Dual-BONCAT Reveals Distinct Day- and Night-Active Microbial Populations in Salt Marsh Sediments

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Abstract

Bio-Orthogonal Non-Canonical Amino Acid Tagging (BONCAT) has emerged as a prominent molecular technique that enables microbial ecologists to visualize and identify metabolically active taxa within complex microbial communities. To date, researchers have used just one non-canonical amino acid (ncAA) in a given experiment; here, we validate a novel approach using two different ncAAs in a single experiment. This advancement facilitates the detection of differentially active subpopulations within the same experimental context, thereby reducing the uncertainty and variability associated with parallel treatments, and providing precise spatial information about organisms that are active under distinct conditions or at different times. We show that both ncAAs can be taken up by *E. coli* cultures and by constituents of the Little Sippewissett Salt Marsh (LSSM) microbiome, resulting in fluorescence signals that are significantly higher than background and ncAA-free control experiments. As a proof of concept experiment, we implemented this "Dual-BONCAT" approach in LSSM sediments, adding one ncAA during daytime hours and the other at night. Subpopulations of cells that were anabolically active during the day and/or night were distinguishable by both fluorescence microscopy, and by fluorescence-activated cell sorting. Subsequent high-throughput 16S rRNA gene amplicon sequencing of active subpopulations revealed that *Methylobacterium*, potentially feeding on plant exudate carbon molecules, was preferentially active during the day, while sulfur-cycling taxa dominated the night-active population. Dual-BONCAT offers an important advancement in multiplexing substrate-analog probing techniques, providing a more realistic understanding of metabolic activity under distinct environmental conditions.

Main Text

Understanding the metabolic activity of microbes in environmental communities is a major goal for microbiologists. Recently, substrate analog probing techniques have been adapted for use in environmental communities, utilizing analog molecular constituents to track assimilatory processes (1–3). One such approach is BONCAT, which relies on the cellular uptake of the non-canonical amino acids (ncAAs) *L*- azidohomoalanine (AHA) and *L*-homopropargylglycine (HPG), which can be incorporated into newly synthesized proteins in place of *L*-methionine without substantially affecting cell function (2,4). AHA and HPG possess chemically modifiable azide and alkyne groups, respectively, to which fluorescently tagged molecules can be bound through "click chemistry" reactions (5,6).

BONCAT is therefore able to distinguish anabolically active (or more precisely, protein-synthesizing) cells from dormant cells. Downstream analysis through microscopy or sequencing (often following isolation via Fluorescence-Activated Cell Sorting, or FACS), enables an array of ecophysiological interpretations. For instance, BONCAT has been used to show carbon use preferences of distinct taxa in hot springs (7) and salt marsh sediment (8), active microbes in the soil microbiome (9), and symbiotic relationships between anaerobic methane-oxidizing archaea (ANME) and sulfate-reducing bacteria in the deep sea (2).

Despite the substantial advances made possible by BONCAT in the study of environmental microbial communities, all known applications of BONCAT to date have used only one of the ncAAs - HPG or AHA - in a given experiment. Here, we present a novel approach making use of

both ncAAs in a single experiment, which we refer to as "Dual-BONCAT". This technique offers additional versatility for more complex experimental designs: by selectively labeling two distinct protein subsets, Dual-BONCAT offers multiplexing capabilities that enable the labeling and analysis of two protein populations within the same cell or sample. We first validate the approach with cell cultures, and then present a proof-of-concept study differentiating salt marsh sediment microbial communities that are active during the day or night.

For our two ncAA-dye combinations, we selected the AHA-DBCO strain-promoted click reaction and the HPG-Azide Cu(I)-catalyzed cycloaddition reaction. Performing the AHA-DBCO reaction first is recommended to avoid potential false positive signal from unbound azide dyes reacting with alkyne dyes (were the AHA-Alkyne dye pairing selected), as well as false negatives from AHA azides reacting with HPG alkynes (were the HPG-Azide dye reaction performed first).

We performed all optimization and experimental procedures on cell cultures (*Escherichia coli*) as well as an environmental system hosting a complex microbial community (surface sediment from the Little Sippewissett Salt Marsh (LSSM) on Cape Cod, MA). Initial Mono-BONCAT tests, using a single ncAA per experiment, established optimal ncAA and dye concentrations that yielded the highest fluorescence signal compared to ncAA-free controls. The following concentrations were used throughout the study: 10 μM for both ncAAs, 25 μM for both dyes in *E. coli* culture; and 50 μM for both ncAAs, 25 μM for the azide dye, and 1 μM for the DBCO dye in LSSM sediment.

In Dual-BONCAT experiments testing the ability of *E. coli* cells to incorporate both ncAAs, the first ncAA (ncAA#1) was introduced at the same time as the 1:100 inoculation of a 10 mL culture (using cells in exponential phase), the second ncAA (ncAA#2) was introduced after 10 hours, and growth was stopped after 24 hours. In our *ex situ* Dual-BONCAT incubations with LSSM sediment, the different ncAAs were introduced 12 hours apart, and the incubation was stopped after 24 hours. With both cultured bacterial cells and environmental communities, we detected successful uptake of both ncAAs using fluorescence microscopy (Figs. 1, S1, S2; see SI for protocol details).

Fluorescence intensities for experimental treatments were substantially higher than the no-ncAA controls regardless of which ncAA was added first. In both *E. coli* permutations, the signal associated with ncAA#1 was significantly brighter than that from ncAA#2; the opposite relationship was observed for both LSSM conditions (Fig. S2; p-values provided in Table S1). The relative brightness of ncAA#1 and ncAA#2 likely depends on several factors such as taxon-specific ncAA uptake kinetics and proteome composition (10), as well as the degree of anabolic activity during the different experimental phases.

To demonstrate the utility of the Dual-BONCAT technique, we sought to identify which microbes in LSSM sediment were active (synthesizing proteins) during sunlit daytime hours and which were active during nighttime darkness. Critically, this approach did not require separate incubations, which obviates concerns about different microbial communities or distinct physicochemical conditions in separate treatments.

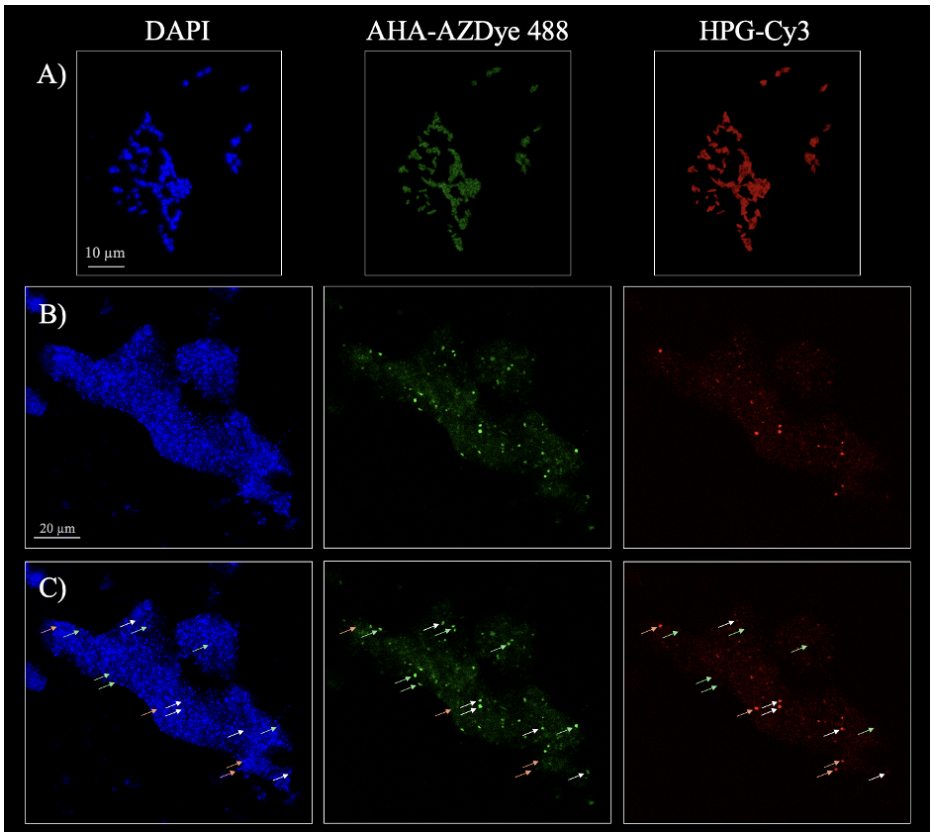


Figure 1: Representative fields of view of Dual-BONCAT experiments applied to A) E. coli cultures and B), C) LSSM environmental microbial communities. The columns correspond to emission windows of 420-490 nm for DAPI, 505-545 nm for AHA-AZDye 488, and 560-650 nm for HPG-Cy3. In A), 10 µM HPG was introduced at the start of a 24-hour experiment, and 10 µM AHA was added 10 hours later. Dye concentrations were 2.5 ng/µL DAPI, 25 µM DBCO-AZDye 488, and 25 µM azide-Cy3. In B) & C), 50 µM HPG was added at the start of a 24-hour experiment, and 50 µM AHA was added 12 hours later. Dye concentrations were 2.5 ng/µL DAPI, 1 µM DBCO-AZDye 488, and 25 µM for the azide-Cy3. B) and C) show the same field of view; the latter row is annotated with colored arrows to highlight differentially-expressed dyes. White arrows show cells that incorporated both ncAAs; green and pink arrows show cells that only incorporated AHA and HPG, respectively.

Two lab-based experimental incubations of shallow (0-3 cm) LSSM sediment were performed. In Condition 1, HPG was added at sunrise and AHA was added 12 hours later, when the incubation was moved to a dark environment. In Condition 2, the order of ncAA addition was reversed. We also included a control treatment that that did not receive any ncAAs but did receive both dyes. For both experiments, we detected cells with only the daytime ncAA (ncAA#1), both ncAAs (ncAA#1&2), and only the nighttime ncAA (ncAA#2) (e.g., Fig. 1C; S2).

We interpret the three distinct cell populations (ncAA#1, ncAA#1&2, and ncAA#2) as follows. 1) The ncAA#1 population was active only during the day. 2) The ncAA#1&2 population exhibited activity either during both day and night, or only at night. (Because ncAA#1 was

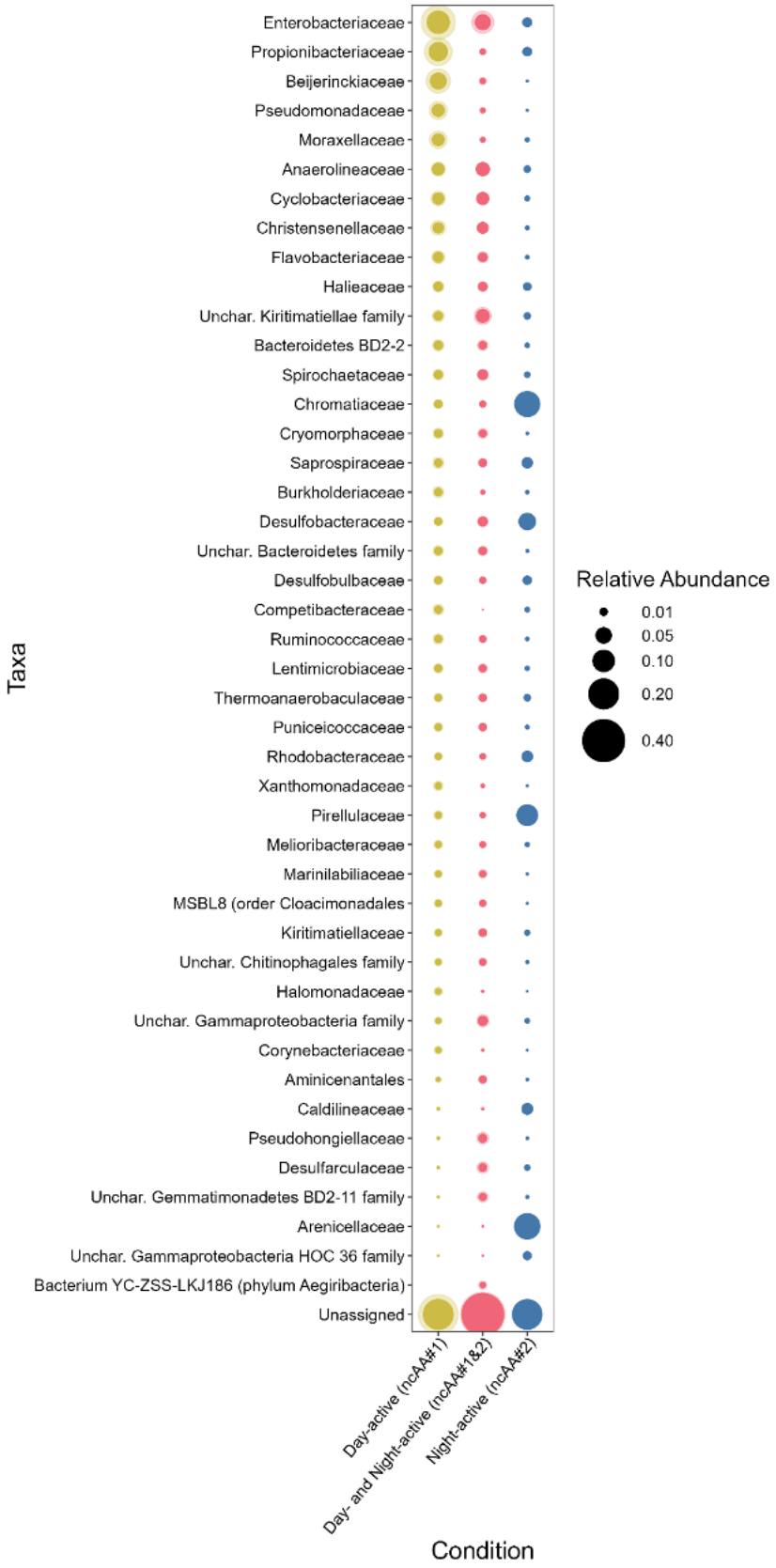
present throughout the incubation, it could be utilized during either time period.) 3) The ncAA#2 population was active only at night. (We suggest that the lack of ncAA#1 incorporation in this population could be due to more favorable uptake of ncAA#2 based on kinetic properties or relative concentrations during the nighttime phase of the experiment. If these cells were anabolically active when only ncAA#1 was present, it would have been incorporated.) These interpretations are predicated on previous observations that all taxa queried to date are capable of using each of the two ncAAs ((10) and references therein).

The three BONCAT-active populations from each of the two incubations were isolated using Fluorescence-Activated Cell Sorting (FACS), and the microbial community composition of each fraction was assessed using high-throughput 16S rRNA gene amplicon sequencing. (Note that for Condition 1 ncAA#2, the "night-only" population, sequencing was not possible due to cell sorting limitations.)

Among the taxa that were most prominent within the day-active (ncAA#1) population (Figs. 2, S3; Tables S2, S3), the *Beijerinckiaceae* genus *Methylobacterium* is commonly associated with plants, and can use one-carbon compounds, including methanol in plant exudate, as a carbon source (11,12). At LSSM, roots of the cordgrass *Spartina alterniflora* permeate the sediment and release a chemically diverse array of organic molecules. Heterotrophs feeding on exudate, such as *Methylobacterium*, were likely stimulated by the *Spartina*'s daytime photosynthetic activity; a similar relationship was observed with nitrogen-fixing microorganisms surrounding *Spartina* roots, whose activity was substantially enhanced within an hour of light exposure (13).

Sulfur-cycling taxa were highly abundant members of the night-active community (ncAA#2 population). *Chromatiaceae*, the purple sulfur bacteria that comprise a key constituent of the syntrophic "pink berry" consortia (14), accounted for 14% of the night-active population; while this taxon is photosynthetic, its dark-phase respiration metabolism (15,16) may be more tightly coupled with protein synthesis. *Arenicellaceae*, whose close relative UBA868 may play a foundational role in marine sulfur oxidation (17), and the sulfate-reducing bacteria family *Desulfobacteraceae* were both preferentially active at night, perhaps responding more favorably than other anaerobic taxa to expanding anoxic zones that accompany nighttime decreases in oxygenic photosynthesis (18). Other abundant taxa, such as the heterotrophic *Anaerolineaceae* and *Cyclobacteriaceae*, did not exhibit night-specific activity patterns; these organisms likely operate independent of diurnal cycles and depend more on ever-present organic matter rather than shifting redox stratification or specific molecules provisioned during light or dark metabolic phases.

Dual-BONCAT offers a new capability for the visualization and identification of differentially active subpopulations within any microbiome of interest. By revealing organisms that are anabolically active under distinct conditions within the same physical environment, novel syntrophic or antagonistic relationships can be queried, and a more realistic understanding of the condition-specific activity of microbial constituents can be attained.


Taxa
Enterobacteriaceae
Propionibacteriaceae
Beijerinckiaceae
Pseudomonadaceae
Moraxellaceae
Anaerolineaceae
Cyclobacteriaceae
Christensenellaceae
Flavobacteriaceae
Halieaceae
Unchar. Kiritimatiellae family
Bacteroidetes BD2-2
Spirochaetaceae
Chromatiaceae
Cryomorphaceae
Saprospiraceae
Burkholderiaceae
Desulfobacteraceae
Unchar. Bacteroidetes family
Desulfobulbaceae
Competibacteraceae
Ruminococcaceae
Lentimicrobiaceae
Thermoanaerobaculaceae
Puniceicoccaceae
Rhodobacteraceae
Xanthomonadaceae
Pirellulaceae
Melioribacteraceae
Marinilabiliaceae
MSBL8 (order Cloacimonadales
Kiritimatiellaceae
Unchar. Chitinophagales family
Halomonadaceae
Unchar. Gammaproteobacteria family
Corynebacteriaceae
Aminicenantales
Caldilineaceae
Pseudohongiellaceae
Desulfarculaceae
Unchar. Gemmatimonadetes BD2-11 family
Arenicellaceae
Unchar. Gammaproteobacteria HOC 36 family
Bacterium YC-ZSS-LKJ186 (phylum Aegiribacteria)
Unassigned
Day-active (ncAA#1)
Day- and Night-active (ncAA#1&2)
Night-active (ncAA#2)
Condition
Relative Abundance
0.01
0.05
0.10
0.20
0.40

205

Figure 2: Relative abundances of family-level taxonomic groupings derived from Dual-BONCAT-FACS-16S analyses of LSSM sediments. Each row corresponds to a family that comprised at least 1% of the overall relative abundance in at least one of the five sorted and sequenced fractions. Each column and color corresponds to a given ncAA condition: yellow indicates cells that incorporated only ncAA#1 (regardless of which ncAA was added first), red indicates cells that incorporated both ncAAs (regardless of which was added first), and blue indicates cells that incorporated only ncAA#2 (when HPG was added second; the other condition did not result in sufficient cells for sequencing). The size of each circle indicates the mean relative abundance for a given family and ncAA condition; the faint "halo" around each data point conveys the standard deviation when two communities were sequenced for a given condition. See Table S2 for the data used to generate this figure.

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Data Availability Statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request. Raw sequence reads have been archived at the National Centre for Biotechnology Information GenBank under the Bioproject ID PRJNA1188625.

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