Chapter 5

TOWARDS CELL-SPECIFIC PROTEOMICS OF THE GUT MICROBIOTA

5.1 Abstract

After developing cell-specific BONCAT in mice to study host-pathogen interactions (Chapter 3), we sought to extend the system to study a commensal microbe that lives within the gut. *Bacteroides fragilis* thrives within the crypts and mucus layers of the mammalian gut, promotes the development of a healthy immune system, and secretes numerous proteins involved in colonization. We utilized a mutant phenylalanyl-tRNA synthetase along with the noncanonical amino acids 4-azido-L-phenylalanine (Azf) and 4-ethynyl-L-phenylalanine (Ef) to tag proteins synthesized by the bacteria within the guts of living mice. This work demonstrates the first use of cell-selective BONCAT within an obligate anaerobe.

This work was a collaborative effort with Judy Shon, Gregory P. Donaldson, Kristie Yu, and Sarkis K. Mazmanian.

5.2 Introduction

Mammals and their resident microbes have formed strong alliances throughout their evolution together over time (1). The mammalian system supplies numerous molecules to its denizens, which produce signaling factors of their own to communicate with host organs (2, 3). Identifying these signaling molecules and interrogating their communication has become an important endeavor in the quest to improve human health: For example, a common gram-negative resident of the gut, *Bacteroides*, has been shown to regulate the host immune system and suppresses disease with its capsular polysaccharide (PSA) (4-6). A global proteomic study of these resident microbes in their host environment would be invaluable, but experimentally challenging due to the immense quantity of host and food proteins in the environment and the technical limitations of shotgun proteomics (7, 8).

To achieve targeted proteomic studies of *Bacteroides* within the gut, we developed cell-selective bioorthogonal noncanonical amino acid tagging (BONCAT) for use with this commensal microbe. The technique relies on the expression of a mutant tRNA-synthetase (aaRS) that can recognize a noncanonical amino acid (ncAA) and incorporate it in a residue-selective manner into newly-synthesized proteins (9). In this example, *Bacteroides* successfully expressed a PheRS mutant engineered to incorporate Azf or Ef ncAAs, effectively labeling its proteins with a bioorthogonal tag for detection and enrichment. Other proteins from the mouse or food will not have this tag and can be removed from the sample prior to analysis. This tool allows researchers to specifically label *Bacteroides* proteins synthesized *in vivo* in an effort to better understand the methods of

communication and survival this commensal microbe leverages in the harsh environment of the gut.

5.3 Results

Adaptation of cell-specific BONCAT to *Bacteroides fragilis*. We inserted the *E. coli* coding sequence of either a mutant MetRS (9, 10) or a mutant PheRS (11) into an *E. coli* – *Bacteroides* shuttle vector (pFD340) (12). Attempts to chromosomally incorporate these tRNA-synthetases using newer vectors were unsuccessful (13, 14). After conjugation of these constructed plasmids or an empty plasmid control into *B. fragilis*, we grew the strains anaerobically. After a 1:100 refresh into supplemented brain heart infusion (BHIS) media, we added PBS containing either 1 mM azidonorleucine (Anl), 200 μM 4-azido-phenylalanine (Azf), or 200 μM 4-ethynyl-phenylalanine (Ef) for 16 hours. The cultures were lysed and subjected to the copper-catalyzed azide-alkyne cycloaddition (CuAAC) with either alkyne-TAMRA (for the azide ncAAs Anl and Azf) or azide-TAMRA (for the alkyne ncAA Ef). While the strains expressing the mutant MetRS (+MetRS) did not show any incorporation of Anl (Fig S5.1), the strain expressing the mutant PheRS (+PheRS) was able to robustly incorporate both Azf and Ef (Fig 5.1C).

Notably, exposure to the ncAA azidohomoalanine (Aha), which does not require expression of a mutant aaRS as it is recognized by the endogenous methionyl-tRNA synthetases of most organisms, resulted in very strong labeling of *B. fragilis* in culture (Fig S5.1B). While Aha is consistently incorporated by endogenous MetRS, labeling

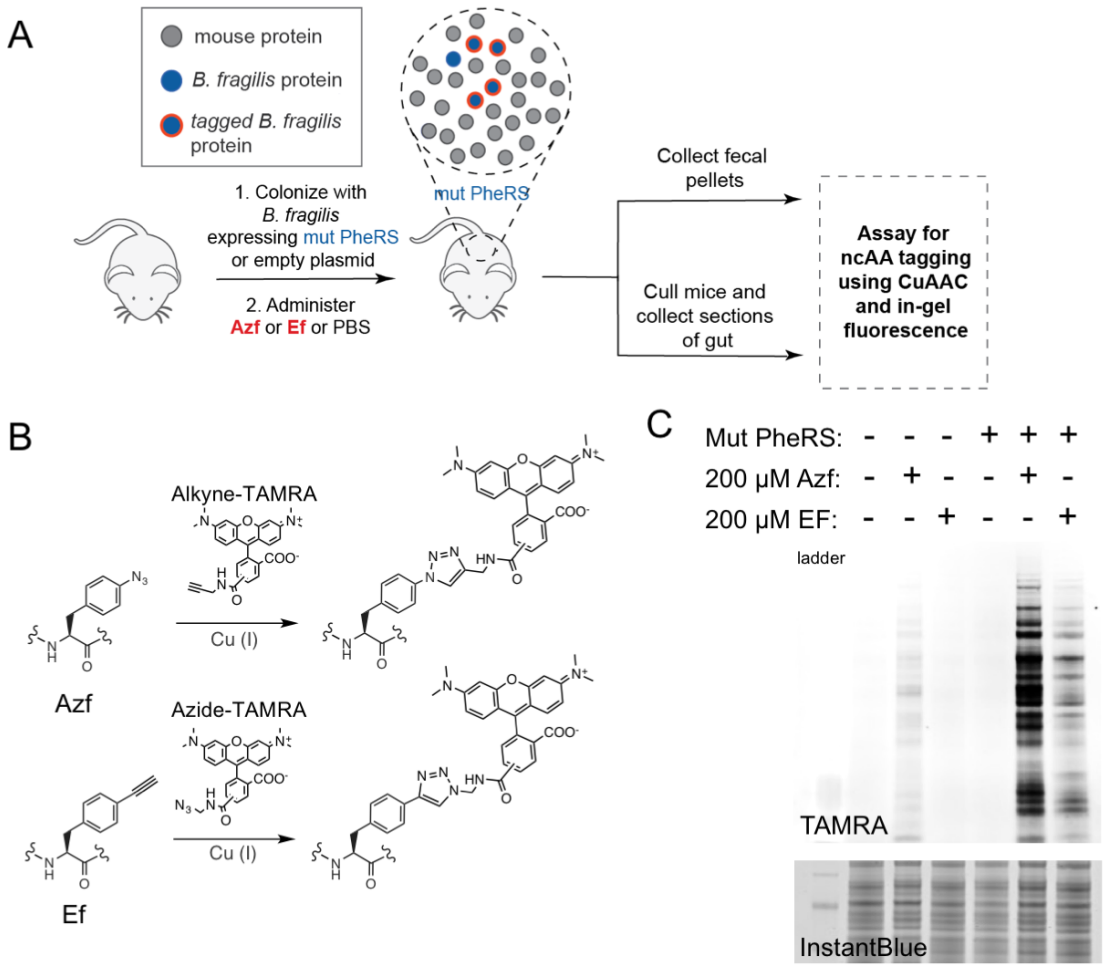


Figure 5.1: A) Scheme used to detect proteins synthesized by *Bacteroides fragilis* within the mouse gut. After colonization of mice with strains expressing a mutant phenylalanyl-tRNA synthetase (PheRS) or not, we administer azidophenylalanine (Azf) or ethynylphenylalanine (Ef). We then collect fecal pellets and homogenize prior to assessing for azide incorporation into proteins. B) Scheme of copper-catalyzed azide-alkyne cycloaddition (CuAAC) with Azf or Ef and the corresponding TAMRA. C) SDS-PAGE gel of *B. fragilis* after 16 hours of labeling during anaerobic growth in BHIS. Only cells that expressed the mutant PheRS and were labeled with Azf or EF showed fluorescence. Azf appears to have slightly higher background than Ef. InstantBlue shows equal protein loading across samples.

usually requires an environment with a depleted supply of methionine; *B. fragilis* showed very strong labeling with Aha even in the supplemented BHIS media without any

methionine depletion. Differences in the species' endogenous protein synthesis machinery, such as sequences of the MetRS or associated tRNAs may explain the difference in AHA incorporation observed between *B. fragilis* and *E. coli*, and the apparent incompatibility with the expressed *E. coli* mutant MetRS: in this case, the *B. fragilis* MetRS shares only 35% sequence homology with the *E. coli* version. Whenever adapting cell-selective BONCAT for a new species, we recommend characterizing labeling of both the PheRS and the MetRS mutant synthetases, and picking the one that shows strong labeling without notably affecting growth.

Colonization of germ-free (GF) mice with *B. fragilis* strains. Mice that had been maintained germ-free were colonized with the +PheRS strain of *B. fragilis* or the empty plasmid control. We allowed the colonization to expand within the gut over 2-4 weeks, then collected fecal pellets and verified by both serial dilution and colony PCR that each plasmid was maintained (Fig 5.2).

Labeling of *B. fragilis* within the gut of GF mice. An important consideration when performing *in vivo* cell-selective BONCAT is the method of delivery of the ncAA in proper concentrations to the cells of interest. Previously, we directly injected the site of infection with the ncAA to label bacteria (Chapter 3), but delivering the ncAA to the gut presented new challenges. Little is known about the pharmacokinetics of Azf and Ef ncAAs within animals and whether they would be stable in the harsh conditions of the stomach and gut. We assessed two different methods of delivering the ncAA to the gut: oral gavage (PO, or force feeding) and adding the ncAA to the mice's drinking water.

We gavaged the mice with 100 μL of 0, 0.5, 5.0, and 50 mM Azf dissolved in PBS (pH 7.5), then collected fecal pellets six hours and 24 hours after. These fecal pellets were resuspended in PBS, lysed, and subjected to CuAAC with an alkyne-TAMRA. Only the mice colonized with the +PheRS *B. fragilis* and gavaged with 50 mM Azf displayed signal six hours post-gavage via in-gel fluorescence (Fig 5.2C). After 24 hours, this signal disappeared, indicating that the Azf had been cleared from the system by this time. Importantly, without protease inhibitors in the lysis buffer, the proteins in solution were found to degrade very rapidly. Even with 10x protease inhibitors in the lysis buffer, we observed a very low signal associated with intact *B. fragilis* proteins, with the strongest TAMRA and InstantBlue protein labeling appearing in a broad band of low molecular weight fragments. The function of the gut is to break down protein: thus, high amounts of protease inhibitors must be used in these experiments in order to minimize protein degradation prior to analysis.

We also tested whether supplying the mice with Azf dissolved in drinking water would induce labeling. The mice were given opaque 50 mL conical tubes that had been converted to water bottles, with 25 mM Azf and sweetened with 2% sucrose to encourage consumption. After two days, we observed that they were only drinking ~1 mL/day, which is significantly less than mice's standard consumption of 3-5 mLs/day. We hypothesized that this could be due to the known bitter taste of phenylalanine and its derivatives (15), although we did not test this hypothesis with our own taste receptors. We collected fecal pellets from the mice that had been given this water and assessed labeling using the

CuAAC reaction. Two days after introduction of Azf to the water we observed strong labeling via in-gel fluorescence in the mice that were colonized with the +PheRS strain (Fig 5.2D). Although we successfully labeled *B. fragilis* proteins in 2 days, we reasoned that this method was not ideal as a constant supply of drinking water would 1) require a large quantity of Azf and 2) render us unable to label the proteins with sufficient time-resolution, as we did not monitor when each mouse drank from the water bottle. Oral gavage addresses both of these issues, but further experiments will have to be performed to assess the time resolution of this method and whether we can increase labeling intensity.

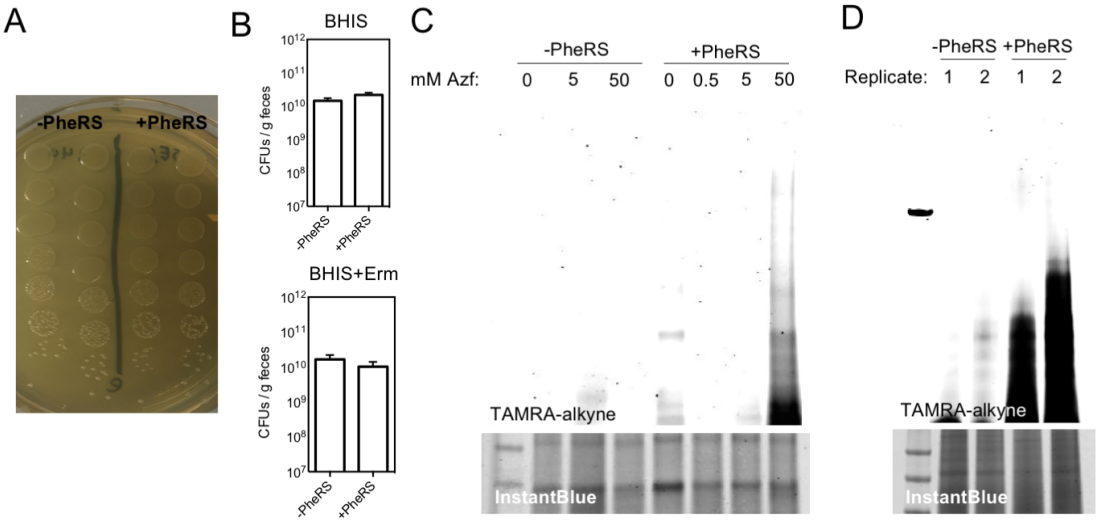


Figure 5.2: A) Dilution series of *B. fragilis* from fecal pellets 2 weeks post colonization of GF mice on erythromycin (Erm) plates. B) Bar graphs showing total CFU counts and Erm-resistant CFU counts. C) In-gel fluorescence gel of alkyne-TAMRA clicked fecal pellet obtained from GF mice 6 hours post 100 µL gavage of noted solutions D) Similar to C except 48 hours post introducing 25 mM Azf in water bottles.

5.4 Discussion and Future Directions

Surprisingly, the endogenous *B. fragilis* MetRS was able to incorporate Aha to a higher degree than other organisms even without depletion of available methionine. A comparison of charging rates of Aha onto tRNA for both mice and *Bacteroides* may be useful: If *Bacteroides* incorporate Aha at a significantly higher rate than the host, feeding Aha to mice may result in semi-specific labeling of the *Bacteroides*' proteins without the necessity to create a genetically-altered strain. In addition, the expression of a mutant *E. coli* MetRS did not show labeling with Anl, suggesting an incompatibility with either the Anl ncAA or between the exogenous synthetase and endogenous tRNA. Sequence analysis of the tRNA recognition sites may explain this incompatibility. Overall, these results highlight the value of having more than one choice of enzyme to perform cell-selective BONCAT. Testing different synthetases (aaRS) and ncAAs whenever it is adapted to a new species will be useful for future studies.

We developed cell-selective BONCAT for use in *Bacteroides*, an important constituent of the mammalian gut microbiota. Expression of a mutant PheRS enabled incorporation of Azf or Ef into microbial proteins while in the gut of mice, which could then be detected using click chemistry. This system can be used to label, visualize, and identify proteins made by the microbiota within a mammalian host, and complements other methods used to study host-microbe interactions *in vivo* (16, 17).

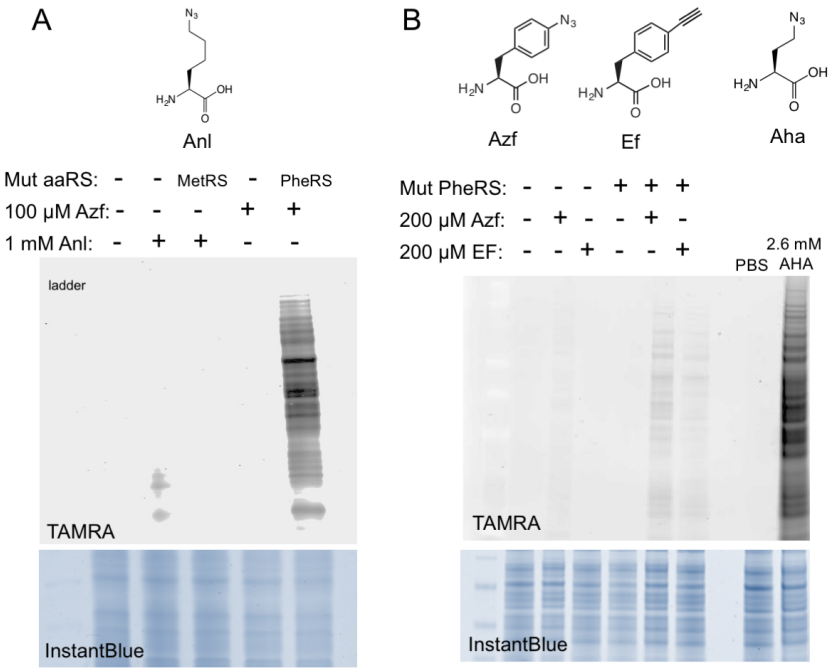
5.5 Experimental Procedures

Bacterial strains and media. *E. coli* were aerobically grown in Luria-Bertani broth (LB) at 37 °C and *B. fragilis* was grown anaerobically in brain heart infusion broth supplemented with vitamin K (0.5 μg/mL) and hemin (5 μg/mL) at 37 °C. Ligation-independent cloning was performed in *E. coli* DH10B cells and positive colonies were selected using ampicillin (100 μg/mL). Conjugations were into wild-type *B. fragilis* following protocols of Lee *et al*. (12) and selected for using erythromycin (10 μg/mL) and gentamicin (100 μg/mL).

Mice. 8-10 week-old germ-free (GF) Swiss Webster mice were purchased from Taconic Farms and bred in flexible film isolators. For colonization experiments, GF mice were transferred to freshly autoclaved microisolator cages, fed *ad libitum* with a standard autoclaved chow diet and given autoclaved water supplemented with 10 μg/mL of erythromycin and 100 μg/mL of gentamicin. All procedures were performed in accordance with the approved protocols using IACUC guidelines of the California Institute of Technology.

Colonization experiments. 8-12 week-old GF Swiss Webster mice were exposed to the *B. fragilis* strain of interest by adding 2 mL of an overnight culture to their bedding. After two weeks and four weeks, fresh fecal samples were collected, weighed, homogenized and serially diluted in PBS for plating on selective media to determine bacterial colony forming units (CFU) per gram of feces. Colonies from these plates were subsequently assayed by PCR to confirm the identity of the plasmid.

Labeling experiments *in vivo*. Colonized mice were gavaged with 100 µL of an Azf solution or PBS (as indicated), and fresh fecal samples were collected as in Colonization Experiments, except with the addition of 10X EDTA-free protease inhibitors (Roche). The resuspended bacterial solution in PBS was brought to 1% SDS and boiled for 10min to lyse the cells. The click reaction was performed on ~10 µg of protein from each sample using a Click-It Kit (Thermo Scientific) and the samples were run on an 8-12% Bis-Tris SDS-PAGE gel, followed by imaging on a Typhoon gel scanner.



Supplemental Figure S5.1: A) *B. fragilis* expressing the mutant MetRS shows no incorporation of azidonorleucine (Anl) after 18 hours of anaerobic growth in the presence of the ncAA. B) The addition of 2.6 mM azidohomoalanine (Aha) to BHIS over 18 hours of anaerobic growth leads to strong labeling. The entirety of the gel is included to compare the Aha incorporation to azidophenylalanine (Azf) or ethynylphenylalanine (Ef) incorporation.

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