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MICROBIAL ECOLOGY

Seeing growth without culture

Protein-synthesizing bacterial and archaeal cells can now be visualized by an adaptation of the BONCAT method, and sorted from complex samples for sequencing. A demonstration on the uncultivated, slow-growing methane-oxidizing consortia shows the high potential of this new method.

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Earth hosts an astronomical number of microbial taxa, most of which are so far unseen, uncultivated and unknown. Brave environmental microbiologists are sailing the seven seas, drilling deep, and probing ice, boiling springs, brines and acid pools, to discover what lives on Earth. But even in our backyards we can find novel types of microorganisms that transform the way we think about life's versatility in tapping into energy resources, in shaping element cycles or in steering biological interactions. The discovery of bacterial and archaeal gene variants has become so fast that the tree of life grows like duckweed. But the next steps in deciphering the roles and functions of tiny life are often far more cumbersome. To observe microbial life in its complex environment and to cultivate different ecotypes takes time. Many of the recently discovered microbial taxa live a life in the slow lane. With their doubling times of months to years, they try the patience of microbiologists, and defy the timelines of PhD theses and project funding. Now, writing in *Proceedings of the National Academy of Sciences*, Roland Hatzenpichler, Victoria Orphan and colleagues report that they have developed an approach that allows us to visualize the slow-growing microorganisms, without a culture in place[1]. Their new high-throughput method substantially expands the toolkit for the microbiological explorer by combining fluorescence-labelling of *de novo* synthesized proteins and of taxon-specific ribosomal ribonucleic acid (rRNA). It allows us to visualize, sort and sequence so-called 'uncultivables' that are active components of complex microbial communities.

The basic method underlying this procedure is the tagging of cellular proteins during *de novo* synthesis, that is, in the process of translation. This method is called BONCAT, short for bioorthogonal noncanonical amino acid tagging. It was originally invented to label, purify and identify proteins of mammalian cells by chromatography and mass spectrometry[2]. The BONCAT method was recently adapted for the visualization of translationally active environmental bacteria and archaea, including marine and freshwater samples, the mouth microbiome and a variety of isolates[3,4]. Here, the artificial amino acids L-azidohomoalanine or L-homopropargylglycine are added in relatively low quantities (nano- to micromolar range), and are readily loaded onto the native transfer RNA of bacteria and archaea, replacing the common amino acid L-methionine[5]. All pure cultures so far tested have incorporated the synthetic amino acids in their proteins without changing any cellular functionalities. By the concept of click chemistry, the incorporated synthetic amino acids can then be specifically labelled with fluorescent molecules (Fig. 1). This lets the growing cells glow under the epifluorescence microscope after only 2–10% of their doubling time[1].

In their new paper, Hatzenpichler *et al.* have now transferred this method to the visualization of the infamously

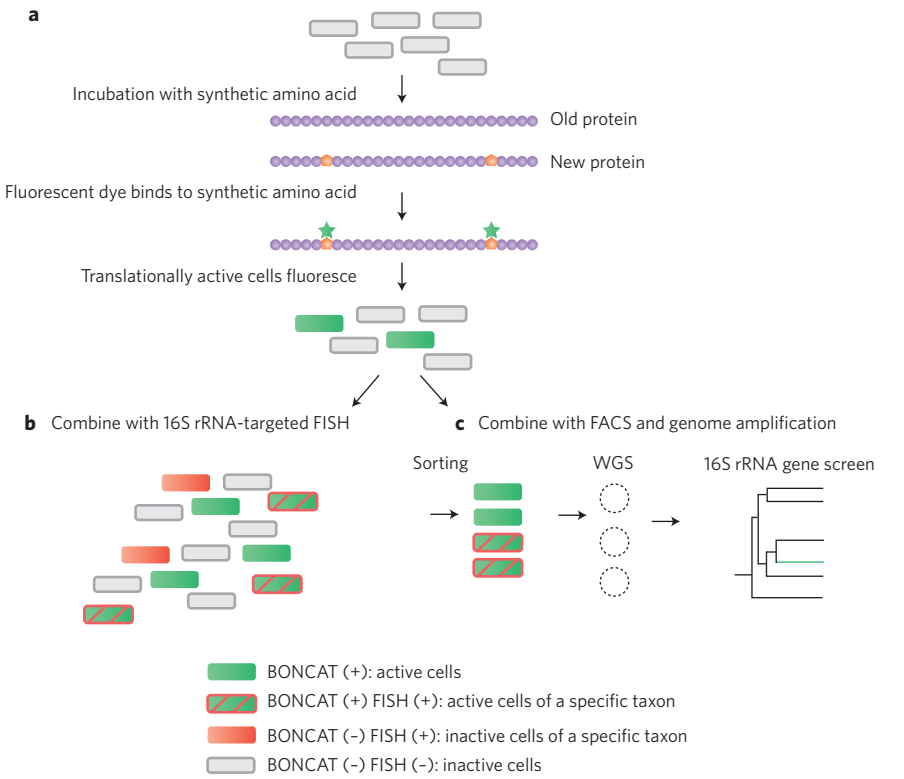

Figure 1 | Concept for the visualization, identification and sorting of BONCAT-labelled cells. **a**, Step 1: a synthetic amino acid is incubated with an environmental sample under defined conditions, and incorporated into a newly formed protein. Step 2: the tagged protein is fluorescently labelled via click chemistry. Step 3: translationally active cells can be detected by epifluorescence microscopy. **b**, rRNA-targeted FISH is performed to identify active taxa (co-labelling by BONCAT and the FISH probe). **c**, Step 1: labelled cells are sorted by FACS. Step 2: cells are lysed and their genomes amplified using multiple displacement amplification. Step 3: genomes are taxonomically screened via amplification and sequencing of the 16S rRNA gene. WGS, whole genome sequencing.

slow-growing cell aggregates of methanotrophic archaea (ANME) and their partner bacteria[1], which together perform the anaerobic oxidation of methane (AOM). AOM consortia are globally relevant catalysts of methane consumption in the seabed. But in their cold deep-sea home, these microbial consortia have generation times rivalling those of elephants, placing them into the realm of 'uncultivables'[6]. Hence, despite their relevance to seabed biogeochemistry, the oceans and climate, we still cannot answer questions regarding the niches of these anaerobic methanotrophs on land and in the sea, or the role of their substantial local and global diversity. The slow growth of AOM consortia, with doubling times of months even in sediment-free hot enrichments, makes it nearly impossible to decipher characteristic ecophysiological traits, which could explain selection by environmental processes. We know next to nothing about their selection of partner bacteria, and why certain consortia types dominate some environments and are missing from others.

With the new BONCAT method[7], combined with a protocol to concentrate AOM consortia from naturally enriched seafloor samples, thousands of protein-synthesizing aggregates of ANME and partner bacteria can be visualized in incubations as short as a week[1]. In their new study, Hatzenpichler *et al.* were also able to combine the visualization of growing consortia by BONCAT with whole-cell fluorescence *in situ* hybridization (FISH) targeting the specific 16S rRNA-based diversity of ANME and partner bacteria. This allowed them to directly visualize the effect of adding and removing methane on the translational activity of different ANME clades and their specific partner cells. The taxonomically and morphologically different, co-occurring consortia were similarly active under the same incubation conditions. Finally, the authors also demonstrate the use of BONCAT for fluorescence-activated cell sorting (FACS), coupled to whole-cell genome amplification. AOM consortia typically consist of a few hundred to a few thousand aggregated cells of two specific populations: ANME and a sulfate-reducing partner bacterium. The sorting and sequencing of active BONCAT-labelled AOM consortia now show a greater variety in biological interaction. Consortia formed by the same ANME population can grow with different families of sulfate-reducing bacteria, and even associate with Verrucomicrobia. The authors provide stunning micrographs of these cell associations and present a number of ecophysiological studies on AOM, enabled by the combination of these different method steps. They also critically assess the limits of BONCAT with regard to quantifying growth rates and identifying specific metabolic and biogeochemical functions. Growth rates cannot be assessed quantitatively with BONCAT, as the rate of methionine exchange and the protein content of cells remains unknown. In this regard, a surprising finding is the differential response of individual AOM consortia to the labelling; some are just faster than others, and even without methane as an energy source, some translational activity was observed.

Clearly, this new combination of methods has great potential for ecophysiological studies in the absence of cultures. A major innovation lies in the high-throughput capacity for testing growth factors for whole-cell assemblages (BONCAT) or specific taxa (BONCAT–FISH), such as different substrates, electron acceptors or other physiological conditions. The visualization of active cells interacting is a prerequisite to solve questions regarding specific associations in symbiosis and pathogenesis. Also, the independence from expensive instruments, such as NanoSIMS and Raman microspectroscopy (with limited availability to most labs), is an important benefit of the method. A further enhancement of high-throughput BONCAT-based methods could come from combining them with low-throughput, quantitative single-cell methods. For example, the new Raman method based on labelling of cellular biomass during growth with heavy (deuterium-rich) water[8] has the advantage of not at all changing the composition of the natural substrate pool. In combination with BONCAT, it would be ideal to test sensitivity, and exclude potential effects of the synthetic amino acids on microbes adapted to oligotrophic conditions. Another important next step in furthering the use of the BONCAT–click chemistry principle in microbial ecology would be the combination with isotope-labelling studies. For example, a combination of BONCAT with NanoSIMS[9,10] could help with deciphering timescales and variations in carbon, nitrogen and sulfur assimilation into different cellular components of translationally active consortia. $^{14}CO_2$-labelling and scintillation counting may allow us to quantify bulk growth rates of communities or picked cells[11]. For sure, with their new study, Hatzenpichler *et al.* have further opened the window into the world of the 'uncultivable majority' that we need to know so much better to understand our environment and the Earth system. ❐

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References

1. Hatzenpichler, R. *et al. Proc. Natl Acad. Sci. USA* **113,** E4069–E4078 (2016).
2. Dieterich, D. C., Link, A. J., Graumann, J., Tirrell, D. A. & Schuman, E. M. *Proc. Natl Acad. Sci. USA* **103,** 9482–9487 (2006).
3. Samo, T. J., Smriga, S., Malfatti, F., Sherwood, B. P. & Azam, F. *Front. Marine Sci.* http://dx.doi.org/10.3389/fmars.2014.00048 (2014).
4. Hatzenpichler, R. *et al. Environ. Microbiol.* **16,** 2568–2590 (2014).
5. Hatzenpichler, R. & Orphan, V. J. in *Single-Cell and Single-Molecule Methods, Hydrocarbon and Lipid Microbiology Protocols* Vol. 7 (ed. McGenity, T. J.) 145–157 (Springer, 2015).
6. Knittel, K. & Boetius, A. *Annu. Rev. Microbiol.* **63,** 311–334 (2009).
7. Hatzenpichler, R. & Orphan, V. J. Detection of protein-synthesizing microorganisms in the environment via bioorthogonal non-canonical amino acid tagging (BONCAT) *protocols.io* http://go.nature.com/2b8lwxz (24 July 2016).
8. Berry, D. *et al. Proc. Natl Acad. Sci. USA* **112,** E194–E203 (2015).
9. Dekas, A. E., Poretsky, R. S. & Orphan, V. J. *Science* **326,** 422–426 (2009).
10. Milucka, J. *et al. Nature* **491,** 541–546 (2012).
11. Dyksma, S. *et al. ISME J.* **10,** 1939–1953 (2016).