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Chemoenzymatic Labeling of Proteins for Imaging in Bacterial Cells

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Supporting Information

ABSTRACT: Reliable methods to determine the subcellular localization of bacterial proteins are needed for the study of prokaryotic cell biology. We describe here a simple and general technique for imaging of bacterial proteins in situ by fluorescence microscopy. The method uses the eukaryotic enzyme *N*-myristoyltransferase to modify the N-terminus of the protein of interest with an azido fatty acid. Subsequent strain-promoted azide−alkyne cycloaddition allows conjugation of dyes and imaging of tagged proteins by confocal fluorescence microscopy. We demonstrate the method by labeling the chemotaxis proteins Tar and CheA and the cell division proteins FtsZ and FtsA in *Escherichia coli*. We observe distinct spatial patterns for each of these proteins in both fixed and live cells. The method should prove broadly useful for protein imaging in bacteria.

Bacteria exercise exquisite control of protein localization as they orchestrate processes such as cell division, chromosome segregation, and environmental sensing.[1] Recent advances in super-resolution microscopy, including stochastic optical reconstruction microscopy (STORM)[2] and photoactivated localization microscopy (PALM),[3] along with new techniques in electron microscopy (e.g., correlated, cryogenic-PALM)[4] allow researchers to visualize molecular ultrastructures in bacterial cells with nanometer-scale resolution. Although fluorescent proteins (FPs) have found widespread use in labeling of proteins for imaging studies,[5] the large size of the FP tag can perturb protein localization.[6] Because organic fluorophores are smaller and generally brighter than FPs,[7] site-specific protein modification with biophysical probes remains an active area of research.[8]

Building on prior studies from the Gordon laboratory and others[9] including our own,[10] we report here a simple and general method for imaging of bacterial proteins in fixed and live cells. By coexpressing human *N*-myristoyltransferase (NMT) and a target bacterial protein outfitted with a short NMT recognition sequence,[11] we append azido fatty acid **1** or **3** to the N-terminus of the target (Figure 1). The azide-labeled protein is then tagged with a fluorophore (**2**) via strain-promoted azide−alkyne cycloaddition.[12,13]

Several features of the NMT system make it an effective tool for labeling of recombinant proteins in bacterial cells and specifically in *Escherichia coli*: neither azido fatty acids nor NMT are normally present in *E. coli* cells;[14] the azido functionality provides a site for facile bioorthogonal ligation; and the fatty acyl-coenzyme A synthetase (FACS) machinery of

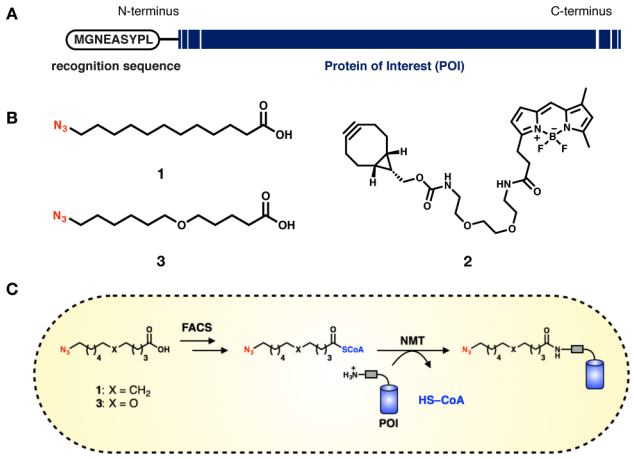

Figure 1. Site-specific labeling strategy in bacterial cells. (A) A genetically encoded N-terminal amino acid sequence serves as the recognition element for NMT on the protein of interest (POI). (B) Structures of fatty acid probes **1** and **3** and BODIPY fluorophore **2**. (C) Metabolic incorporation of fatty acid surrogate **1** or **3** allows site-specific labeling with NMT. Probe **1** or **3** is added directly to bacterial cultures and is converted into an active thioester for NMT recognition. FACS = fatty acyl-coenzyme A synthetase; NMT = *N*-myristoyltransferase; HS−CoA = coenzyme A.

E. coli is known to convert **1** into an active substrate for NMT.[15] To the best of our knowledge, however, there has been no prior report on use of the NMT labeling system for protein imaging in bacteria.

We chose the bacterial chemotaxis proteins Tar and CheA and the cell division proteins FtsZ and FtsA as test substrates for NMT labeling and fluorescence imaging. The transmembrane protein Tar and cytoplasmic protein CheA localize at the poles of *E. coli* in clusters believed to enhance chemotactic signaling.[16,17] FtsZ and FtsA have been shown to localize at the septum and to play important roles in cell division.[18,19] To label these proteins, we introduced at each N-terminus the nonapeptide sequence MGNEASYPL derived from calcineurin B, a protein that is naturally myristoylated in mammalian cells.[20] NMT appends fatty acid substrates to the N-terminal glycine residue of the recognition sequence following cleavage of the initiator methionine by methionyl aminopeptidase.[21] Target proteins were expressed from modified pQE80-L plasmids under control of the bacteriophage T5 promoter[22] (Table S1). Cells were outfitted with a dual-

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plasmid expression system, with one plasmid (pHV738-NMT-MetAP)[23] directing expression of NMT and methionyl aminopeptidase and a second coding for inducible expression of one of the four bacterial protein targets (Figure S1). *E. coli* cultures were grown at 37 °C to an optical density at 600 nm (OD_{600}) of 0.5. Protein expression was then induced by addition of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG), and N-terminal labeling was accomplished by addition of 500 μM **1**. After 4 h, cells were harvested by centrifugation, washed twice with phosphate-buffered saline (PBS), and lysed. Crude lysates were alkylated with 1 mM iodoacetamide and subsequently treated with 2 μM BODIPY conjugate **2**, which carries a reactive bicyclononyne unit,[24] at 37 °C for 0.5 h.

We verified by gel electrophoresis that protein labeling occurred selectively. For each of the bacterial proteins, in-gel fluorescence detection (488 nm excitation, 520 nm emission) showed a distinct band with apparent molecular weight corresponding to that of the intended target (Figure 2). To

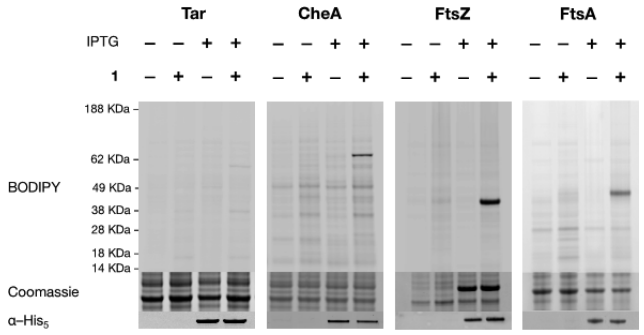

Figure 2. SDS–PAGE analysis of *E. coli* lysates prepared from cells expressing target proteins encoded on modified pQE80-L plasmids (Table S1). Protein expression was achieved by addition of 1 mM IPTG. Cultures were labeled with **1** at the time of induction. Cells were lysed, and the lysates were treated with **2**. Western blot analysis against a C-terminal His_5 epitope tag with a primary antibody conjugated to Alexa Fluor 647 confirmed protein expression.

provide a measure of the sensitivity of the method, we compared the intensity of this band in the inverted grayscale image to the background grayscale intensity associated with the same region of a gel prepared from uninduced cells treated with **1** and **2** (Figure S2). For cells expressing Tar, CheA, FtsZ, and FtsA, we found band intensities 2.4-, 6.6-, 6.7-, and 5.7-fold above background, respectively. We verified conjugation of a single copy of **1** to each protein of interest by mass spectrometry (Figures S3–S6 and Tables S4–S7). In cells labeled with **1**, we observed complete modification of each target protein; the unmodified forms were not observed. CheA was found in its phosphorylated form[25] in the absence of **1**. In cells expressing NMT and FtsZ or FtsA but not treated with **1**, we observed modification by endogenous myristic acid;[26] endogenous myristoylation was not detected for Tar or CheA.

We screened a range of concentrations of **1** and **2** in cellular labeling experiments (Figure S7); concentrations of 250 μM **1** and 20 μM **2** were found to be sufficient. At these concentrations, we observed no effect on cell growth (Figure S8). We found no chain length redistribution of **1** in cells (Figure S9), but we did find evidence for incorporation of **1** into the phosphatidylethanolamine fraction of cellular lipids (Figures S10–S12). Although reaction of **2** with modified membrane lipids might be expected to contribute to diffuse background labeling in imaging experiments, we did not encounter problems of this kind.

For in situ imaging experiments, target proteins were outfitted with C-terminal myc tags for immunofluorescence labeling and expressed from modified pBAD24 plasmids (Table S2) under control of the tightly regulated P_{BAD} promoter[27] (Figure S13). Cells were treated with 0.2% (w/v) arabinose and 250 μM **1** at 37 °C for 2 h, fixed with 4% paraformaldehyde, and permeabilized with 0.3% (v/v) Triton X-100. Permeabilized cells were incubated with 20 μM **2** and an anti-myc antibody conjugated to Alexa Fluor 647 for two-color fluorescence labeling. In fluorescence confocal microscopy, cells were excited at 488 nm and the emission was monitored from 505 to 550 nm for detection of the BODIPY fluorophore. The same cells were excited at 633 nm and monitored at 650 nm for immunofluorescence detection. We found clear evidence of polar localization for the bacterial chemotaxis proteins Tar and CheA and septal localization for the cell division proteins FtsZ and FtsA (Figure 3). Control experi-

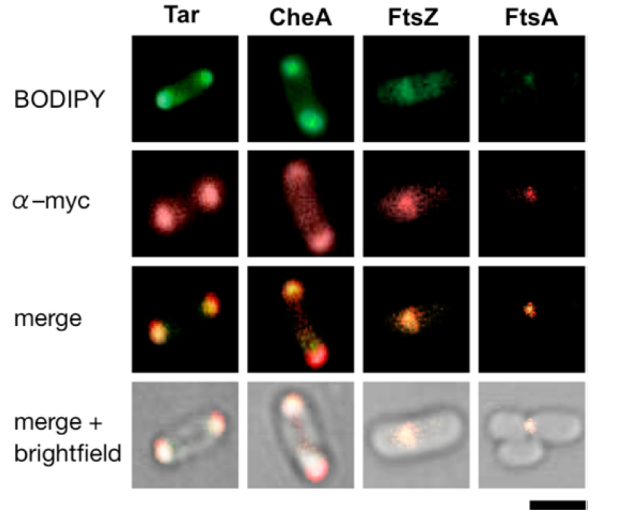

Figure 3. Imaging of proteins in fixed cells. Polar localization of Tar and CheA can be visualized by N-terminal fluorescence labeling with **2** and by immunofluorescence. Septal localization of FtsZ and FtsA can be visualized in similar fashion (scale bar = 1 μm).

ments confirmed that addition of the hydrophobic fatty acid **1** did not interfere with localization of the target proteins, nor was there significant interference from labeling of **1** in the absence of induction of the target protein (Figures S14 and S15).

We then asked whether the methods introduced here could be used for protein imaging in live cells. Cells harboring both pHV738-NMT-MetAP and one of the modified pBAD24 plasmids for inducible expression of target proteins (Table S2) were treated with 250 μM **1** at the time of induction and then grown at 37 °C for 2 h. Cells were collected by centrifugation, resuspended to an OD_{600} of 2, and rinsed three times with PBS. A 100 μL aliquot of the cell suspension was treated with 20 μM **2** at 37 °C for 30 min in the dark, and the cells were washed three times with PBS and mounted on agarose slides for imaging. When we labeled cells with **2** without fixation or permeabilization, we again found clear evidence of polar localization of each of the chemotaxis proteins and septal localization of each of the cell division proteins (Figure 4). Analysis of fluorescence from cells expressing Tar, CheA, FtsZ, and FtsA showed labeling 2.7-, 9.8-, 10.8-, and 7.1-fold above

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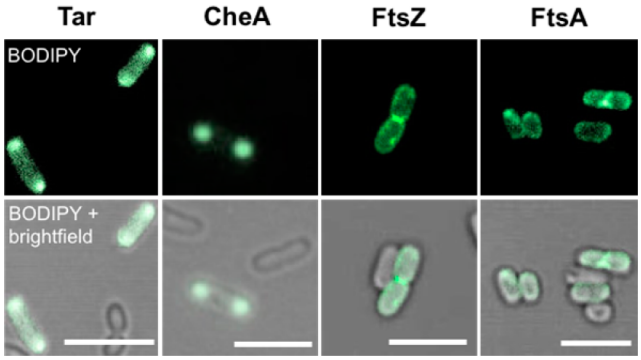

Figure 4. Live-cell images for chemotaxis and cell division proteins labeled with **1** and **2** (scale bars = 2 μm).

background, respectively (Figure S16). Labeling of FtsZ was also observed at lower concentrations of arabinose (Figure S17). In control experiments, cells did not show significant fluorescence in the absence of expression of the target protein or treatment with **1** (Figure S18). These results suggest that the NMT labeling system and fluorophore **2** should be broadly useful for live-cell imaging.

Finally, we asked whether both the *E. coli* FACS[28] and NMT could process other fatty acid substrates and expand the toolbox of probes for protein imaging. To demonstrate the feasibility of using alternative fatty acids, we prepared the more hydrophilic fatty acid **3**. Analysis of crude bacterial lysates prepared from cells treated with **2** and **3** confirmed selective modification of Tar, CheA, FtsZ, and FtsA (Figures S19–S21). The extent of modification by **3** was determined by mass spectrometry (Figures S22–S25 and Tables S4–S7). Addition of **3** did not affect cell growth (Figure S8). Degradation of **3** in cells was not observed (Figure S9), and **3** did not perturb the lipidome (Figure S26).

Live cells labeled with **3** yielded clear evidence of polar localization for Tar and CheA as well as septal localization for FtsZ and FtsA (Figure S27); the extent of labeling above background was similar to that observed with **1** (Figure S16). These results, along with the known promiscuity of NMT, suggest that other fatty acids bearing bioorthogonal functionality might be useful for protein labeling and imaging in live bacterial cells.

The results reported here demonstrate the feasibility of using NMT to modify membrane-associated and cytosolic bacterial proteins for fluorescence imaging. Although the method is unlikely to be applicable to proteins that bear N-terminal localization signals (roughly 10% of the *E. coli* proteome),[29] it may be especially useful in experiments in which fusion to a fluorescent protein disrupts localization of the protein of interest. With the advent of super-resolution imaging in bacterial systems,[30] we anticipate that the methods described here may be useful for conjugation of bright, photoswitchable fluorophores to bacterial proteins. We are currently working to combine the NMT labeling system with super-resolution techniques for live-cell protein imaging in bacteria.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b07067.

Experimental details, synthesis of probes **1**–**3**, mass spectrometry data, and additional microscopy images (PDF)

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Notes

The authors declare no competing financial interest.

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