

Chapter 2

Cell Surface Labeling of *Escherichia coli* via Copper(I)-Catalyzed [3+2] Cycloaddition

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Introduction

The display of proteins and polypeptides on the surfaces of cells is an important technology that has been used extensively¹ for applications ranging from screening of antibody fragments^{2,3} to the creation of whole cell bioremediation agents.^{4,5} These approaches are powerful, but are limited by the fact that the proteins and peptides displayed on the cell surface are composed of just the canonical amino acids. Expansion of the range of chemical functionality available on the mammalian cell surface has been demonstrated elegantly by the Bertozzi group,^{6,7,8} and a recent paper from the Schultz laboratory has shown the addition of the ketone functional group to the surface of bacteria.⁹ In this report we demonstrate the display and selective modification of the azido functional group on the surface of *Escherichia coli*. The use of azidohomoalanine (**1**) as a methionine surrogate as well as its selective modification via the Staudinger ligation has been reported previously.¹⁰ Here we show that **1** can be metabolically incorporated into outer membrane protein C (OmpC), one of the abundant outer membrane porins of *E. coli*,¹¹ and that selective modification of the azide functional group can be realized via copper-mediated [3+2] azide-alkyne cycloaddition.¹² The utility of this chemistry in biological contexts has recently been established^{13,14} and thus inspired us to explore the prospects for labeling intact bacterial cells. The specificity of the modification was verified by Western blotting and flow cytometry.

Results and Discussion

Naturally occurring *E. coli* OmpC contains three methionine residues, and hence only three potential sites for replacement with azidohomoalanine. According to a

published model⁴ of *E. coli* OmpC, only one of these methionine sites is exposed to the extracellular milieu. To increase the number of sites for functionalization on the surface of the cell, six additional methionine residues were engineered into OmpC by site-directed mutagenesis. The residues chosen are all exposed on the outside of the cell according to the published model. When possible, residues with similar size and hydrophobic character were replaced with methionine.¹⁵ When expressed in media supplemented with **1**, the mutant protein was produced at a level comparable to that of the wild-type (Figure 1). Furthermore, the mutant OmpC is targeted to the outer membrane of the cell, even when expressed in media supplemented with **1**.

Cells expressing either wild-type OmpC or mutant OmpC containing azidohomoalanine (OmpC-AHA) were prepared by using a medium shift procedure as previously described.¹⁶ The cells were then washed and subjected to the [3+2] reaction with the biotinylated alkyne reagent **2** (Scheme 1). The concentrations of CuSO₄, tris(carboxyethyl)phosphine (TCEP) and triazole ligand **3** were ten-fold more dilute than in previous reports^{13,14} because of the low solubility of **3** in aqueous media. Cells expressing OmpC containing methionine (OmpC-met) and cells that were induced in media supplemented with only 19 amino acids (no methionine) were prepared and subjected to similar reaction conditions as controls. As demonstrated in Figure 1, functionalization is specific toward both wild-type OmpC-AHA and mutant OmpC-AHA; we cannot detect background staining of either recombinant OmpC-met or naturally-expressed OmpC in the Western blot. Furthermore, OmpC-AHA is the only band detected in the Western blot; although incorporation of **1** into other cellular proteins must occur, labeling of such proteins is not detected.

A series of optimization experiments was performed on the mutant in order to achieve balance between a high level of functionalization and maintenance of intact cells. Western blotting was performed to examine modulation of protein expression conditions and [3+2] reaction conditions. Among the factors tested were concentration of the inducer IPTG, concentration of the copper catalyst, reaction time, and addition of the triazole ligand **3**. Vigorous induction of OmpC-AHA (*i.e.*, addition of 1 mM IPTG) was found to be necessary for extensive cell-surface labeling, though biotinylation was still detectable when cells were induced at 0.1 mM IPTG. Decreasing the concentration of CuSO₄ to 1 μM or 10 μM resulted in a complete loss of biotinylation, while the reaction proceeded well with 100 μM or 1 mM CuSO₄. After one hour of reaction with cells induced at 1 mM IPTG and functionalized in 100 μM CuSO₄, essentially no biotinylation was observed by Western blotting. A small amount of biotinylation was observed after 2 h or 4 h, but extensive modification required overnight reaction (16 h). A recent paper¹⁴ reported that similar [3+2] reactions proceed to high yield without the triazole ligand **3**. However, when **3** was omitted from the whole cell reaction, essentially no biotinylation was detected by Western blotting. Through these experiments, we converged on optimal conditions for OmpC expression and functionalization. The conditions are as follows: induction at 1 mM IPTG and reaction with 100 μM CuSO₄ for 16 h at 4°C (see Supporting Information for details). These conditions were used to generate functionalized cells both in the Western blot experiments shown in Figure 1 and in the flow cytometry experiments described below.

Cells expressing either wild-type or mutant versions of OmpC-met or OmpC-AHA, and cells that were induced in the presence of all amino acids except methionine,

were biotinylated and subsequently stained with an avidin-Alexa Fluor 488 conjugate and subjected to flow cytometric analysis. Unlabeled cells were analyzed initially, and this population was placed in the first decade of the fluorescence channel by adjusting photomultiplier tube voltage. Cells expressing OmpC-met and cells induced in the presence of 19 amino acids have the same mean fluorescence as the unlabeled cells, indicating that there is essentially no non-specific staining of the cells by the avidin-Alexa Fluor conjugate. Given the Western blot data above, it was surprising that cells expressing wild-type OmpC-AHA yield only background fluorescence intensity (Figure 2). This result can be attributed to the fact the naturally occurring methionine in OmpC is sterically encumbered according to the published model.⁴ While it may be possible for azidohomoalanine at that position to be biotinylated, the residues surrounding the azidohomoalanine hinder binding of the bulky avidin molecule. In contrast to the wild-type OmpC, cells expressing the mutant OmpC-AHA display an approximately ten-fold increase in mean fluorescence over background (Figure 2). When cells expressing mutant OmpC-AHA are mixed with cells induced in the presence of 19 amino acids, cytometry reveals two populations with essentially baseline separation (Figure 2). Thus the mutant OmpC is a useful tool for detection of unnatural amino acid incorporation on an individual cell basis.¹⁷

The results reported here demonstrate that recombinant *E. coli* OmpC can be functionalized with azidohomoalanine and targeted to the outer membrane of the cell. We further illustrate that a cell surface that displays azide functionality can be chemoselectively modified with a biotin-bearing reagent by means of a copper-mediated azide-alkyne cycloaddition. Furthermore, after staining with fluorescent avidin, cells

incorporating the unnatural amino acid can be readily distinguished from cells lacking the unnatural amino acid. Such a flow cytometric assay may be used in a variety of applications, including screening for the *in vivo* incorporation of new, reactive unnatural amino acids. Such studies are currently underway in our laboratory.

Materials and Methods

Strains and Expression Plasmids. The strain M15MA was constructed using P1 phage-mediated generalized transduction. Briefly, P1 phage were used to infect strain CAG 18491 (Yale *E. coli* Genetic Stock Center), which contains the *metE::Tn10* mutation. The phage collected from infected CAG 18491 cells were used to transduce strain M15 (Qiagen Corp., Valencia, CA). A stabilization procedure¹⁸ was followed to eliminate the transposon and create a stable auxotroph. The gene encoding OmpC was amplified from M15MA genomic DNA using the following primers: OmpC for, 5'-CTGCGCCTGGTCTCACATGAAAGTTAAGTACTG-3' and OmpC rev, 5'-CCGAAGCTTTTATTAGAACTGGTAAACCGG-3'. The PCR product was sequentially digested with *Hind*III and *Bsa*I and subsequently ligated to pQE-60 (Qiagen), which was digested with *Nco*I and *Hind*III. The resulting plasmid, pQE-60 OmpC, was transformed into competent M15MA cells harboring the plasmid pREP4, generating the expression strain M15MA[pQE-60/OmpC].

Site-Directed Mutagenesis. Mutations introduced to OmpC to enrich its methionine content were as follows: V50M, N88M, T187M, A230M, L271M, and L306M. The

mutations were introduced using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's recommendations.

Synthesis of Azidohomoalanine and Biotin-PEG-Propargylamide.

Azidohomoalanine (**1**) was prepared as previously described.¹⁹ Biotin-PEO-propargylamide **2** was prepared by dissolving TFP-PEO-Biotin (32 mg, Pierce, Rockford, IL) in excess neat propargylamine. After 10 minutes, the solution was added dropwise to ethyl ether. A white precipitate formed and was collected by centrifugation. The precipitate was dried *in vacuo* yielding 22 mg (82 %) of **2**. This material was characterized by ¹H and COSY NMR to confirm the formation of an amide bond. ¹H NMR (CD₃OD): δ 1.38-1.50 (m, 2H), δ 1.51-1.88 (m, 8H), δ 2.19 (t, 4H, *J* = 7.3 Hz -CO-CH₂-CH₂-CO-), δ 2.46 (t, 2H, *J* = 2.7 Hz, -CH₂-CO-NH-) δ 2.58 (t, 1H, *J* = 2.7 Hz, -NH-CH₂-C≡C-H), δ 2.89-2.96 (dd, 2H, *J*₁ = 5.1 Hz, *J*₂ = 12.5 Hz, CH-CH₂-S), δ 3.17-3.28 (m, 5H), δ 3.44-3.69 (m, 12 H), δ 3.93 (d, 2H, *J* = 2.7 Hz, -NH-CH₂-C≡C-H), δ 4.27-4.35 (dd, 1H, *J*₁ = 5.1 Hz, *J*₂ = 7.9 Hz, -HN-CH-CH₂-), δ 4.46-4.54 (dd, 1H, *J*₁ = 4.5 Hz, *J*₂ = 7.9 Hz, -HN-CH-CH-).

Metabolic Incorporation of Azidohomoalanine into OmpC. A single colony of M15MA[pQE-60/OmpC] was grown overnight in M9 minimal medium supplemented with all twenty natural amino acids and the antibiotics ampicillin and kanamycin. A small amount of this starter culture was added to fresh M9 medium supplemented with all of the natural amino acids. Upon reaching an OD₆₀₀ of 1, the cells were pelleted and resuspended in M9 medium containing all natural amino acids except for methionine.

After agitation at 37 °C for 10 minutes, the cells were pelleted again and resuspended in M9 medium with 19 amino acids (no methionine). The cells were divided into three equal portions: one for methionine, one for azidohomoalanine, and one for no analog. Methionine and azidohomoalanine were added to the appropriate cultures at a concentration of 40 mg/L. Isopropyl- β -D-thiogalactoside (IPTG) was added to a final concentration of 1 mM to induce expression of OmpC. Induction at 37 °C was for three hours.

[3+2] Cycloaddition Chemistry on Whole Cells. Cells producing recombinant OmpC (1 mL culture) were pelleted and washed once with PBS (pH 7.4) and resuspended in 1 mL PBS. The [3+2] cycloaddition conditions were essentially as described previously.¹³ Specifically, CuSO₄ was added to a final concentration of 100 μ M while TCEP and the triazole ligand were added to a final concentration of 200 μ M. The final concentration of biotin-PEO-propargylamide was 50 μ M. Given that each cell expresses $\sim 10^5$ copies of OmpC,⁴ and the reaction was performed on $\sim 10^9$ cells in a volume of 1 mL, this concentration of biotin-PEO-propargylamide represents approximately a 100-fold excess of alkyne to azide. The reaction was allowed to proceed for 16 h at 4 °C with agitation. At the conclusion of the reaction, the cells were pelleted and washed twice with PBS to remove excess reagents.

Purification of Outer Membrane Proteins and Western Blotting. The outer membrane protein fraction of cells functionalized with biotin-PEO-propargylamide was prepared as previously described.⁴ Purified outer membrane fractions were

electrophoresed (12 % tris-tricine gel, 150 V) and transferred to a nitrocellulose membrane (30 V, 1 h at 4 °C). The membrane was blocked with a 5 % milk solution in PBS/Tween for 1 h. Following washing, the membrane was probed with an avidin-HRP conjugate (Amersham Biosciences, Piscataway, NJ), and the bands were visualized with detection reagents (Amersham Biosciences).

Flow Cytometry. Analysis of cells by flow cytometry was carried out on a DakoCytomation MoFlo cell sorter (Fort Collins, CO) equipped with a 488 nm laser. Cells functionalized with biotin-PEO-propargylamide were incubated with an avidin-Alexa Fluor 488 conjugate (Molecular Probes, Eugene, OR) for two hours at 4 °C. After avidin staining, the cells were washed three times with PBS and diluted to a density of approximately 2×10^7 cells/mL. Between 20,000 and 50,000 events were gathered in each experiment. The data was analyzed using Summit Software (DakoCytomation, Ft. Collins, CO).

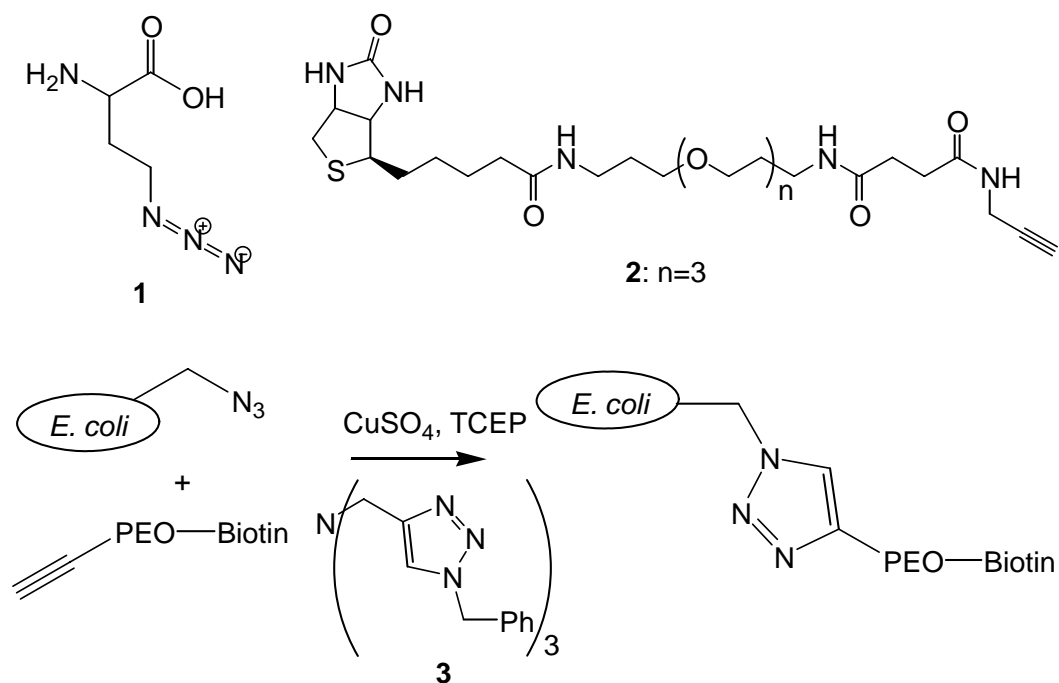
Acknowledgements

We thank K. Barry Sharpless and Valery Fokin for their gift of the triazole ligand and for helpful advice on the azide-alkyne cycloaddition. We also thank Isaac Carrico, Pin Wang, and Rochelle Diamond for helpful discussions and Rich Roberts for use of his flow cytometer. This work was supported by the NSF Center for the Science and Engineering of Materials at Caltech. A.J.L. is an NSF Graduate Research Fellow.

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- (15) The mutations introduced were V50M, N88M, T187M, A230M, L271M, and L306M. The exposed methionine in wild-type OmpC is at residue 142.
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Scheme 1: Structure of azidohomoalanine **1** and biotin-PEO propargylamide **2**. Biotinylation reaction of whole *E. coli* via [3+2] Cu-mediated azide-alkyne cycloaddition.

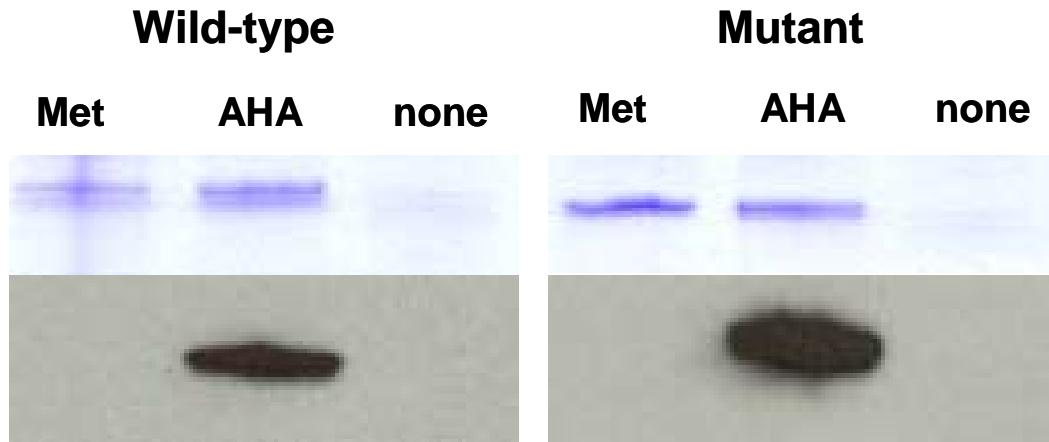


Figure 1: Coomassie-blue stained gel (top) and Western blot (bottom) of outer membrane fractions after [3+2] biotinylation. Met: OmpC containing methionine, AHA: OmpC containing azidohomoalanine, none: OmpC produced when cells are induced in the presence of 19 amino acids (no methionine). The amount of protein expressed and targeted to the outer membrane is similar for cells expressing OmpC-met and OmpC-AHA. Only OmpC-AHA is detected by avidin-HRP for both the wild-type and mutant proteins.

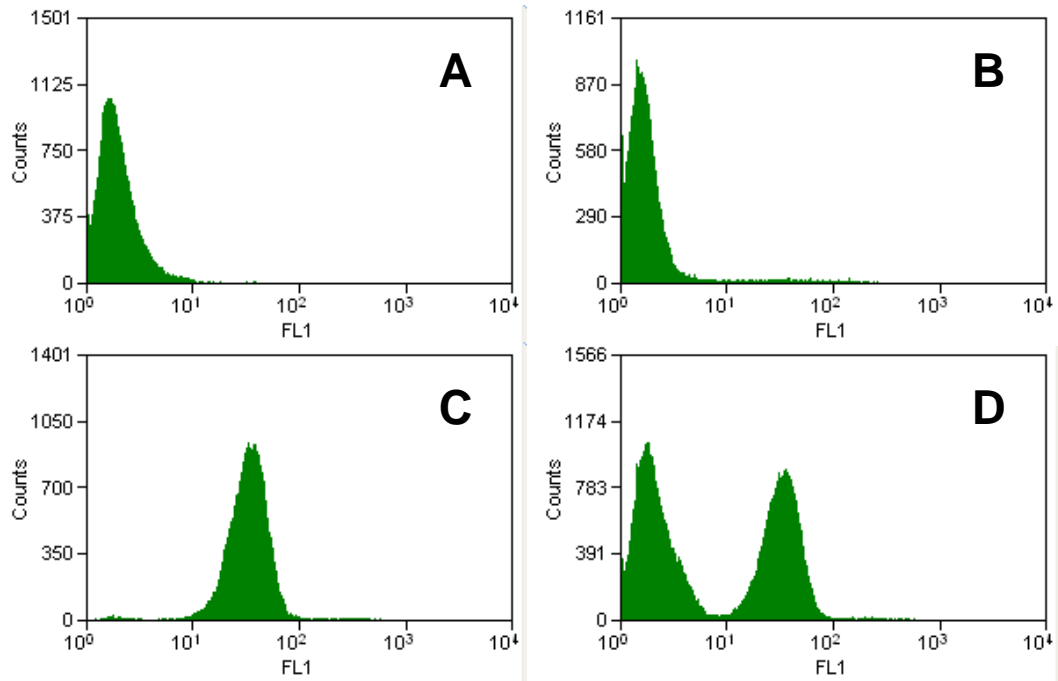


Figure 2: Flow cytometry data from A: mutant cells bearing OmpC-met, B: wild-type cells bearing OmpC-AHA, C: mutant cells bearing OmpC-AHA, D: a mixed population of mutant cells bearing OmpC-AHA and cells induced in medium supplemented with only 19 amino acids.