Protein Identification

DOI: 10.1002/anie.201002050

Orthogonal Alkynyl Amino Acid Reporter for Selective Labeling of Bacterial Proteomes during Infection**

*Markus Grammel, Mingzi M. Zhang, and Howard C. Hang**

Bacterial pathogens have evolved sophisticated mechanisms to evade host defenses and cause disease.[1] The emergence of new and antibiotic-resistant bacterial pathogens demands a better understanding of virulence mechanisms for antibacterial-drug discovery. Although the discovery of bacterial toxins, quorum sensing, and protein-secretion pathways has revealed some key virulence mechanisms, the precise mechanisms by which intracellular bacterial pathogens subvert host immune responses are still unclear.[1] The analysis of individual virulence factors has demonstrated that bacterial pathogens alter their protein expression to infect and replicate in host tissues.[2] However, the system-wide identification and analysis of bacterial proteins that are uniquely expressed or secreted during infection is paramount for understanding mechanisms of bacterial pathogenesis.[2,3] Comparative genomics and mutagenesis studies have revealed bacterial genes that are important for infection, but their precise biochemical mechanisms and temporal expression patterns can be elusive as a result of posttranscriptional regulation.[2,3] Direct biochemical analysis of bacterial proteomes during infection is needed.[3] The large excess of host proteins in mixed pathogen–host lysates presents a significant challenge for proteomic analysis of bacterial proteins during infections,[3] and even after the physical isolation of intact bacteria, significant amounts of host proteins still remain.[4,5] This factor is particularly important since many bacterial virulence factors are often expressed at low levels.[2] New strategies are therefore required to selectively enrich bacterial proteins from host proteomes for their analysis during infection.

The incorporation of nonnatural amino acids in bacteria has provided new methods to differentiate bacterial proteins from host proteomes. For example, the incorporation of phenylalanine analogues in mycobacteria by amber-stop-codon-suppression technology has enabled the selective labeling of green fluorescent protein expressed in *Mycobacterium tuberculosis* during the intracellular infection of macrophages.[6] Alternatively, alkyne- or azide-functionalized methionine (Met) surrogates can be incorporated by the endogenous methionyl-tRNA synthetase (MetRS) into bacterial proteomes.[7,8] These amino acid chemical reporters enable the metabolic labeling and detection of newly synthesized proteins using bioorthogonal ligation methods, ssuch as the copper(I)-catalyzed azide–alkyne cycloaddition.[10] Additionally, MetRS mutants have been identified that can incorporate azidonorleucine (ANL, Figure 1 a),[11,12] a Met surrogate that is not efficiently activated by the wild-type (wt) MetRS or other endogenous aminoacyl-tRNA synthetases.

ANL can therefore be used as an orthogonal amino acid reporter to selectively label proteins in nonpathogenic *Escherichia coli* by the bacterial expression of MetRS mutants in the presence of mammalian cells.[13] Although these studies have demonstrated the selective targeting of bacterial proteomes in the presence of host cells,[6,13] the use of nonnatural amino acid reporters for the analysis of endogenously expressed bacterial proteins during infection has not been reported. Herein, we report a new orthogonal alkynyl amino acid reporter for the specific imaging and enrichment of bacterial proteomes during the infection of mammalian cells with the Gram-negative intracellular bacterial pathogen *Salmonella typhimurium* (Figure 1 b).

On the basis of the reported superior selectivity of azide- over alkyne-functionalized secondary CuAAC reagents[14] and our own experience with fatty-acid chemical reporters, we evaluated whether an alkynyl isostere of ANL, 2-amino-octynoic acid (AOA, Figure 1 a), could be accepted by previously reported MetRS mutants in *S. typhimurium*.[13,15,16] MetRS mutants were generated from the *E. coli metG* gene (the *S. typhimurium metG* gene is 95% identical to *E. coli metG*) by site-directed mutagenesis and ligated into the low-copy-number plasmid pWSK29 under the expression control of the *lac* promoter, which provides constitutive expression in *S. typhimurium*. All plasmids were transformed into the *S. typhimurium* strain IR715. AOA was synthesized by the alkylation of diethyl acetamidomalonate with hex-5-ynyl-4-methylbenzenesulfonate and sequentially deprotected to yield the racemic product (see Scheme 1 in the Supporting Information). For the in vitro analysis of the *S. typhimurium* strains expressing MetRS mutants, bacteria were grown in full Luria–Bertani (LB) medium to the stationary phase and diluted into minimal medium containing Met or AOA. Protein lysates were reacted with the azido-rhodamine (az-Rho)[15] detection tag by CuAAC and analyzed by SDS-PAGE and in-gel fluorescence scanning. AOA selectively and

[*] M. Grammel, M. M. Zhang, Dr. H. C. Hang
Laboratory of Chemical Biology and Microbial Pathogenesis
The Rockefeller University
1230 York Avenue, New York, NY 10065 (USA)
Fax: (+1) 212-327-7276
E-mail: hhang@rockefeller.edu

[**] We thank The Rockefeller University Proteomics Resource Center for mass spectrometry analysis, James Flexner for help with the azidonorleucine synthesis, and members of the Hang Laboratory for providing secondary detection reagents. M.M.Z. is supported by A*STAR, Singapore. H.C.H. acknowledges support from The Rockefeller University, the Irma T. Hirschl/Monique Weill-Caulier Trust, the Lerner Trust, and the Northeastern Biodefense Center NIH/NIAID (2 U54 AI057158-06).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201002050.

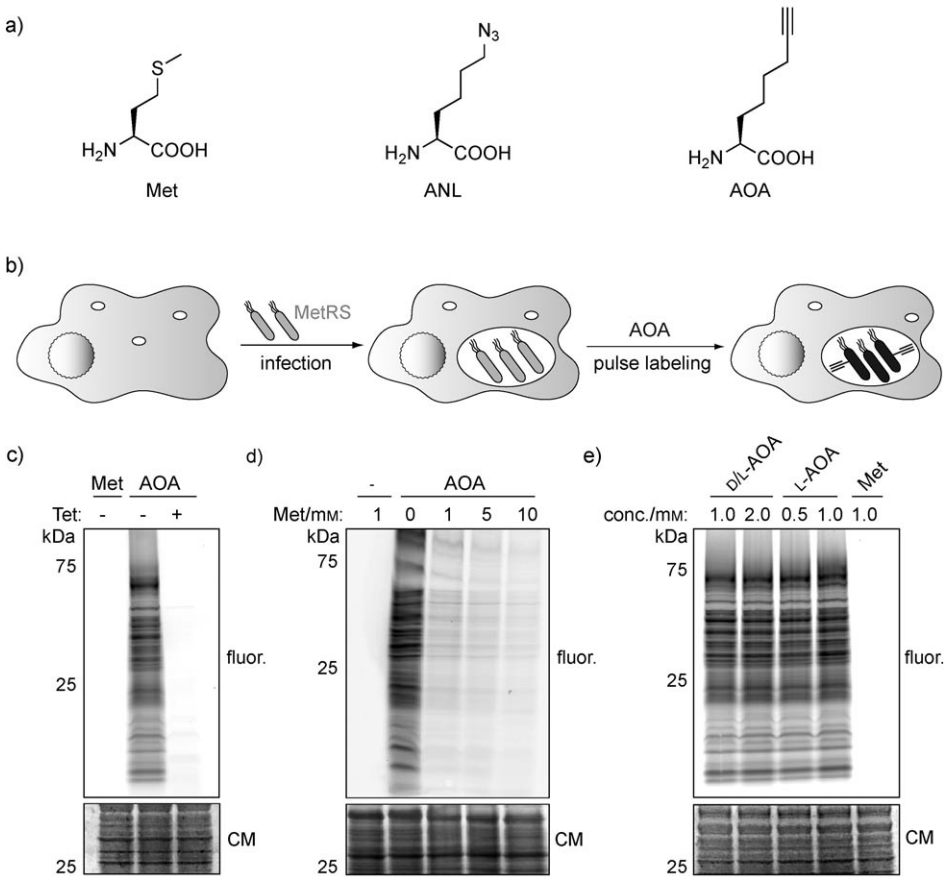

Figure 1. Selective labeling of bacterial proteomes with orthogonal amino acid reporters during infection. a) Structures of methionine (Met), azidonorleucine (ANL), and 2-aminooctynoic acid (AOA). b) Infection of host cells with bacterial pathogens expressing a mutant methionyl-tRNA synthetase (MetRS), followed by pulse labeling with the orthogonal amino acid reporter AOA, enables the specific imaging and proteomic analysis of bacterial proteomes by using CuAAC or "click chemistry". c) MetRS-NLL *S. typhimurium* was treated with Met (1 mM) or AOA (2 mM), in the presence or absence of tetracycline, for 30 min. The cell lysates were subjected to CuAAC with az-Rho and analyzed by in-gel fluorescence. d) Competition experiment with Met for labeling with AOA (2 mM). e) Comparison of the labeling efficiency of D/L-AOA and L-AOA. CM = Coomassie; "fluor." indicates in-gel fluorescence scanning.

efficiently labeled proteins in *S. typhimurium* expressing the MetRS-NLL mutant (L13N-Y260L-H301L; Figure 1c).

To confirm the selectivity of AOA for newly synthesized Met-containing proteins, we conducted protein synthesis inhibition and Met-competition experiments. The pre- and coincubation of MetRS-NLL *S. typhimurium* with the protein synthesis inhibitor tetracycline (Tet) effectively abolished AOA labeling (Figure 1c), and Met competed efficiently against AOA incorporation in a dose-dependent manner (Figure 1d).

Since the synthetic route to AOA results in a racemic mixture, we investigated whether the presence of the D-AOA isomer in the racemate impairs *S. typhimurium* labeling. Pig acylase I was used for the kinetic resolution of the racemic mixture of AOA[17] to afford enantiomerically pure L-AOA (see Figure 1 in the Supporting Information). The activity of racemic AOA at a 2 mM concentration was compared to that of 1 mM L-AOA. Since both preparations showed identical labeling efficiencies at comparable effective concentrations (Figure 1e), we decided to use the racemic AOA preparation for subsequent experiments. These results demonstrate that AOA can selectively label newly synthesized Met-containing proteins in Gram-negative bacterial pathogens, such as MetRS-NLL *S. typhimurium*.

To determine the most efficient combination of MetRS mutant and orthogonal amino acid reporter for the selective labeling of bacterial proteomes during the infection of host cells, we evaluated other reported MetRS mutants with ANL and AOA in *Salmonella*. We generated three previously reported MetRS mutants with high activation efficiency for ANL to evaluate their utility for the metabolic labeling of *S. typhimurium* proteins with orthogonal amino acid reporters: L13G (MetRS-L13G), L13N-Y260L-H301L (MetRS-NLL), and L13P-Y260L-H301L (MetRS-PLL).[11] All three MetRS mutants permit the incorporation of ANL and AOA into *Salmonella* proteomes (Figure 2a). Neither ANL nor AOA were greatly incorporated into *Salmonella* transformed with control plasmid or overexpressing wt MetG. However, AOA displayed some background incorporation by the endogenous protein translation machinery (Figure 2a). The expression levels of all MetRS mutants were comparable, as judged by S-tag western blot analysis of the epitope tag fused to all MetRS constructs (see Figure 2 in the Supporting Information). Both triple mutants (NLL, PLL) enabled more efficient incorporation of ANL and AOA over the single mutant (L13G). Furthermore, AOA showed superior signal-to-noise ratios relative to labeling with ANL for all three MetRS mutants (see Figure 3a in the Supporting Information). These results demonstrate that both orthogonal amino acid reporters function in *Salmonella* expressing MetRS mutants, but MetRS-NLL in combination with AOA affords the optimal orthogonal enzyme–substrate pair for the metabolic labeling of *Salmonella* proteins.

We then evaluated the efficiency of the ANL and AOA labeling of *S. typhimurium* proteomes during the intracellular infection of mammalian cells. To analyze the selective incorporation of orthogonal amino acid reporters in intracellular bacterial pathogens, we infected Raw264.7 murine macrophages with MetRS-NLL *S. typhimurium* at a multiplicity of infection (MOI) of 100 for 30 min. Following

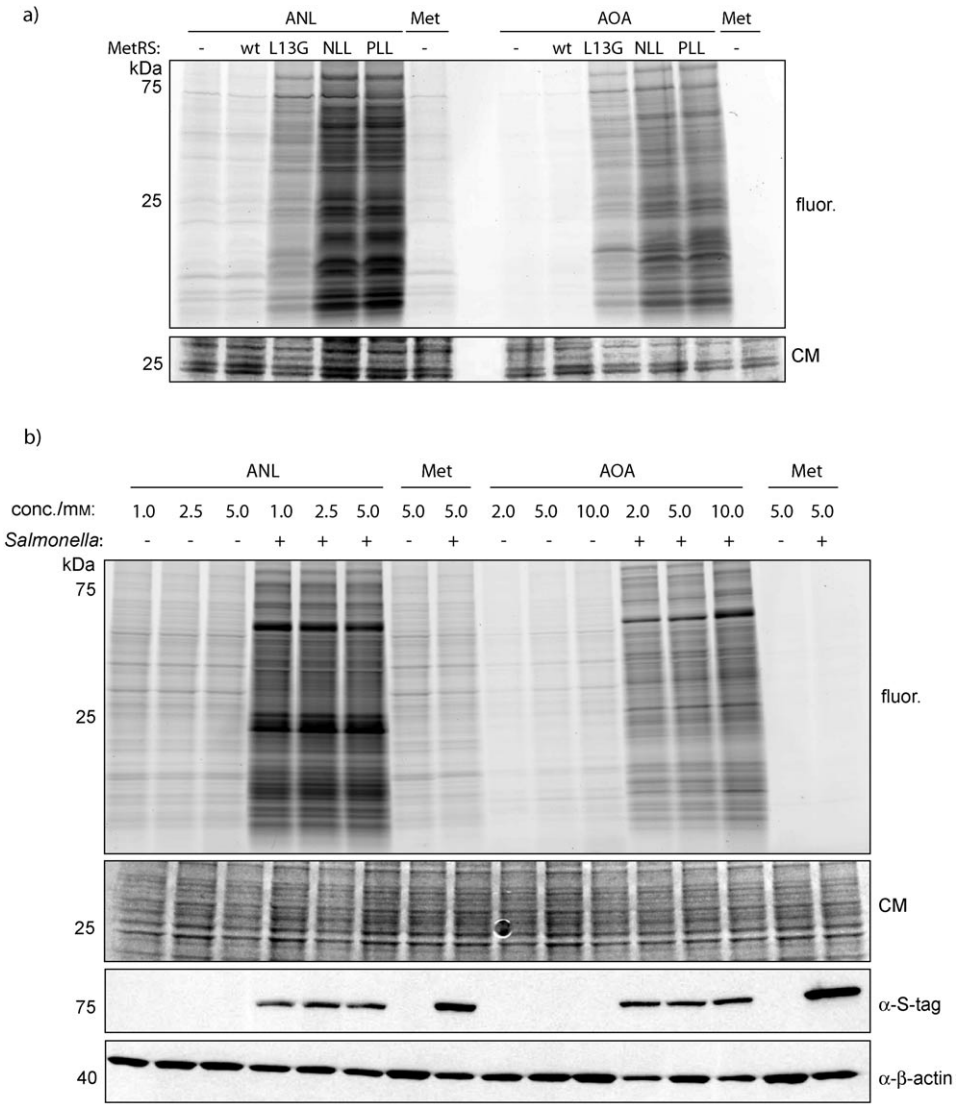

Figure 2. Comparative analysis of orthogonal amino acid reporters and MetRS mutants in *S. typhimurium*. a) *S. typhimurium* expressing different MetRS mutants was labeled with AOA (2 mM) or ANL (1 mM) in minimal medium. Cell lysates were analyzed by CuAAC with az- or alk-Rho and in-gel fluorescence scanning. b) Raw264.7 cells infected with MetRS-NLL *S. typhimurium*, or uninfected, were labeled with ANL or AOA at different concentrations 2 h post infection. The total cell lysates were analyzed by CuAAC with az- or alk-Rho and in-gel fluorescence scanning. Western blot analysis was performed for mutant MetRS expression and levels of the host protein β-actin. CM = Coomassie; "fluor." indicates in-gel fluorescence scanning.

infection, extracellular bacteria were killed by the addition of gentamicin, a cell-impermeable antibiotic, to the cell-culture medium. After 2 h, macrophages infected with MetRS-NLL *S. typhimurium* were labeled with different concentrations of Met, ANL, or AOA in the growth medium for 3 h. Cell pellets containing bacteria and mammalian cells were lysed with 4% SDS lysis buffer, treated with alk-Rho (alkyne-rhodamine)[15] or az-Rho, and analyzed by in-gel fluorescence.

Samples infected with MetRS-NLL *S. typhimurium*, as validated by S-tag Western blot analysis, and labeled with ANL or AOA displayed a strong fluorescence signal above background in contrast to the uninfected samples labeled with ANL or AOA (Figure 2b). Comparison of the respective Met controls for samples treated with AOA or ANL demonstrates the considerably higher nonspecific background signal of the alkyne-functionalized detection tag (Figure 2b), as observed earlier in vitro (Figure 2a). Close inspection of AOA-treated uninfected macrophages revealed marginal concentration-dependent labeling of mammalian proteomes that was not observed with ANL (Figure 2b). Given the higher reactivity of alkyne detection reagents (Figure 2a,b), low levels of ANL labeling in mammalian cells are probably undetectable above the background. Quantification of the relative fluorescence intensities of ANL and AOA suggests that AOA, at a concentration of 2 mM, affords the most sensitive labeling of *Salmonella* proteins during infection (see Figure 3b in the Supporting Information). These results show that ANL and AOA can be selectively incorporated into *S. typhimurium* during intracellular infection by the bacterial expression of MetRS-NLL. We found that AOA, together with azide detection reagents, displayed superior labeling sensitivity at all tested concentrations (see Figure 3b in the Supporting Information).

To confirm the selective labeling of *Salmonella* with AOA inside mammalian cells, we performed fluorescence-imaging studies of infected cells. HeLa cells were infected with MetRS-NLL *S. typhimurium* (MOI = 100), 16 h post-infection cells were pulse-labeled with AOA or Met for 1 h, fixed, permeabilized, reacted with az-Rho, and stained with anti-*Salmonella* serum. Imaging of rhodamine fluorescence demonstrated robust and selective labeling of intracellular bacteria with AOA (Figure 3a). No rhodamine fluorescence was observed in host cells or MetRS-NLL *S. typhimurium* infected cells treated with Met under these conditions (Figure 3a). Colocalization of AOA-labeled *S. typhimurium* with anti-*Salmonella* serum (Figure 3a) validated the selective incorporation of AOA into the bacterial proteome and exclusion from host proteins. We also analyzed *S. typhimurium* infected Raw264.7 macrophages by staining with a

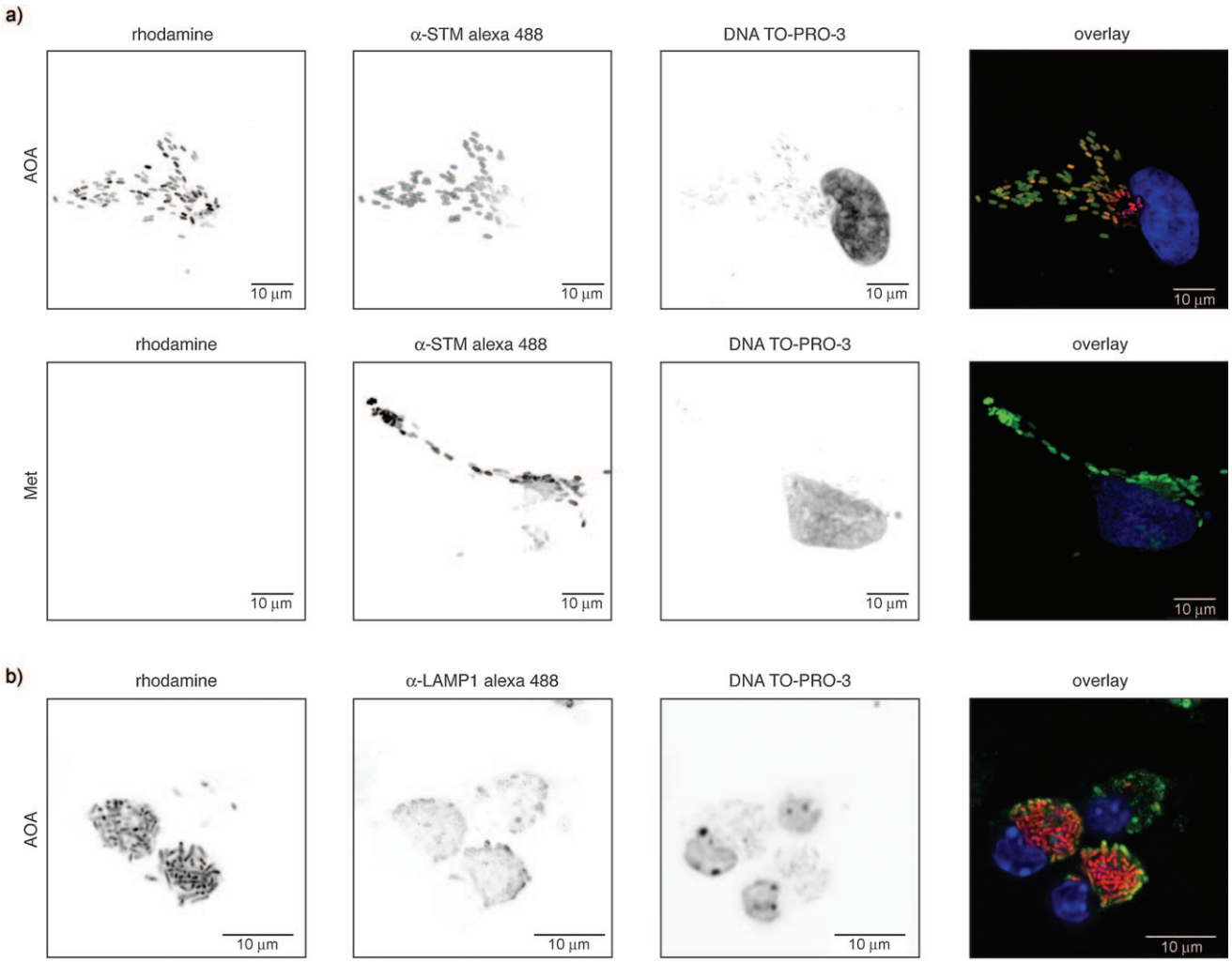

Figure 3. Fluorescence microscopy of AOA-labeled MetRS-NLL *S. typhimurium* infected mammalian cells (scale bar: 10 μm). a) HeLa cells were infected with MetRS-NLL *S. typhimurium* and were labeled 16 h postinfection with AOA (2 mM) or Met (1 mM). Fixed cells were stained for *S. typhimurium* (α-STM alexa 488, green) and DNA (TO-PRO-3, blue) after CuAAC with az-Rho (rhodamine, red). b) Raw264.7 cells were infected with MetRS-NLL *S. typhimurium* and were labeled 16 h postinfection with AOA (2 mM). Fixed cells were stained for LAMP-1 (α-LAMP-1 alexa 488, green) and DNA (TO-PRO-3, blue) after CuAAC with az-Rho (rhodamine, red).

fluorescently labeled LAMP-1 antibody. LAMP-1 serves as a marker for *Salmonella*-containing vacuoles of infected host cells.[2] AOA-labeled MetRS-NLL *S. typhimurium* inside Raw264.7 macrophages were enclosed in LAMP-1-positive compartments (Figure 3b), which suggests that AOA labeling does not significantly disturb intracellular trafficking of *Salmonella* in host cells. These results further validate the utility of AOA for the selective metabolic labeling of *Salmonella* proteins inside mammalian cells.

After the selectivity and efficiency of AOA labeling had been established, we compared *Salmonella* proteins that are metabolically labeled by AOA during growth in a liquid culture with *Salmonella* proteins that are labeled during the infection of macrophages. For this comparative analysis, MetRS-NLL *S. typhimurium* grown in a liquid culture (in vitro) and Raw264.7 macrophages infected with MetRS-NLL *S. typhimurium* (16 h postinfection) were pulse-labeled with AOA or Met for 1 h. Cell lysates were then treated with azido-diazo-biotin,[18] affinity-purified with streptavidin, and eluted from beads for protein identification by gel-based proteomics (see Figure 4 in the Supporting Information).

The generated raw data was searched against a concatenated mouse–*Salmonella* database for protein identification (see the Supporting Information). Only proteins that contained two unique peptides and were not detected in the Met control were considered for this study. The analysis of infected Raw264.7 macrophages identified a total of 218 proteins that met our filter criteria (see Table 1 in the Supporting Information). Of these proteins, 185 were assigned to the *Salmonella* proteome (85%), and the remainder were mouse proteins (Figure 4a). We also identified 472 proteins from MetRS-NLL *S. typhimurium* pulse-labeled with AOA in minimal medium (see Table 2 in the Supporting Information). Between the in vitro and intracellular *Salmonella* datasets, we could identify 96 common proteins, whereas 89 proteins (48%) were observed exclusively in the sample derived from infected macrophages (Figure 4b). Interestingly, at least five *Salmonella* proteins

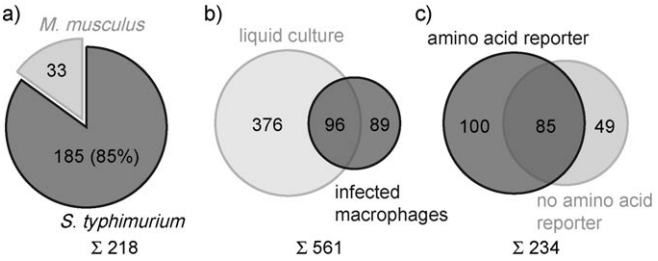

Figure 4. Proteomic analysis of AOA-labeled MetRS-NLL *S. typhimurium* infected mammalian cells. a) A total of 218 *S. typhimurium* proteins were identified from infected Raw264.7 cells. Of these proteins, 185 (85%) were *Salmonella* proteins and 33 were mouse proteins. b) Comparison of *Salmonella* proteins identified from infected macrophages and *Salmonella* grown in minimal liquid culture: 376 proteins were identified in liquid culture only, 89 proteins were identified in infected macrophages only, and 96 were identified in both samples. c) Comparison of proteins identified from AOA-pulse-labeled infected Raw264.7 cells (amino acid reporter) and a previously published dataset of *Salmonella* proteins identified from infected Raw246.7 cells under comparable conditions without AOA labeling (no amino acid reporter):[4] 100 proteins were exclusively identified in our dataset, 85 proteins were identified in both datasets, and 49 proteins were only identified in the previously published dataset.[4]

(SodM, SsrB, SseA, PipB2, and PhoP) from infected Raw264.7 cells were previously described as virulence factors.[19]

When compared with other proteomic datasets generated from macrophages infected with *S. typhimurium*,[4] our results demonstrate that AOA pulse labeling in conjunction with bioorthogonal ligation enables greater enrichment of bacterial proteins (identification of 85% of *Salmonella* proteins with AOA labeling in comparison to approximately 45% under comparable conditions without AOA)[4] and reveals additional bacterial proteins that may be expressed preferentially during infection (Figure 4c). Although the use of AOA and MetRS-NLL only targets Met-containing proteins, 96% of the *S. typhimurium* LT2 proteome contains one or more Met residues in addition to the N-terminal initiator Met residue. Hence, the majority of the *Salmonella* proteome is amenable to labeling with AOA (see Figure 5 in the Supporting Information). Our preliminary proteomic studies demonstrate that AOA pulse labeling in combination with CuAAC enables the efficient enrichment and identification of endogenously expressed bacterial proteins that are synthesized differentially by *Salmonella* during the infection of host cells.

In summary, we report a new and efficient orthogonal amino acid reporter for the selective labeling of bacterial proteomes during infection. The combination of CuAAC with AOA pulse labeling enables the imaging of bacteria within mammalian cells as well as the enrichment and proteomic analysis of endogenously expressed *Salmonella* proteins from infected mammalian cells. The application of orthogonal amino acid reporters should open new and exciting opportunities for the imaging and proteomic investigation of different bacterial pathogens during infection.

Received: April 6, 2010
Published online: July 14, 2010

Keywords: bacterial pathogenesis · click chemistry · nonnatural amino acids · protein labeling · proteomics

[1] A. P. Bhavsar, J. A. Guttman, B. B. Finlay, *Nature* **2007**, *449*, 827.
[2] A. Haraga, M. B. Ohlson, S. I. Miller, *Nat. Rev. Microbiol.* **2008**, *6*, 53.
[3] K. D. Rodland, J. N. Adkins, C. Ansong, S. Chowdhury, N. P. Manes, L. Shi, H. Yoon, R. D. Smith, F. Heffron, *Future Microbiol.* **2008**, *3*, 625.
[4] L. Shi, J. N. Adkins, J. R. Coleman, A. A. Schepmoes, A. Dohnkova, H. M. Mottaz, A. D. Norbeck, S. O. Purvine, N. P. Manes, H. S. Smallwood, H. Wang, J. Forbes, P. Gros, S. Uzzau, K. D. Rodland, F. Heffron, R. D. Smith, T. C. Squier, *J. Biol. Chem.* **2006**, *281*, 29131.
[5] D. Becker, M. Selbach, C. Rollenhagen, M. Ballmaier, T. F. Meyer, M. Mann, D. Bumann, *Nature* **2006**, *440*, 303.
[6] F. Wang, S. Robbins, J. Guo, W. Shen, P. G. Schultz, *PLoS ONE* **2010**, *5*, e9354.
[7] K. E. Beatty, J. C. Liu, F. Xie, D. C. Dieterich, E. M. Schuman, Q. Wang, D. A. Tirrell, *Angew. Chem.* **2006**, *118*, 7524; *Angew. Chem. Int. Ed.* **2006**, *45*, 7364.
[8] K. L. Kiick, E. Saxon, D. A. Tirrell, C. R. Bertozzi, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 19.
[9] M. Meldal, C. W. Tornøe, *Chem. Rev.* **2008**, *108*, 2952.
[10] D. C. Dieterich, A. J. Link, J. Graumann, D. A. Tirrell, E. M. Schuman, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 9482.
[11] I. C. Tanrikulu, E. Schmitt, Y. Mechulam, W. A. Goddard III, D. A. Tirrell, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 15285.
[12] A. J. Link, M. K. Vink, N. J. Agard, J. A. Prescher, C. R. Bertozzi, D. A. Tirrell, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 10180.
[13] J. T. Ngo, J. A. Champion, A. Mahdavi, I. C. Tanrikulu, K. E. Beatty, R. E. Connor, T. H. Yoo, D. C. Dieterich, E. M. Schuman, D. A. Tirrell, *Nat. Chem. Biol.* **2009**, *5*, 715.
[14] A. E. Speers, B. F. Cravatt, *Chem. Biol.* **2004**, *11*, 535.
[15] G. Charron, M. M. Zhang, J. S. Yount, J. Wilson, A. S. Raghavan, E. Shamir, H. C. Hang, *J. Am. Chem. Soc.* **2009**, *131*, 4967.
[16] K. E. Beatty, D. A. Tirrell, *Bioorg. Med. Chem. Lett.* **2008**, *18*, 5995.
[17] H. K. Chenault, J. Dahmer, G. M. Whitesides, *J. Am. Chem. Soc.* **1989**, *111*, 6354.
[18] Y. Y. Yang, J. M. Ascano, H. C. Hang, *J. Am. Chem. Soc.* **2010**, *132*, 3640.
[19] J. Yang, L. Chen, L. Sun, J. Yu, Q. Jin, *Nucleic Acids Res.* **2008**, *36*, D539.

