

## Highlight

# Click-chemistry tagging of proteins in living cells: new possibilities for microbial (meta) proteomics

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On page 2568 of this issue, Hatzenpichler and colleagues describe the application of BONCAT (bioorthogonal non-canonical amino acid tagging) (Hinz *et al.*, 2013), a click-chemistry method originally developed to study protein synthesis and localization in neuronal cells, to pure-culture and environmental bacteria and archaea. Click chemistry and other bioorthogonal reactions have been intensively studied by chemists and some biologists for the past 15 years but have been little used as yet by environmental microbiologists. The authors show that click-chemistry amino acid analogues can be taken up by and detected in a range of pure-culture and environmental bacteria and archaea; that cells identified as translationally active by BONCAT are generally also metabolically active by the independent criterion of ammonia incorporation; and that production of proteins induced by an environmental change (heat shock) can be followed over time. BONCAT and other click-chemistry methods offer a promising route towards minimally invasive, cultivation-free investigations of the *in situ* enzymatic capabilities of microbes in diverse communities.

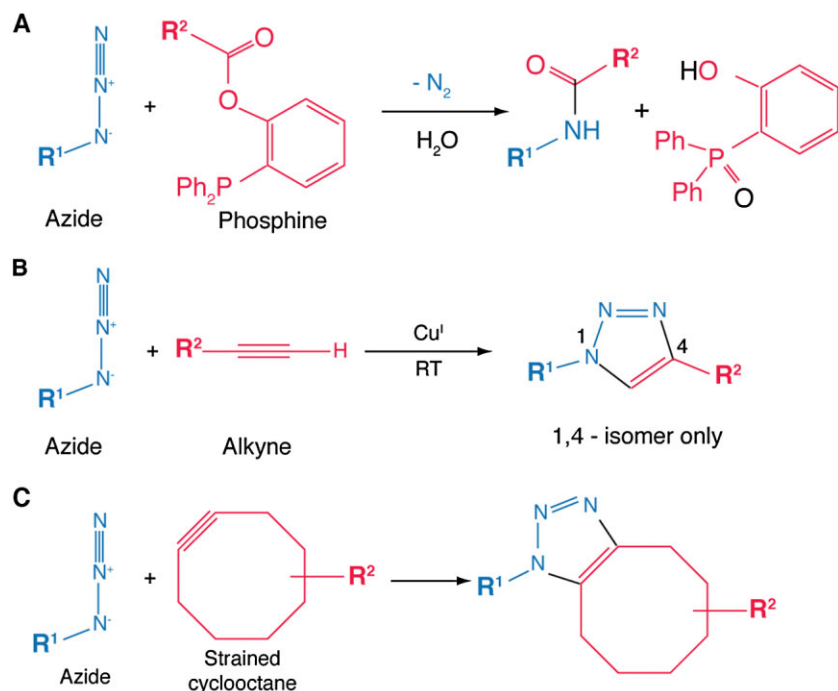
'Bioorthogonal' describes reactions that occur independently of cellular enzymes and do not affect non-target biomolecules (see Patterson *et al.*, 2014 for a recent overview). In BONCAT and related methods, alkyne–azide additions are used (Fig. 1). Samples are incubated with an amino acid analogue bearing an azide or alkyne group, which is taken up from the medium and incorporated into growing polypeptide chains – ideally without significant toxicity or growth effects. Some analogues (such as the *L*-azidohomoalanine used here) are recognized by wild-type transfer RNA synthetases, albeit with reduced efficiency, while others can only be incorporated by specially

engineered target species (reviewed in Ngo and Tirrell 2011). The azide (or alkyne) group is then a substrate for ring strain-promoted, copper-catalysed or photocatalysed 'click' addition of an alkyne- (or azide-) containing fluorescent label or capture tag (e.g. biotin), allowing microscopic identification of translationally active microbial cells or the isolation and identification of newly synthesized proteins. In general, the azide group is made part of the internalized compound, as the extreme scarcity of intracellular azides minimizes the risk of background reactions.

Biochemical applications of click chemistry reactions have been the subject of several recent reviews (e.g. Baskin and Bertozzi, 2007; Best, 2009; Ostrovskis *et al.*, 2013; Rudolf and Sieber, 2013; Yu and Wang, 2013; Lang and Chin, 2014). Emphasis here will be on the potential for protein-targeted studies of natural microbial communities, but DNA, RNA, lipids and polysaccharides can also be studied by click-chemistry methods (e.g. Jao and Salic, 2008; Raghavan *et al.*, 2008; Dommerholt *et al.*, 2010; Darzynkiewicz *et al.*, 2011; Chang and Bertozzi, 2012; Hagemeyer *et al.*, 2012; Neef *et al.*, 2012). With some of the necessary reagents becoming commercially available, these techniques are increasingly accessible to biologists.

One basis of click chemistry is the Staudinger ligation, developed by Bertozzi and co-workers. In their first application, cell-surface azides were produced via an engineered biosynthetic pathway and ligated with biotinylated phosphine, which in turn was detected by fluorescently labelled avidin (Saxon and Bertozzi, 2000). The reaction was soon refined to a 'traceless' variant (Fig. 1A), in which the phosphine group is eliminated from the ligation product (Saxon *et al.*, 2000; Saxon and Bertozzi, 2000). While still widely used, the Staudinger ligation is relatively slow, and phosphines are subject to air oxidation. The goal of subsequent work has been to find fast, highly specific reactions that can be carried out in aqueous solutions at physiological temperature and pH, without toxicity to cells or excessive perturbation of the molecules under study, at least over the experimental time course (reviewed in Debets *et al.*, 2013). The target and label molecules must also be able to reach the correct cellular compartment(s), whether by diffusion or uptake.

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**Fig. 1.** Click chemistry reactions discussed. Adapted from Liang and Astruc (2011) and Lang and Chin (2014). See Lang and Chin (2014) for additional possibilities.

A. Traceless Staudinger ligation.  
 B. Copper (I)-catalysed alkyne–azide cycloaddition (CuAAC).  
 C. Strain-promoted alkyne–azide cycloaddition (SPAAC).

Copper(I)-catalysed alkyne–azide cycloaddition (Fig. 1B) (Kolb *et al.*, 2001; Rostovtsev *et al.*, 2002; Meldal and Tornøe, 2008), a modification of the Huisgen azide–alkyne cycloaddition, is faster than the Staudinger ligation and has also found many applications; however, copper toxicity limits its *in vivo* usefulness. This can be minimized with organic copper ligands (Rudolf and Sieber, 2013), but these may in turn interfere with cell access. Strain-promoted alkyne–azide cycloadditions (Fig. 1C), such as the BONCAT reaction, were first adapted for click chemistry by Bertozzi and colleagues (Sletten and Bertozzi, 2009) and have since been further developed (reviewed in Debets *et al.*, 2013). These can be quite fast and require no catalyst. Some reported challenges are that the more reactive of the strained cyclooctynes commonly used may be unstable or react with cellular thiol groups and that their hydrophobicity can make synthesis difficult. Click reactions can also be catalysed by light (reviewed in Tasdelen and Yagci, 2013), offering precise spatial and temporal control. However, the requirement for exposure to light, generally at potentially damaging UV wavelengths, may limit its applicability in environmental samples. Finally, with exposure to ultrasound, click reactions can be catalysed by metallic copper (Cintas *et al.*, 2010).

In the experiments reported here (Hatzenpichler *et al.*, 2014), incorporation of the methionine analogue *L*-azidohomoalanine (AHA) in growing cells was subsequently detected in fixed cells with alkyne-containing fluorescent dyes. Detection was much more efficient with

fixed than with live cells; the method is not yet suitable for imaging living cells in environmental samples. By combining ribosomal RNA (rRNA)-targeted fluorescence *in situ* hybridization (rRNA-FISH) with BONCAT, translationally active single cells could be phylogenetically classified. Good labelling was achieved with several bacterial and one archaeal pure culture, oral microbiome samples and pond water, with most FISH-labelled cells detectably incorporating AHA. This was not true for an anoxic marine sediment sample; it is not yet known whether this is due to intrinsically low protein synthesis rates or some other cause, such as inability to take up AHA or interaction of AHA with sediment or porewater constituents. In a mixture of pure-culture cells, AHA labelling correlated well with ammonia incorporation as detected by nanoscale secondary ion mass spectroscopy, an independent measure of metabolic activity. And, in encouraging news for studies of specific enzymatic activities, fluorescently labelled proteins from harvested cells could be detected by SDS-PAGE and excised for sequencing; in *Escherichia coli*, labelled heat-shock proteins were observed in samples taken within 2 min of heat treatment. While much work remains to be done, the authors have outlined an agenda that could (and should) be taken up by many other environmental microbiologists.

One logical next step would be the application of bioorthogonal chemistry to activity-based proteome profiling (ABPP) (reviewed in Willems *et al.*, 2011) of environmental samples. Untangling the complex mixture of proteins in (meta)proteomes is an extreme analytical

challenge and one not necessarily worth undertaking for microbial ecologists with specific questions – for example, what is the diversity of glycolytic enzymes in a given sample? What extracellular lipases are present, and which species produce them? An activity-based probe bound covalently to the active site and bearing a clickable group allows the attachment of capture or detection labels to a specific subset of the proteome. Cell-associated or extracellular proteins with capture tags can be isolated for identification, while cell-associated proteins with detection labels (e.g. fluorescent dyes) can be localized, and in combination with rRNA-FISH could in principle be assigned to phylogenetic groups.

Applications of bioorthogonal reactions to ABPP have so far been mainly in eukaryotes. For example, they have been used to validate intracellular drug targets (Ghosh and Jones, 2014); to separately label different serine proteases in a rat tissue proteome (Haedke *et al.*, 2012); to examine the activity and regulation of tyrosine phosphatases, which as transmembrane signalling molecules play a role in human diseases (Krishnamurthy and Barrios, 2009); to identify viral, bacterial and human sialidases (Tsai *et al.*, 2013); and for subcellular imaging of lipases in mammalian cells (Viertler *et al.*, 2012). Perhaps most ambitiously, Willems and colleagues (2012) used three mutually orthogonal (non-crossreacting) bioorthogonal reactions to simultaneously label three different proteasome components in mammalian cell extracts and living cells. As this selection illustrates, tools are already available for a wide range of enzyme classes. Their adaptation to microbial communities will likely not be trivial, but should provide a much-needed counterpart to DNA- and RNA-based studies.

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