Electronic Supplementary Material (ESI) for Chemical Communications

Supplementary Information

Cell-selective labeling of bacterial proteomes with an orthogonal phenylalanine amino acid reporter

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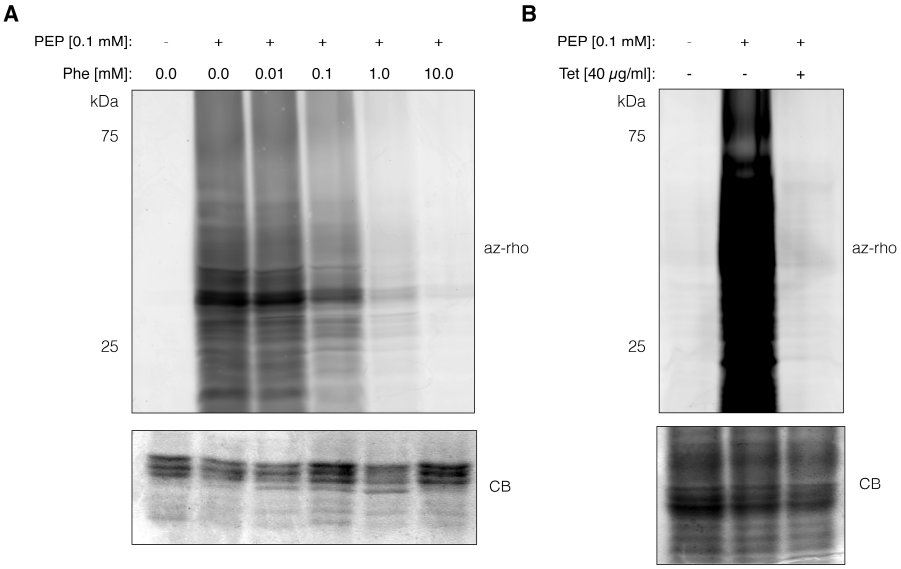
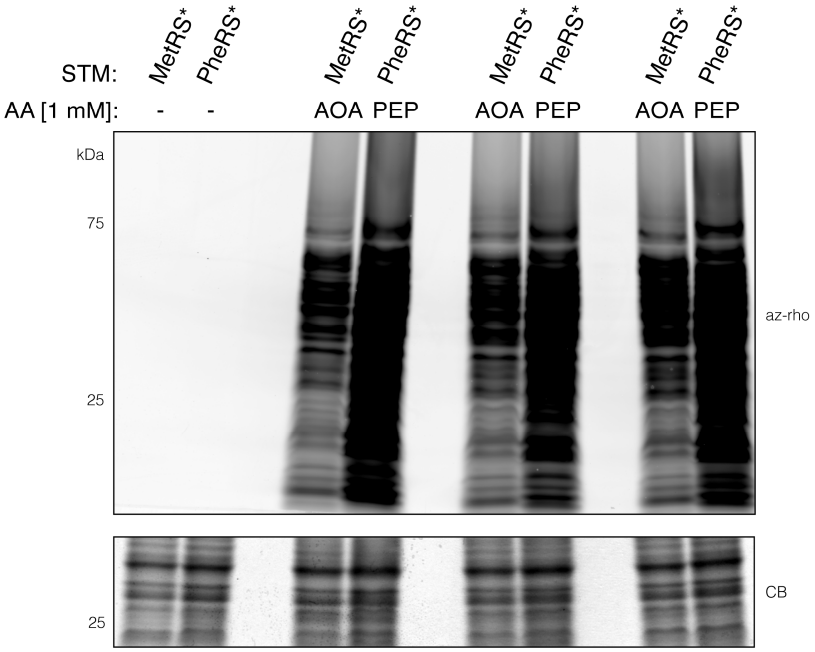
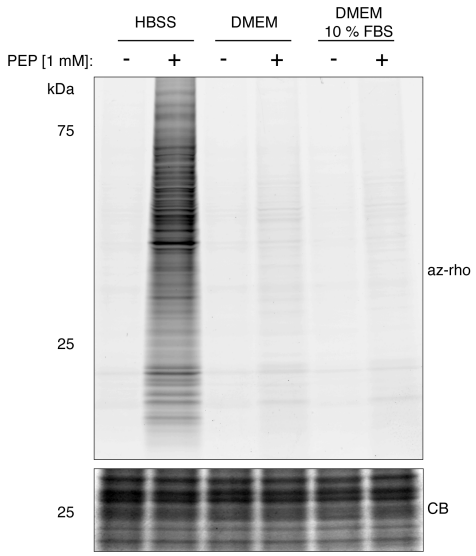


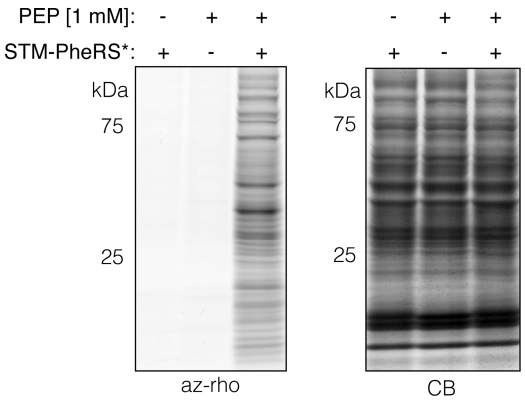
Suppl. Fig. 1 A) In-gel fluorescence analysis of phenylalanine (Phe) competition with para-ethynylphenylalanine (PEP). B) In-gel fluorescence analysis of tetracycline inhibition of PEP incorporation. az-rho: azido-rhodamine fluorescence, CB: coomassie blue, Tet: tetracycline.



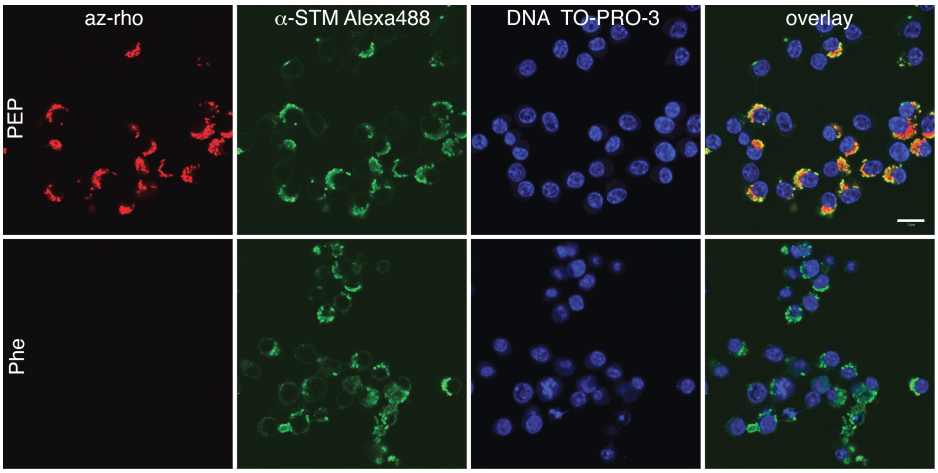
Suppl. Fig. 2 In-gel fluorescence analysis of 3 replicates of AOA and PEP labeling of *Salmonella typhimurium* in liquid culture. AA: amino acid, STM: *Salmonella typhimurium*, MetRS*: mutant methionyl-tRNA synthetase (NLL)[1], PheRS*: phenylalanyl-tRNA synthetase (A294G), az-rho: azido-rhodamine fluorescence, CB: coomassie blue, AOA: aminooctynoic acid, PEP: para-ethynylphenylalanine.



Suppl. Fig. 3 In-gel fluorescence analysis of para-ethynylphenylalanine (PEP) labeling of RAW264.7 murine macrophages. HBSS: Hank's buffered saline solution, DMEM: Dulbecco's Modified Eagle Medium, FBS: fetal bovine serum, az-rho: azido-rhodamine fluorescence, CB: coomassie blue.



Suppl. Fig. 4 In-gel fluorescence analysis of PEP-labeled STM-PheRS*-infected Raw264.7 murine macrophage cell lysates. PEP: para-ethynylphenylalanine, STM-PheRS*: *S. typhimurium* expressing A294G *E. coli* alpha chain of phenylalanyl-tRNA synthetase, az-rho: azido-rhodamine fluorescence, CB: coomassie blue.



Suppl. Fig. 5 Fluorescence microscopy of PEP-labeled STM-PheRS*-infected Raw264.7 murine macrophages. PEP: para-ethynylphenylalanine, STM-PheRS*: *S. typhimurium* expressing A294G *E. coli* alpha chain of phenylalanyl-tRNA synthetase, Phe: phenylalanine, az-rho: azido-rhodamine fluorescence (red), α-STM Alexa488: anti-*S. typhimurium* antibody stain visualized with secondary Alexa488-conjugated antibody (green), DNA TO-PRO-3: DNA stain (blue), scale bar indicates 10 µm.

Materials and Methods

All chemical reagents were purchased from Sigma-Aldrich. Azido-rhodamine (az-rho), alkne-rhodamine (alk-rho), and TBTA (tris((1-benzyl-1H-1,2,3-triazol-4-yl)methyl) amine) were generated by the Hang laboratory as previously described.[2] PEP was generated as previously described.[3]

Plasmids and Bacterial strains

Salmonella typhimurium IR715 was transformed with pWSK29 encoding the A294G mutant of the *E. coli pheS* gene. *PheS* was amplified from *E. Coli* K12 by PCR (GAGCTCCATGTCACATCTCGCAGAACT-GG, GGTACCCGCATCGCTATCAATCGCC) and sub-cloned into pJET1.2. The resulting fragment contained a mutation relative to the reported UniProt sequence (P08312, D258N, GAC to AAC), which was re-mutated to the reference sequence together with the introduction of mutation (A294G) by use of the QuickChange Multi Site-Directed Mutagenesis Kit (Stratagene, gaaccttctgcagaagtggacgtcatgggtaaaaacg, gtttactctggtttcggcttcgggatggggatg). The fragment was excised from pJET1.2 and inserted into pWSK29 with SacI and KpnI.

Bacterial pulse labeling *in vitro*

Overnight cultures of bacteria were grown in full LB with 100 µg/ml Carbenicillin (Cb). These cultures were then diluted 1:30 into fresh LB (Cb) and grown to stationary phase. Bacteria were collected by centrifugation (10,000 rpm, 3 min) and resuspended in M9 (1x M9 salts, 20 mM glucose, 2 mM $MgSO_4$, 100 µM $CaCl_2$) minimal medium (Cb). Bacteria were grown in minimal medium for 15 min and collected by centrifugation. Bacterial pellets corresponding to 1 ml of culture volume were resuspended in M9 minimal medium (Cb) containing the chemical reporter. Bacteria were labeled for 1 hour and collected by centrifugation, washed once in PBS, and stored at -80°C. For tetracycline (Tet) protein synthesis inhibition, bacteria were grown for 30 min in LB (40 µg/ml Tet) before being labeled for 30 min in M9 minimal medium (40 µg/ml Tet).

Infection of mammalian cells and pulse labeling

For fluorescence microscopy experiments, $300x10^3$ RAW264.7 cells were split into 12 well plates 24 h before infection on round glass cover slips. For subsequent cell lysis and in-gel fluorescence experiments, $1.5x10^6$ RAW264.7 cells were split into 6 well plates 24 h before infection. An LB overnight culture of *Salmonella* was diluted 1:30 into fresh LB with 100 µg/ml carbenicillin and grown to stationary phase (OD600 > 2.0). Bacteria were collected by centrifugation and washed once with cold PBS. For a multiplicity of infection (MOI) of 100, an appropriate volume of bacteria in PBS was added to DMEM (10% FBS) and put on ice for 30 min. Bacteria in DMEM (10% FBS) were added to RAW264.7 cells and centrifuged at 1000g for 5 min at 4°C. The infection was allowed to proceed for 25 min at 37°C at 5% CO_2. The wells were then washed 4 times with PBS before the cells were covered in DMEM (10% FBS) with 100 µg/ml gentamicin and incubated or 90 min at 37°C at 5% CO_2 to kill all extracellular bacteria. Subsequently, wells were washed 2 times with PBS and filled with DMEM (10% FBS) with 12.5 µg/ml gentamicin. The cells were further incubated for 14.5 h at 37°C at 5% CO_2. The cells were pulse labeled with 1 mM PEP in full medium (DMEM 10% FBS) for 1 h at 37°C at 5% CO_2. After 1 h the cells were washed 4 times with cold PBS.

Cell lysis, click-chemistry, and in-gel fluorescence analysis

For cell lysis, infected RAW264.7 cells were collected by scraping in ice-cold PBS and centrifugation at 1000g for 5 min at 4°C. Cell pellets were lysed by resuspending them in 0.1% SDS in PBS with Roche complete protease inhibitor, 1 mM PMSF, and 1 µl benzonase/ml (Sigma E1014), and then sonicating them for 30 s in a water bath sonicator, followed by 10 min incubation on ice. Samples were filled up with 12% SDS in PBS to a final concentration of 4% SDS and sonicated again. Then, remaining debris was removed by centrifugation at 20,000g for 10 min at room temperature. Protein concentrations were determined by BSA assay (Pierce). For click-chemistry, 50 µg of cell lysate from each sample was filled up to the smallest

common volume with 4% SDS in PBS. A click chemistry master mix was prepared and added to each sample:

Reagent	Stock Concentration	Comments	Final Concentration
Azido-rhodamine	10 mM	Stock solution in DMSO	100 µM
TCEP	50 mM	Freshly prepared in water	1 mM
TBTA	2 mM	Stock solution in 4:1 n-butanol:DMSO	100 µM
$CuSO_4$	50 mM	Freshly prepared in water	1 mM

TCEP - tris(2-carboxyethyl)phosphine, TBTA - tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine

The components were added in the order listed above to the master mix and vigorously vortexed. Then the master mix was added to each individual protein sample to yield a final volume of 50 µl at the final concentrations indicated. The samples were incubated for 1 h at room temperature.

The samples were precipitated after click chemistry to remove small molecules. For this purpose, 50 µl of water was added to each sample, followed by 400 µl of methanol, and 100 µl of chloroform. The samples were vigorously vortexed and 300 µl of water was added. The samples were vortexed again and centrifuged at 20,000g for 5 min at room temperature. The upper phase was removed carefully and 1 ml of ice-cold aetone was added. The samples were vortexed and centrifuged at 20,000 g for 15 min at 4°C. The organic supernatant was discarded and the samples briefly dried on air. Then, the samples were resuspended in 30 µl 4% SDS in PBS and briefly sonicated. 10 µl of 4 times Laemmli buffer was added to each sample together with 2 µl of beta-mercaptoethanol, samples were incubated at 95 °C for 5 min, and run for analysis on a 4-20% Tris-HCl Criterion™ Precast Gel (Biorad). Protein gels were rinsed with deionized water and incubated for multiple hours in 50% water, 40% methanol, 10% acetic acid at 4°C. Before in-gel fluorescence analysis, protein gels were transferred to deionized water and incubated for at least 30 min at room temperature. Protein gels were scanned on an Amersham Bioscience Typhoon 9400 variable mode imager (excitation 532 nm, 580 nm filter, 30 nm bancd-pass). All image adjustments were done on the entire depicted image for all samples equally in Adobe® Photoshop® CS3. After fluorescence scanning, protein gels were stained with GelCode Blue Stain Reagent (Thermo) or Coomassie Brilliant Blue (BioRad). Both stains are indicated as coomassie blue (CB) in all figures.

Sample preparation for fluorescence microscopy

For fluorescence microscopy, cells were fixed and permeabilized in 3.7% paraformaldehyde, 0.2% Triton X-100 in PBS for 15 min at room temperature. The cells were then washed at least 4 times for 5 min each with PBS. A click-chemistry master mix was prepared from stock solutions listed above to yield the following final concentrations in PBS (20 µM az-rho, 1 mM TCEP, 100 µM TBTA, 1 mM $CuSO_4$). The cells were covered in click chemistry master mix and incubated for 1 h at room temperature. Then, the cells were blocked for 1 h with 2% FBS, 1% Tween 20 in PBS at room temperature. The cells were washed once with PBS and anti-*S. typhimurium* serum was added (1:250) in 0.2% saponin in PBS for 30 min at room temperature. The cells were washed 4 times with PBS and subsequently incubated with anti-rabbit Alexa488-conjugated antibody in 0.2% saponin in PBS for 30 min at room temperature. Cells were washed in PBS and finally incubated with TO-PRO-3 DNA stain (1:1000) in 0.2 % saponin in PBS. The cells were washed once with 0.2% saponin in PBS and 4 times in PBS before mounting on microscope slides in ProLong Gold (Invitrogen). The images were taken on an upright LSM510 laser scanning confocal microscope (Zeiss) equipped with a Krypton/Argon laser with 488 and 568 lines and a HeNe laser with a 633 line, using a 100x oil immersion objective. .

References:

1. M. Grammel, M. M. Zhang, and H. C. Hang, *Angew. Chem. Int. Ed. Engl*, 2010, **49**, 5970-5974.
2. G. Charron, M. M. Zhang, J. S. Yount, J. Wilson, A. S. Raghavan, E. Shamir, and H. C. Hang, *J. Am. Chem. Soc*, 2009, **131**, 4967-4975.
3. B. Kayser, J. Altman, and W. Beck, *Tetrahedron*, 1997, **53**, 2475–2484.