requiring antibiotic treatment courses of up to 2 years (often with severe side-effects).21 One new therapy – the first in many years – was recently approved for drug-resistant forms of Mtb: pretomanid in combination with bedaquiline and linezolid. Whilst this is laudable – as is the clinical development of other Mtb active agents – the demand for new treatments for Mtb and other intracellular pathogens currently far outstrips supply.22 Mtb is not the only intracellular pathogen causative of high-mortality/morbidity disease. Other intracellular pathogens of clinical concern include Salmonella enterica, Yersinia pestis, Chlamydia trachomatis, Listeria monocytogenes and Coxiella burnetii to name but a few.23 Whilst not dis-
Bacterial pathogens have acquired highly effective mechanisms for infecting- and surviving inside hosts. These mechanisms lead to increased virulence and persistence within the host environment, along with suppression of the host immune system. Due to their importance for bacterial cell viability and infectivity inside the host, bacterial defense mechanisms at the host–pathogen interface are a promising target for novel antibiotics. Thus, effective chemical tools to study the host–pathogen interface at the molecular level are of paramount importance in deepening our understanding of the relationship between the fundamental biochemistry of the host–pathogen and their virulence. This in turn can provide new information in the fight against antibiotic resistance. To provide clinically significant data, these chemical tools must be useable in cell culture and in vivo in pathogenic bacteria. Moreover, the cellular processes they report on must be relevant to bacterial cell survival and/or virulence.

Recently, novel chemical probes and approaches have been developed that allow for the profiling of various metabolic processes as well as in cell wall and protein labeling. In metabolic profiling a chemical probe is exogenously supplied to an organism, which subsequently incorporates the chemical probe into its cellular architecture by means of endogenous enzymes. Novel chemical probes have been developed that carry fluorescent dyes allowing for facile, one-step metabolic labeling of bacterial structures of interest such as the peptidoglycan (PG) cell wall, or the mycomembrane of corynebacteria. Moreover, the introduction of bioorthogonal chemical labeling strategies and the subsequent improvements made to these probes and labeling strategies while also highlighting several examples of important findings achieved using these methods. Specifically, we highlight the use of d-amino acids (DAs) for metabolic labeling of PG in the bacterial cell wall and the labeling of the mycomembrane of corynebacteria using trehalose-analogues. Also covered is the use of bioorthogonal non-canonical amino acid tagging for the labeling of new protein synthesis in bacteria and the application of so-called activity based probes that target enzyme activities specific to the bacterial pathogen of interest.

**Metabolic labeling of peptidoglycan**

Peptidoglycan (PG) is the major constituent of the bacterial cell wall. PG consists of linear glycan strands that are cross-linked through short peptides, creating a dense macromolecular network that is vital to bacterial survival. The bacterial cell wall provides structural integrity for the cell and protects it against the osmotic pressure established by high intracellular concentrations of proteins and other biomolecules. The PG cell wall also functions as an anchor for important macromolecules of the cell envelope, such as teichoic acids, and Braun’s lipoprotein. Due to its vital role in bacterial survival, relative accessibility at the outside of the cell, and absence in eukaryotic cells, PG biosynthesis is a prime target for antibiotics. Many widely used antibiotics including the β-lactams exemplified by penicillin, glycopeptides such as vancomycin, and the lipopeptide bacitracin function by inhibiting various aspects of bacterial cell wall biosynthesis. However, due to misuse and overuse, bacteria are acquiring resistance against these antibiotics at an alarming rate. Nonetheless, the bacterial cell wall remains a very promising target for the development of novel antibiotics.

PG metabolism has been studied using various methods, such as metabolic labeling with radiotopes. Other methods include labeling of the bacterial cell wall using fluorescently tagged antibiotics as well as by the incorporation of a fluorescently labeled tripeptide into the cell wall by exploiting the PG recycling machinery of Escherichia coli (E. coli). These techniques have multiple limitations: (1) labeling techniques that are dependent on the PG recycling pathway of E. coli can only be used to study this specific organism; (2) radiolabeling of PG is known to be a very laborious and technically complex task; (3) labeling the bacterial cell wall using fluorescently tagged antibiotics is limited to bacterial species with an accessible cell wall and enzyme machinery tolerant to these large pendant fluorophores; and (4) the use of fluorescently tagged antibiotics has an inhibitory effect on bacterial cell growth, thereby limiting their use.

Recently, metabolic labeling of PG using new d-amino acid (DAA) analogues has led to significant advancements in the understanding of cell wall biosynthesis and recycling of PG. Whereas nearly all life forms use exclusively L-amino acids, most bacteria also use DAs, which are incorporated into the
PG cell wall.48 \( \text{\text{d}}\)-Alanine and \( \text{\text{d}}\)\text{-glutamic acid are the DAAs that are most often incorporated into PG and can thus be found in the cell wall of nearly all bacteria.}48 Interestingly, studies have shown that the biosynthetic machinery involved in PG synthesis is highly tolerant to a variety of different DAAs.49,50 This phenomenon has been exploited to incorporate DAAs and analogues thereof, to act as reporter molecules (one-step metabolic PG labeling) or as chemical handles for the conjugation of reporter molecules (two-step metabolic PG labeling).

Kuru et al.30 were the first to employ fluorescent \( \text{\text{d}}\)\text{-amino acids (FDAA)s for one-step PG labeling and imaging of bacterial cell walls in live cells. Starting from the \( \text{\text{d}}\)-amino acids 3-\( \text{\text{d}}\)-alanine and \( \text{\text{d}}\)-lysine, they reacted reporter molecules 7-hydroxycoumarin-3-carboxylic acid (HCC), 4-chloro-7-nitrobenzofurazan (NBD), fluorescein and carboxytretramethylrhodamine (TAMRA) to synthesize a variety of fluorescent amino acids, such as HADA, NADA, FDL and TDL (Fig. 1).30 Subsequently, they succeeded in labeling a wide range of Gram-negative and Gram-positive bacteria, including, but not limited to, \( \text{E. coli}, \text{Staphylococcus aureus} \) and \( \text{Bacillus subtilis} \), and showed that these FDAA were selectively incorporated into the bacterial cell wall. In \( \text{B. subtilis} \), they showed that these FDAA were incorporated exclusively into PG and not into teichoic acids; which also contain \( \text{\text{d}}\)-alanine residues.30 Because of their broad species applicability and excellent selectivity for PG, FDAA have been used to study cell wall synthesis and its role in pathogenesis,51–59 and to establish the presence of PG in many bacterial species.60–62 An interesting application of FDAA is the construction of so-called ‘virtual time-lapse’ images of PG synthesis in individual organisms. By sequentially adding FDAA with different spectral properties, the growing PG layer can be sequentially labeled with multiple, differently colored FDAA. This creates a multi-colored image where each color represents the location and amount of PG that is synthesized during the time in which FDAA was added.30,63

FDAA owe their broad applicability partially to their low molecular weight; labeling of the Gram-negative bacterial cell wall requires passage through the outer membrane, which is markedly less permeable to molecules over a molecular weight of ~600 Da, such as antibiotics.64 Indeed, when Hsu et al.55 compared the labeling of \( \text{E. coli} \) with FDAA with molecular weights between 300–700 Da, they found that FDAA over a molecular weight of ~500 Da have reduced access to the \( \text{E. coli} \) periplasm and cytoplasm and label the cell wall less efficiently. Today, the available toolkit of FDAA covers the whole visible spectrum, from violet to far-red, providing many options for PG imaging in real time. Amongst these are FDAA that are compatible with stochastic optical reconstruction microscopy (STORM), such as Cy3B and Atto 488, allowing imaging of PG nanostructures with far better spatial resolution.65 Improvements to other FDAA include increased water solubility, increased photostability, and the possibility to excite with far-red light, reducing the phototoxic burden on the bacteria that is being probed. In conclusion, FDAA labeling is an excellent analysis technique to study the bacterial cell wall because of its selectivity for bacterial PG, it’s relative experimental simplicity, and the ubiquity of bacterial PG in the cell walls of all bacterial taxa. Moreover, FDAA only label nascent PG and are therefore an ideal tool to study spatial and temporal aspects of PG synthesis. The expansion of the FDAA toolkit into probes that are suitable for STORM has improved the spatial resolution that can be attained with FDAA labeling and cements the relevance of this technique for the study of the bacterial cell wall.65 Notably, however, FDAA have not yet been applied to the imaging of intracellular pathogenic bacteria.

One downside of the FDAA-approach is that these probes are only incorporated into PG via the extracellular pathways reliant on the \( \text{\text{d}}\)-\text{p} and \( \text{\text{d}}\)-\text{p}-transpeptidases, and are therefore not tolerated by the \( \text{\text{d}}\)-alanine racemase (Alr) and the UDP-N-acetylMuramoyl-tripeptide-\( \text{d}\)-alanyl-\( \text{\text{d}}\)-alanyl ligase (MurF).62,66 These steps of the pathway in most species can, however, be labeled using a two-step labeling approach. The approach is based on metabolic incorporation of a \( \text{\text{d}}\)-amino acid equipped with a specific chemical handle, which, once incorporated into the PG network, can be labeled using a reporter molecule that is reactive to the handle. Two-step PG labeling has a lower risk of causing biological perturbation than one-step PG labeling, as the chemical handle is usually much smaller than the probes used in one-step PG labeling. Presently, bioorthogonal groups are commonly used as chemical handles in two-step PG labeling, because of their high reactivity towards their bioorthogonal reaction partners and their inertness towards biomolecules.34 PG labeling has been performed using \( \text{\text{d}}\)\text{-cysteine as a chemical handle, effectively incorporating thiol groups into the cell wall for coupling to thiol-reactive reporter molecules.67 But, reporter molecules that target \( \text{\text{d}}\)-cysteine do not selectively label PG, as they are also reactive to \( \text{\text{l}}\)-cysteine residues in cellular proteins. To overcome such limitations, the introduction of \( \text{\text{d}}\)-amino acids containing bioorthogonal groups such as azides, alkynes, and nitrones allows for highly selective two-step metabolic labeling of PG.68

Kuru et al.30 and Siegrist et al.31 independently reported the use of DAAs containing bioorthogonal groups for PG labeling; \( \text{R}\)-propargylglycine (also known as ethynyl-\( \text{\text{d}}\)-alanine, EDA) and 2-amino-3-azidopropanoic acid (commonly known as azido-\( \text{\text{d}}\)-alanine, ADA) (Fig. 1). Kuru et al. validated the concept of bioorthogonal two-step metabolic PG labeling by labeling both \( \text{B. subtilis} \) and \( \text{E. coli cells in vitro} \) with EDA and ADA.30 Expanding on this work, Siegrist et al. showed that \( \text{Listeria monocytogenes} \) and \( \text{Mtb} \) could be efficiently labeled by EDA and ADA, \( \text{in vitro} \) and inside infected \( \text{J774} \) macrophages. Notably, this work also demonstrated that macrophages take up sufficient EDA for effective labeling of intracellular bacteria without any apparent toxicity to either the macrophages or bacteria, highlighting the potential of two-step metabolic DAA labeling for cell culture-based applications.17 Combining the promiscuity of EDA labeling with the selectivity of RADA labeling, Garcia-Heredia et al. showed that PG repair also occurs away from the sites of \text{de novo} PG synthesis in various mycobacterial species.66 Since then, Pidgon et al. also reported probes specific for the \( \text{\text{l}}\)-\text{p}-transpeptidase to provide further
tools to study bacterial cell wall synthesis. The possibility of applying these probes to the study of the intracellular lifecycle of pathogens offers a tantalizing opportunity as a tool in the development of drugs against such pathogens.69

The bioorthogonal toolkit for metabolic labeling of PG was further expanded by Shieh et al.70 They synthesized D-amino acids equipped with the strained cyclooctyne and bicyclononyne (BCN) moieties (Fig. 1), for copper-free, strain-promoted alkyne–azide “click” (SPAAC) reactions. Furthermore, they synthesized near-infrared (NIR) azide-functionalized fluorogenic Si-rhodamine dyes whose fluorescence quantum yield greatly increases upon reaction with alkynes. These NIR fluorogenic dyes are very useful for in vivo imaging as near infrared light penetrates deeper through tissue than higher wavelength light and no washing of excess dye is required as the dye becomes vastly more fluorescent upon reaction. The Pezacki group added an additional bioorthogonal reaction to the labeling repertoire by designing nitrone D-amino acids that readily

Fig. 1 (A) Schematic overview of the labeling approaches; (B) D-amino acid probes for metabolic labeling of peptidoglycan (PG); (C) click-reactive muramic acid and acetyl-CoA analogues.
undergo reactions with strained alkynes in a strain-promoted alkyne-nitrone cycloaddition (SPANC) reaction (Fig. 1).68,71

Metabolic incorporation of $\alpha$-amino acid probes is well tolerated in most bacteria, with notable exceptions. For instance, the intracellular bacterium *Chlamydia trachomatis* required additional reagents to enable PG labeling. While circumstantial evidence for the existence of a PG containing cell wall in *C. trachomatis* had existed for decades,72,73 until recently no one had conclusively isolated or characterized PG from this bacterium.74,75 This resulted in a discrepancy called the ‘Chlamydial Anomaly’, where no PG was observed, yet the pathogen was sensitive to PG-targeting antibiotics.76,77 To address the matter Liechti et al. set out to conclusively establish the presence of PG in *C. trachomatis* by labeling with the $\alpha$-amino acid probe EDA, but found that EDA failed to label the bacteria.62 They hypothesized that this was because chlamydial PG synthesis enzymes could not accommodate EDA, but that they might accommodate modified $\alpha$-alanine dimers wherein one of the two amino acids was EDA (EDA-DA). Indeed, after incubating the bacteria with the alkyne containing EDA-DA dipeptide, they were subsequently able to label *C. trachomatis* with Alexa Fluor 488 azide. Interestingly, no labeling was detected when using the dipeptide analogue bearing the bioorthogonal modification on the C-terminal $\alpha$-alanine residue (i.e. DA-EDA). The authors reasoned that the lack of labeling was due to removal of the C-terminal $\alpha$-alanine residue in either transpeptidation or carboxypeptidation reactions. The work of Liechti et al. shows that labeling of PG in some bacteria may require modified $\alpha$-amino acid probes, but also highlights the versatility of $\alpha$-amino acid labeling for the detection and study of bacterial PG.

In addition to the use of unnatural $\alpha$-amino acids, strategies based on other building blocks have been developed to probe PG assembly in bacteria. In this regard, Grimes and coworkers designed metabolic probes based on N-acetyl muramic acid (MurNAc) that allowed for labeling of the carbohydrate moiety of PG.78,79 Although labeling of PG with $\alpha$-amino acids analogues is a powerful and versatile method, there are several benefits to labeling this carbohydrate moiety: firstly, $\alpha$-alanine residues of the PG stem peptide can be removed by transpeptidation and carboxypeptidation reactions, which may decrease the probe’s lifetime, whereas the carbohydrate backbone of PG is not subject to remodeling. Secondly, MurNAc is a superior probe for *de novo* PG synthesis, as it is only incorporated in newly synthesized PG, while $\alpha$-alanine probes can also be incorporated into mature PG by exchange reactions.50,80,81 Finally, MurNAc binding to Nod-like receptors (NLRs) is involved in innate immune activation,82 while $\alpha$-alanine residues are not involved in immune activation, making MurNAc analogues more suitable for probing immune activation by PG.

Metabolic labeling of the carbohydrate moiety in PG was first achieved by Liang et al. using MurNAc analogues with azido- and alkyne modifications at the 2-$N$ acetyl position (Fig. 1).78 In some Gram-negative bacteria, including, amongst others, Pseudomonas putida, MurNAc can be recycled to uridine diphosphate-MurNAc (UDP-MurNAc) by enzymes AmgK and MurU and subsequently incorporated into newly synthesized PG.83 Interestingly, Liang et al. showed that both *E. coli* and *B. subtilis* could incorporate MurNAc analogues into *de novo* synthesized PG after transfection with a vector for *P. putida* AmgK and MurU.78 Their results suggest that metabolic MurNAc labeling could be applied to a wide range of bacteria, both Gram-negative and Gram-positive, if transfected with amgK and murU. Metabolic MurNAc labeling of most bacteria does, however, require genetic modification of the bacteria and is limited to bacteria in which amgK and murU can be introduced, limiting the practical use of this particular metabolic labeling technique.

Building on these approaches, Wang et al. recently reported an elegant method for post-synthetic, metabolic labeling of the PG carbohydrate backbone.79 Their technique exploits the function of the bacterial enzyme peptidoglycan O-acetyltransferase B (PatB), which uses acetyl-CoA as an acetyl donor to O-acetate the 6′ hydroxyl group of MurNAc residues in the carbohydrate moiety of PG.84 Post-synthetic O-acetylation of PG occurs in many intracellular pathogenic bacteria, such as Neisseria meningitidis, Neisseria gonorrhoeae and Staphylococcus aureus,85–87 where it contributes to bacterial virulence by providing resistance against PG degradation by the antimicrobial enzyme lysozyme.88 Wang et al. hypothesized that bioorthogonal groups could be introduced into PG post-synthetically by feeding azide- (SNAz) or alkyne- (SNAk) functionalized acetyl-CoA analogues (Fig. 1). They found that PatB efficiently O-acetylates PG in *B. subtilis* when supplied with SNAz or SNak and in doing so enabled visualization of the incorporated probes by reaction with fluorescent dyes carrying complementary bioorthogonal groups.79 Interestingly, they also showed that the artificially O-acetylated *B. subtilis* was, at least partially, protected from degradation by lysozyme.
Trehalose is exclusively found in members of the suborder of Corynebacterineae, which does not include canonical Gram-negative or Gram-positive bacteria, it opens up the possibility to use metabolic labeling with trehalose-probes for clinical diagnosis of Mtb.

Clinical diagnosis of Mtb in high-burden, developing countries is currently performed using either the Ziehl–Neelsen test or the Auramine Truant stain, both of which are only moderately reliable. Furthermore, these diagnostic tests also do not discriminate between live or dead Mtb cells and thus cannot be used by physicians to determine treatment efficacy. Thus, novel, Mtb-specific diagnostic tools are required to improve TB diagnosis efficacy. Metabolic labeling with trehalose analogues has shown promise as a novel diagnostic approach for the rapid detection of Mtb infection. Trehalose, in the form of TMM and TDM, is anchored non-covalently to the mycobacterial cell wall. Trehalose is synthesized and esterified to form TMM in the cytosol, after which TMM is transported to the periplasm. Once in the periplasm, the mycolyltransferase complex antigen 85 (Ag85) transfers mycolate from TMM to AG, to form a network of covalent mycolates that form the foundation of the MM, or to another molecule of TMM, forming TDM. In both cases, a molecule of trehalose is regained, which is recycled by active transport into the cytosol by the trehalose-specific transporter SugABCLpqY. Based on analysis of the trehalose/Ag85 co-crystal structure, Backus et al. hypothesized that trehalose could be modified with functional groups without affecting substrate activity for Ag85, exploiting a promiscuity that is similar to that of \( \alpha \)-alanine metabolism. They synthesized various trehalose-analogues, including fluorescein isothiocyanate (FITC)-trehalose (FITC-Tre, Fig. 2), and tested their ability to label pathogenic Mtb using a one-step labeling method. FITC-trehalose was efficiently incorporated into growing Mtb mycomembranes by Ag85 in vitro and exclusively labeled live Mtb cells; heat-killed Mtb cells were not labeled, nor were cells of the Gram-negative pathogens Pseudomonas aeruginosa and Haemophilus influenza or Gram-positive Staphylococcus aureus. FITC-Tre could even be incorporated into Mtb inside macrophages; macrophages were infected with Mtb and successfully labeled with FITC-Tre. Backus et al. also demonstrated that Ag85 enzymes tolerate a surprisingly wide range of substrate modifications. Moreover, their work demonstrates that the Ag85 pathway can be hijacked to incorporate exogenous trehalose into TMM and, subsequently, into the MM. They also speculate that, given the conserved nature of Ag85 amongst mycobacteria, the FITC-trehalose probe could presumably label other mycobacterial species equally as well as Mtb. However, Rodriguez-Rivera et al. found that FITC-trehalose labels other mycobacterial with poor efficiency and does not label corynebacteria at a detectable level at all. SAR studies showed that removal of an anomeric methyl group significantly increased labeling efficiency amongst myco- and corynebacteria, as did repositioning of the FITC functionality on the trehalose scaffold. Rodriguez-Rivera et al. applied metabolic trehalose labeling to assess the influence of ethambutol treatment on mycomembrane fluidity using fluorescence recovery after photobleaching (FRAP) experiments. They showed that ethambutol can increase MM fluidity at sub-inhibitory concentrations, thereby enhancing antibiotic drug accessibility. One-step metabolic labeling is an excellent method for metabolic incorporation of trehalose analogues into the mycomembrane via extracellular incorporation by the Ag85 complex. However, an important aspect of trehalose metab-

Fig. 2 Fluorescent and fluorogenic trehalose analogues for labeling of the mycomembrane.
olism occurs intracellularly, where trehalose is formed and subsequently incorporated into various metabolites, which together constitute the ‘trehalose’. The importance of trehalose for bacterial survival calls for chemical probes that can provide insight into intracellular trehalose metabolism. Swarts et al. hypothesized that the trehalose-recycling pathway could be hijacked to actively transport azido-trehalose analogues into the mycobacterial cytosol through the trehalose-specific transporter SugABClpQY, after which the trehalose-analogue would be incorporated into the ‘trehalome’ and subsequently labeled with azide-reactive reporter molecules. Trehalose-analogues with azido-groups (TreAz analogues) at the 2-, 3-, 4- or 6-position (Fig. 2) were added to a culture of Mycobacterium smegmatis, a non-pathogenic Mycobacterium that is frequently used as a model organism for Mtb, and then exposed to a fluorescent dye coupled to a cyclooctyne group leading to labeling via a strain-promoted [3 + 2] cycloaddition. They found that the TreAz analogues labeled M. smegmatis efficiently and that the route of trehalose incorporation was dependent on the structure of the TreAz analogue; 2-, 4- and 6-TreAz were incorporated via the recycling pathway, while 3-TreAz was incorporated only extracellularly by Ag85. This incorporation was also possible for pathogenic Mtb and Mycobacterium bovis. The recent development of chemoenzymatic routes towards unnatural trehalose analogues is expected to expand the application of this method by facilitating access to the new analogues.

Two-step metabolic labeling with trehalose analogues has also been applied to study mycolation of AG and O-mycolated proteins that are found in the MM. The Swarts group hypothesized that the substrate specificity of Ag85 as a mycolyltransferase would extend to a 6-heptynoyl trehalose analogue (O-AlkTMM, Fig. 2), based on previously reported Ag85 activity studies. They hypothesized that O-AlkTMM could be taken up into the periplasm, where Ag85 would transfer the 6-heptynoyl group to mycolyl acceptors such as AG and subsequently labeled with azide-reactive reporter molecules. Trehalose-analogues with azido-groups (TreAz analogues) at the 2-, 3-, 4- or 6-position (Fig. 2) were added to a culture of Mycobacterium smegmatis, a non-pathogenic Mycobacterium that is frequently used as a model organism for Mtb, and then exposed to a fluorescent dye coupled to a cyclooctyne group leading to labeling via a strain-promoted [3 + 2] cycloaddition. They found that the TreAz analogues labeled M. smegmatis efficiently and that the route of trehalose incorporation was dependent on the structure of the TreAz analogue; 2-, 4- and 6-TreAz were incorporated via the recycling pathway, while 3-TreAz was incorporated only extracellularly by Ag85. This incorporation was also possible for pathogenic Mtb and Mycobacterium bovis. The recent development of chemoenzymatic routes towards unnatural trehalose analogues is expected to expand the application of this method by facilitating access to the new analogues.

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DMN-Tre could indeed be used for high-content image-based screening of the effect of various drugs on intracellular Mtb. Recently, new solvochromatic trehalose probes based on 3-hydroxychromone fluorophores (3HC, Fig. 2) were reported that show a 10-fold increase in fluorescence enhancement compared to DMN. This allowed the detection of Mtb cells within 10 minutes of treatment and may well represent a major step towards on-site testing for Mtb infection. Hodges et al. recently reported the use of a different strategy for fluorogenic Mtb cell wall detection. Their approach employs a trehalose variant dubbed “quencher-trehalose-fluophore” (QTF, Fig. 2) consisting of a lipid-BODIPY construct and a DABCYL quencher attached to a trehalose scaffold. Esterase Ag85 cleaves the lipid-fluorophore from the quencher-trehalose, leading to enhancement (turn-on) of fluorescence (Fig. 2). Finally, the Swarts-group recently reported a similar approach, in which cleavage of a quencher group from a construct containing fluorescein and a DABCYL quencher by the mycomembrane remodeling hydrolase Tdmh, led to the enhancement of green fluorescence.
cally active cells. As such, they can only be used to diagnose dividing \textit{Mtb} and monitor treatment efficacy. The metabolically dormant forms of \textit{Mtb}, however, remain undetected by these technologies. There remains an urgent and unmet need for the development of probes for this subclass of \textit{Mtb}.

**Proteome labeling with BIOORTHOGONAL amino ACIDs**

The methionine tRNA/tRNA synthetase pair of many species are rather promiscuous. At the turn of the millennium, the Tirrell and Bertozzi groups were the first to exploit this promiscuity to introduce bioorthogonal groups into bacterially expressed proteins and label these using click reactions.\textsuperscript{114} This technique was later applied by the Tirrell-lab to the pan-proteome Met-labeling of whole cells. The approach, latter dubbed bioorthogonal non-canonical amino acid tagging (BONCAT),\textsuperscript{115} has subsequently been used for the enrichment, identification, and visualization of exclusively nascent proteins, thus reducing the complexity of proteomic analysis of a given sample.\textsuperscript{116} Moreover, pulse-labeling with BONCAT probes allows for analysis of changes in protein production in response to internal and external cues.\textsuperscript{115} With regards to the study of intracellular pathogens, two BONCAT-based approaches have been taken. Those reliant on the hijacking of existing tRNA/synthetase pairs, and those that genetically incorporate new tRNA/synthetase pairs. Both approaches have been used extensively to interrogate pathogen’s protein expression at the host–pathogen interface.

The first approach, which relies on the promiscuity of the methionine tRNA and its synthetase, have shown that methionine analogues containing alkene,\textsuperscript{117–120} alkyne,\textsuperscript{117,121} and azido\textsuperscript{114} functionalities can be accepted substrates by the methionyl-tRNA synthetase (MetRS) in a variety of pro- and eukaryotes.\textsuperscript{122} Care has to be taken when incorporating these amino acids into new species, as the speed of protein synthesis and cell division can be altered by the presence of such unnatural amino acids, as demonstrated by homopropargylglycine (Hpg, Fig. 3) which shows toxicity towards certain bacteria at higher incubation rates, whereas azidohomoalanine does not (Aha, Fig. 3).\textsuperscript{123} The advantage of this approach is that it provides a facile way to image only the proteome of an intracellular pathogen after uptake by a host cell, without any need for further genetic modification of the species. Reacting these proteins with a clickable biotin- or FLAG-tag can also enable the enrichment of these target proteins for mass spectrometry.\textsuperscript{116} We ourselves have used this strategy to incorporate Aha or Hpg into \textit{E. coli ex vivo} and then co-incubated these bacteria with mouse bone marrow-derived cells (BMDCs) to visualize bacterial cell degradation \textit{in situ}.\textsuperscript{124} For this we used correlated light-electron microscopy (CLEM) imaging, to provide ultrastructural content of the degrading \textit{E. coli} cells within BMDC phagosomes. We have also translated the technique to allow super-resolution correlated light-electron microscopy (STORM-CLEM) of pathogen in BMDCs to show the durability of \textit{Salmonella} bacteria inside the host cell vacuole.\textsuperscript{123} Recently, we also combined the BONCAT labeling of the \textit{Mtb} proteome with the labeling of its PG layer through the use of EDA-labeling with correlative imaging, showing that multiple click reactions can be performed on pathogens simultaneously. We used this approach to assess the heterogeneous effect of various antibiotics on intracellular \textit{Mtb} (see Fig. 4 for an example image).\textsuperscript{125}

Approaches that allow expansion of the genetic code of pathogens have also become powerful tools to study host–pathogen interactions. For example, Ngo et al.\textsuperscript{126} devised a new BONCAT-like strategy that involved genetically modifying the cell type of interest to enable incorporation of NCAA for this cell type only. For this strategy, a mutant MetRS (NLL-MetRS) was developed that can append the NCAA azido-norleucine (ANL; Fig. 3) to endogenous tRNA.\textsuperscript{127} To achieve cell type selective protein labeling with bioorthogonal handles, the cell type of interest is transfected with the NLL-MetRS gene, enabling this cell type to incorporate ANL into newly synthesized proteins. ANL is not compatible with endogenous MetRS, leading the NCAA to be exclusively incorporated into proteins from the pathogen and not the host. Because of these features, BONCAT using NLL-MetRS is highly suited for analyzing pathogen’s nascent protein expression at the host–pathogen interface. The potential of this approach was further demonstrated in the interrogation of host–pathogen interactions by infecting murine macrophages with \textit{E. coli} that constitutively expressed NLL-MetRS and visualizing the bacteria selectively within the macrophage.

Using this cell selective BONCAT method, Mahdavi et al.\textsuperscript{128} were able to enrich and identify several key secreted \textit{Yersinia} outer proteins (Yops) and other secreted factors in an infection model.\textsuperscript{128} To enrich Yops that were secreted into the host cytosol, HeLa cells were infected with NLL-MetRS-expressing \textit{Yersinia} cells and subsequently lysed using digitonin, preser-

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**Fig. 3** BONCAT probes for metabolic labeling of proteins.
ving the integrity of the *Yersinia* cells. Subsequent reaction to TAMRA-alkyne allowed for facile identification of fluorescent Yops in host cytosol fractions. Moreover, by applying BONCAT in a pulse-labeling manner at specific times after infections, the authors were able to determine the order of Yop secretion, demonstrating the potential of BONCAT for elucidating the temporal aspects of virulence factor secretion. The Hang group also reported a similar strategy for the labeling of intracellular salmonella with 2-aminooctynoic acid (AOA, Fig. 3) and showed that the bacterial proteome of *Salmonella enterica* could be labeled and imaged with a high degree of selectivity inside RAW-macrophages. In addition, this approach also enabled for the enrichment and proteomic analysis of endogenously expressed *Salmonella* proteins from infected mammalian cells.

Amber codon suppression – the approach whereby a tRNA/tRNA synthetase pair reactive to the amber stop codon are introduced in a species have also been applied to the study of pathogenic species. Wang et al. reported the application of this strategy to incorporate para-iodophenylalanine into GFP expressed in the *Mtb*-strain H37Ra inside RAW-macrophages. The same approach was later exploited by Touchette et al. for the incorporation of the photo-crosslinkable amino acid *p*-benzoyl-1-phenylalanine (pBPA, Fig. 3) into the mycobacterial outer cell wall of *M. smegmatis* to identify the interaction partners of this protein. It is likely that the application of such techniques to study *Mtb* interactions with the host proteome will deliver new insights. The expanded genetic code variant of *Salmonella* was also recently reported and used to incorporate *p*-azidophenylalanine (Fig. 3) into the bacterial proteome. However, no intracellular labeling was attempted yet by this approach. Intracellular labeling was achieved for the facultative intracellular pathogen *Neisseria meningitides*. Takahashi et al. also incorporated pBPA into the bacterium and used it to elucidate the role of NMB1345 in the infection pathway.

**ABPP CLEM of bacterial enzymes**

A final area which is providing tantalizing new insights into the intracellular biology of the host–pathogen interaction is the use of probes to visualize the activity of specific pathogenic enzymes inside the host, so-called activity-based protein profiling (ABPP). This approach has been used to image host cell alterations upon bacterial infection, but the imaging of pathogenic enzymes – particularly those involved in virulence – is an exciting new area. An early example involved the use of the broad spectrum serine hydrolase probe FP-TAMRA (Fig. 5) to identify the secreted serine hydrolases of *Mtb* and *V. cholera*. In a later study, all four *V. cholera* serine hydrolases were identified using a proteomic approach in an infection model of this pathogen. FP-TAMRA was also used to identify 8 new serine hydrolases in the facultative intracellular pathogen *Staphylococcus aureus*. Lentz et al. used FP-TAMRA to develop activity-based probes [ABPs] for two of these hydrolases FphB and FphF. Using these inhibitors/probes the authors could delineate a role for FphB in the infection of liver and heart cells, although an exact mechanism for this role remains elusive. The recently resolved crystal structure of FphF in complex with the inhibitor may help in elucidating these roles.
intracellular survival of this pathogen under certain conditions. Recently, the group of Bogyo also used this approach to develop a covalent probe that allowed visualization of an \textit{Mtb} hydrolase, namely the hydrolase important for pathogenicity-1 (HIP-1). Using probe CSL173 (Fig. 5), they could observe its activity when spiked into the host proteome to identify HIP-activity.\textsuperscript{143} They have recently described the further development of these types of probes to synthesize a chemiluminescent point-of-care test for \textit{Mtb} that allowed the selective identification of live \textit{Mtb}, providing a useful tool for the study of drug efficacy.\textsuperscript{144}

The electrophilic nature of the beta-lactam scaffold has also been used to image penicillin binding protein (PBPs) activities in pathogens.\textsuperscript{145} Initial approaches were marred by a poor substrate specificity of these probes for the various PBPs. This was recently solved for another facultative intracellular pathogen, \textit{Streptococcus pneumoniae}.\textsuperscript{146} By modifying the beta-lactam core and attaching fluorescein (compound 7FL, Fig. 5) or TAMRA to these modified lactam-cores, the PBPs 1\textsubscript{b} and 2\textsubscript{x} could be selectively labeled and imaged in the dividing bacteria.\textsuperscript{147} With these probes it was found that these PBPs were restricted to a ring surrounding the bacterial division site. What these observations mean in the context of the recently discovered intracellular niche of the pathogen remains to be elucidated.

Conclusion

As these above examples clearly demonstrate, the application of novel chemical approaches to imaging is leading to rapid advances in the study of intracellular pathogens. The labeling of cellular components and individual enzyme activities is leading us to improved point of care tests.

There are, however, areas in which we foresee major developments taking place. An area, which will likely move the field forward is the continuing development of better live-cell compatible chemistries. These will hopefully allow the development of detailed dynamic pictures of the host–pathogen interaction. Reactions, such as the Inverse Electron-Demand Diels–Alder ligation (IEDDA),\textsuperscript{148} can serve as excellent starting points for this development. For the near future, we envisage that the currently existing metabolic cell wall probes, d-alanine- and trehalose analogues, will maintain their relevance, as there are still many lingering questions concerning bacterial cell wall metabolism and growth modes. Here we foresee that the use of these methods, combined with advanced imaging and quantification techniques, will be increasingly deployed to study the efficacy of antibiotics in these host–pathogen interactions. Information on the precise effects on the intracellular live cycle of pathogens will increase understanding of the method of actions of new and existing antibiotics.

Moreover, secreted bacterial proteins, many of which are associated with virulence and persistence in the host environment, remain uncharacterized and their mechanisms of action poorly understood; we envisage that BONCAT-probes can contribute significantly to further the understanding of these secreted factors through the use of new retrieval-mass spectrometry approaches. Together, these findings may lead to novel vaccines, antibiotics and other therapies against bacterial infection.

Conflicts of interest

There are no conflicts to declare.

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