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Article

MS-Based *in Situ* Proteomics Reveals AMPylation of Host Proteins during Bacterial Infection

Theresa Rauh, Sophie Brameyer, Pavel Kielkowski, Kirsten Jung, and Stephan A. Sieber*

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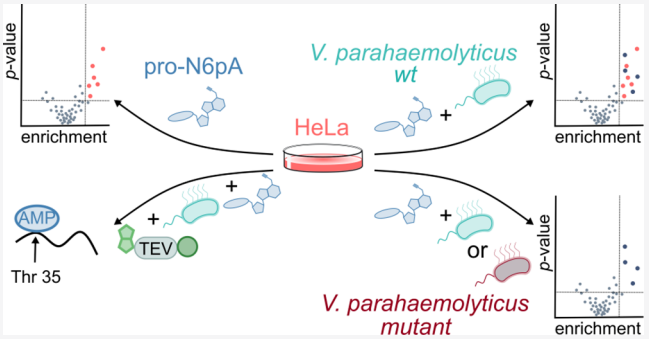
ABSTRACT: Bacteria utilize versatile strategies to propagate infections within human cells, *e.g.*, by the injection of effector proteins, which alter crucial signaling pathways. One class of such virulence-associated proteins is involved in the AMPylation of eukaryotic Rho GTPases with devastating effects on viability. In order to get an inventory of AMPylated proteins, several technologies have been developed. However, as they were designed for the analysis of cell lysates, knowledge about AMPylation targets in living cells is largely lacking. Here, we implement a chemical-proteomic method for deciphering AMPylated host proteins *in situ* during bacterial infection. HeLa cells treated with a previously established cell permeable pronucleotide probe (**pro-N6pA**) were infected with *Vibrio parahaemolyticus*, and modified host proteins were identified upon probe enrichment and LC-MS/MS analysis. Three already known targets of the AMPylator VopS—Rac1, RhoA, and Cdc42—could be confirmed, and several other Rho GTPases were additionally identified. These hits were validated in comparative studies with *V. parahaemolyticus* wild type and a mutant producing an inactive VopS (H348A). The method further allowed to decipher the sites of modification and facilitated a time-dependent analysis of AMPylation during infection. Overall, the methodology provides a reliable detection of host AMPylation *in situ* and thus a versatile tool in monitoring infection processes.



KEYWORDS: *AMPylation, adenylylation, Rho GTPases, Fic, VopS, infection*

With the increasing threat of multiresistant bacteria and the corresponding lack of efficient antibiotics, research into attenuating bacterial pathogenesis (also termed virulence) has attracted major attention as an alternative therapeutic approach. However, as pathogenic bacteria exploit versatile weapons to harm eukaryotic cells, tools to track and uncover their hitherto unknown infection mechanisms are urgently required.[1] Effector protein mediated post-translational modification (PTM) represents a devastating strategy to alter the host proteome and thus to promote the infection process.[2] This provokes large impacts on the host's central signaling pathways or its cellular physiology, benefiting bacterial replication and survival.[3,4]

One representative of this modifying process is AMPylation (also called adenylylation), in which adenosine triphosphate (ATP) donates an adenosine 5′-monophosphate (AMP) moiety to a protein's Ser, Tyr, or Thr side-chain.[5,6] In 2009, the two bacterial effector proteins VopS of *Vibrio parahaemolyticus*[5] and IbpA of *Histophilus somni*[6] were found to AMPylate host Rho GTPases at conserved residues. These strains utilize different secretion systems (type III secretion system in case of *V. parahaemolyticus*) to directly inject effector proteins into the host cytosol (Figure 1A).[5,6] Due to the important role of GTPase signaling in the regulation of actin dynamics, cytokine production, and immune cell signaling,[7] the switch I and switch II regions of these enzymes are preferred targets to impair further downstream signaling.[8] In the case of VopS, AMPylation of Rho GTPases (RhoA, Rac1, and Cdc42) occurs in the switch I region entailing several devastating consequences, among others the inhibition of GTPase binding to the p21-activated kinase (PAK) and inhibition of the NFκB, Erk, and JNK kinase signaling pathways.[9] In recent years, several additional effector proteins—besides VopS and IbpA—were discovered to modify host proteins by AMPylation. For only some of those, the corresponding protein targets as well as the resulting physiological consequences could be elucidated.[10] Examples are the *Legionella pneumophila* effector protein DrrA, which AMPylates Rab1b on a Tyr residue in the switch II region,[11] and the *Bartonella henselae* effector protein BepA, which among others targets the breast cancer anti-estrogen resistance protein 1 (BCAR1) and likely other yet unidentified host proteins.[12,13]

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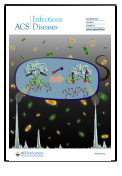

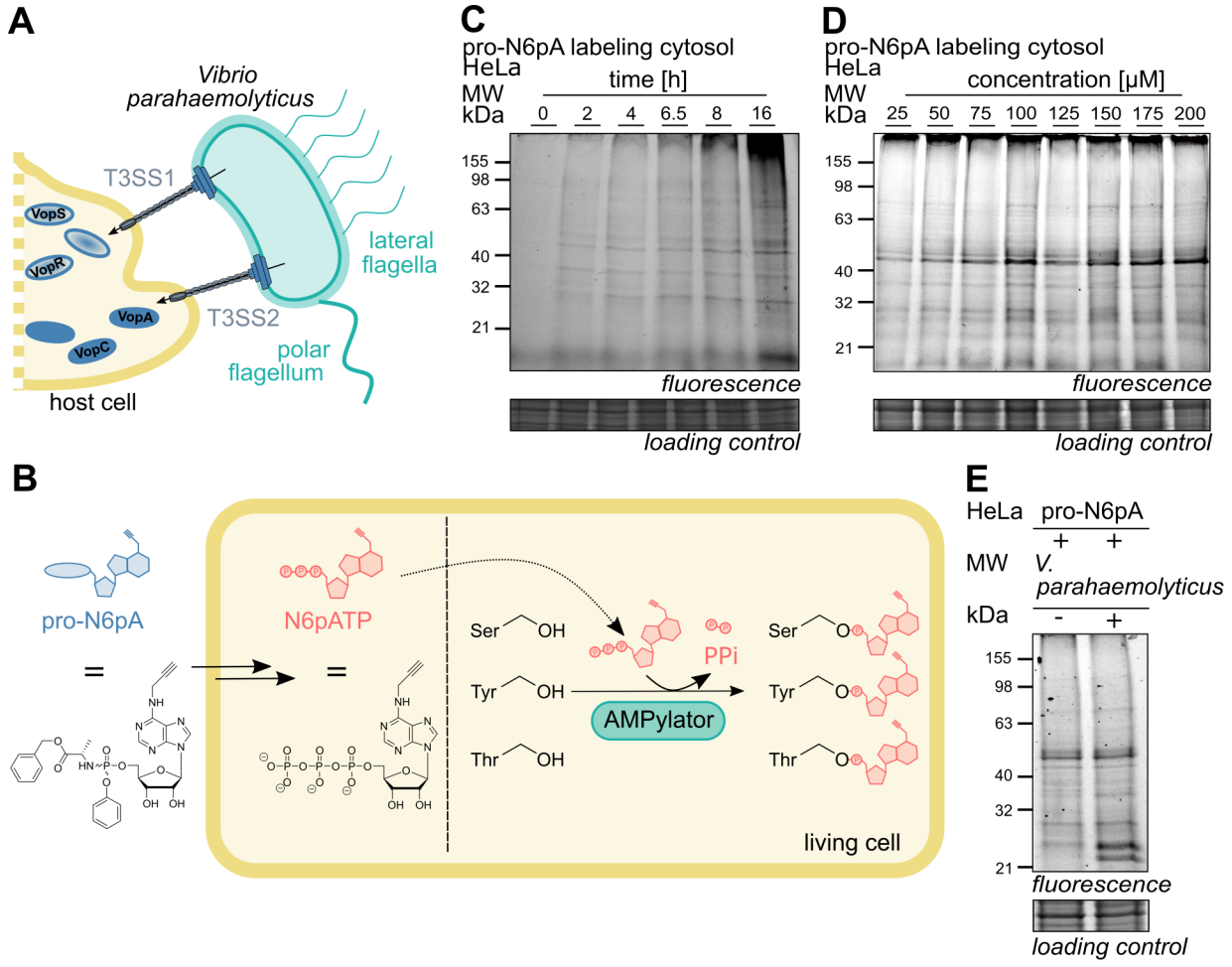

Figure 1. Pro-N6pA allows the tracking of AMPylated proteins: (A) Selected virulence strategies of *V. parahaemolyticus* showing its two type III secretion systems (T3SSs) with T3SS1 secreting VopS. (B) *In situ* activation of **pro-N6pA** to **N6pATP** and its further use in the AMPylation of proteins at Ser, Tyr, or Thr side-chains releasing pyrophosphate. (C,D) SDS-PAGE of *in situ* labeling of HeLa cells with **pro-N6pA** in a time dependent (C) (100 μM **pro-N6pA**) or concentration dependent (D) (16 h) manner. (E) Gel-based analysis of *V. parahaemolyticus* wild type infected as well as uninfected HeLa cells treated with 100 μM **pro-N6pA**.

Overall, bacteria encode more than 2000 Fic-domain (filamentation induced by cAMP) containing proteins[14] with some of them being phylogenetically comparable to VopS or IbpA.[15]

In order to unravel AMPylation during bacterial infection, diverse methods were pursued. Early studies included *in vitro* AMPylation assays[11,15] or the incorporation of radiolabeled ATP in cell lysates.[5,13] Moreover, antibodies directed toward AMPylated amino acid residues[16−18] and various probes, including propargylated ATP (N^6pATP), were developed to identify AMPylated proteins either by in-gel analysis or *via* protein mass-spectrometry (MS).[19−21] MS-based approaches were further fine-tuned toward the analysis of characteristic fragmentation patterns[22] along with optimized methods[23] and its application extended to isotopically labeled proteins.[24] Furthermore, potential targets of VopS were identified using self-assembled *in vitro* human protein (NAPPA) microarrays.[25] NAPPA captures recombinant human proteins on the array surface followed by incubation with VopS in the presence of N^6pATP. Subsequent readout of AMPylation by fluorescence imaging revealed 20 putative targets of VopS (out of 10 000 human proteins).[26] The most recent approach to identify AMPylation targets equips recombinantly expressed Fic enzymes with synthetically produced nucleotide derivatives (TReNDS). These derivatives, which are connected to the recombinant Fic enzyme *via* an artificially introduced cysteine residue, are able to capture their target protein.[12]

However, none of these methods are suitable to directly detect AMPylation in an *in situ* setting.[27] We recently introduced two probes which enabled the *in situ* detection of AMPylation under physiological conditions as well as live-cell imaging. With this strategy, we were able to detect various AMPylated proteins throughout different cell types and track the dynamics of this modification in living cells.[28,29]

In this work, the cell-permeable pronucleotide probe **pro-N6pA** (Figure 1B) was used to implement a chemical-proteomic method for deciphering AMPylated host proteins *in situ* during bacterial infection. Probe treatment of HeLa cells followed by *V. parahaemolyticus* infection and LC-MS/MS analysis revealed several known and unknown targets of VopS along with the cognate AMPylation binding sites. A comparative study between *V. parahaemolyticus* wild type and a mutant expressing an inactive VopS (mutant H348A) further validated these proteins. Moreover, infections of host cells with bacteria such as *Pseudomonas aeruginosa* PAO1 or *Escherichia coli* CFT073, which produce Fic-domain containing proteins with low similarity to virulence-associated AMPylators,

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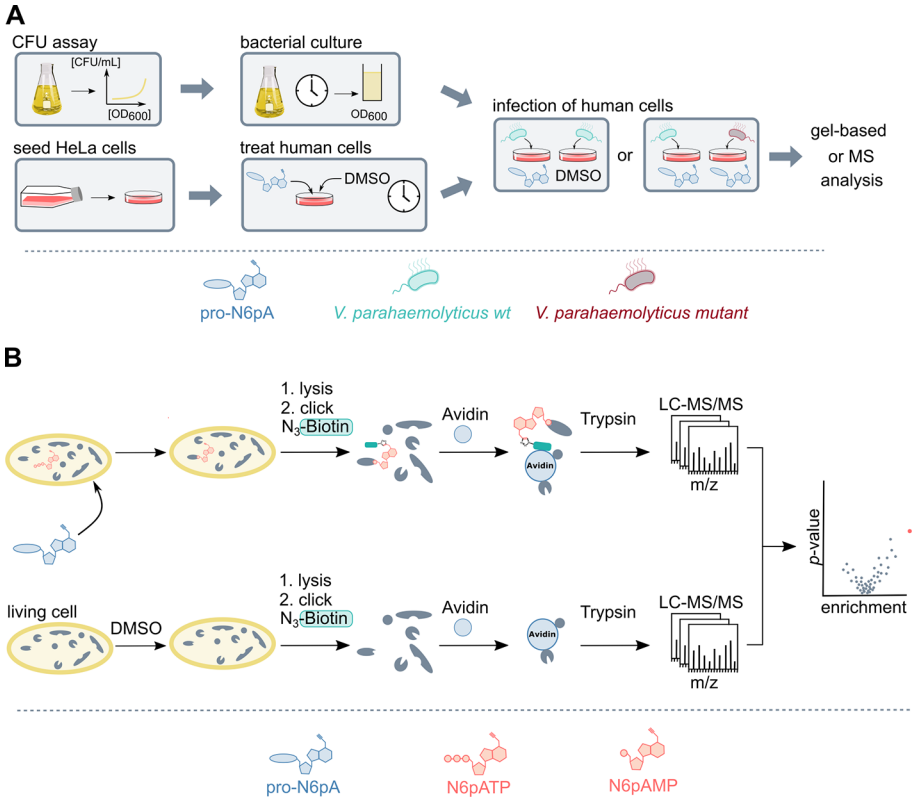

Figure 2. Illustrations of the individual working steps. (A) Step-wise overview of the infection workflow with bacterial growth and probe treatment of HeLa cells. (B) Schematic overview of the proteomic profiling procedure following the infection workflow, with LFQ to identify AMPylated proteins *in situ*.

resulted in a lack of modifications, demonstrating the fidelity of this approach.

RESULTS AND DISCUSSION

Development of an *in Situ* Proteomics Approach to Detect AMPylated host Proteins during Bacterial Infection. With more than 2000 bacterial proteins bearing a Fic-domain, we aimed to develop a tailored platform to decipher putative AMPylation targets *in situ* with major emphasis on those playing a crucial role in host virulence. Since our recently introduced phosphoramidate pronucleotide probe **pro-N6pA** enables the *in situ* identification of AMPylated proteins,[28] we applied this tool toward targeting AMPylated host proteins in bacterial infection experiments. As proteins in general are only partially modified by PTMs,[30] the first goal was to maximize the labeling efficiency of **pro-N6pA**. Therefore, time and concentration-dependent labeling experiments of **pro-N6pA** treated HeLa cells were conducted. Upon treatment with the alkyne probe, cells were lysed, clicked to rhodamine-azide and protein target bands were evaluated by fluorescent gel-based analysis (Figure 1C, 1D). In accordance with previous results,[28] labeling with 100 μM **pro-N6pA** for 16 h was optimal in terms of intensity and number of protein bands.

With these optimized conditions in hand, we commenced toward the MS-based identification of intrinsically AMPylated proteins in HeLa cells as a reference for the later infection experiments. Therefore, a quantitative proteome profiling experiment with intact HeLa cells was performed. Cells were treated with 100 μM **pro-N6pA** or dimethyl sulfoxide (DMSO) as a background control and processed *via* label-free quantification (LFQ) analysis.[31] MS data analysis revealed 27 significantly enriched proteins (Figure S1, Table S1) among them several already known to be modified by AMPylation within human cells.[12,28,29,32] These proteins served as a non-virulence associated reference for further infection experiments.

For infection studies, *V. parahaemolyticus* strain RIMD 2210633 was chosen as a characterized model organism in order to fine-tune and customize conditions for identifying AMPylated host proteins. HeLa cells were co-incubated with bacteria as well as **pro-N6pA** and prior to human cell lysis, bacteria were separated by centrifugation. Subsequently, the proteome was subjected to gel-based or MS analysis. Prior to MS studies, the workflow was adjusted for optimal infection conditions. Gel-based labeling with different multiplicities of infection (MOI) and various incubation times were conducted, which revealed the best labeling conditions at MOIs of 10 for 1.5 h (Figure S2). Probe labeling under this condition clearly revealed two fluorescent bands located around 23 kDa, corresponding to the size of human Rho GTPases (Figure 1E). Next, quantitative LC-MS/MS experiments were performed to unravel the identity of these infection-associated protein targets. To select for the best conditions, the performance of stable isotope labeling by amino acids in cell culture (SILAC), known for its extensive protein coverage and confident results,[33] was compared with LFQ, hallmarked by an accurate and simple analysis even in case of partially missing peptides.[31] To maximize protein quantification in the SILAC experiments,[34] all samples, infected and uninfected, were treated with **pro-N6pA** at 100 μM. To account for changes on the proteome level triggered by invading bacteria, we

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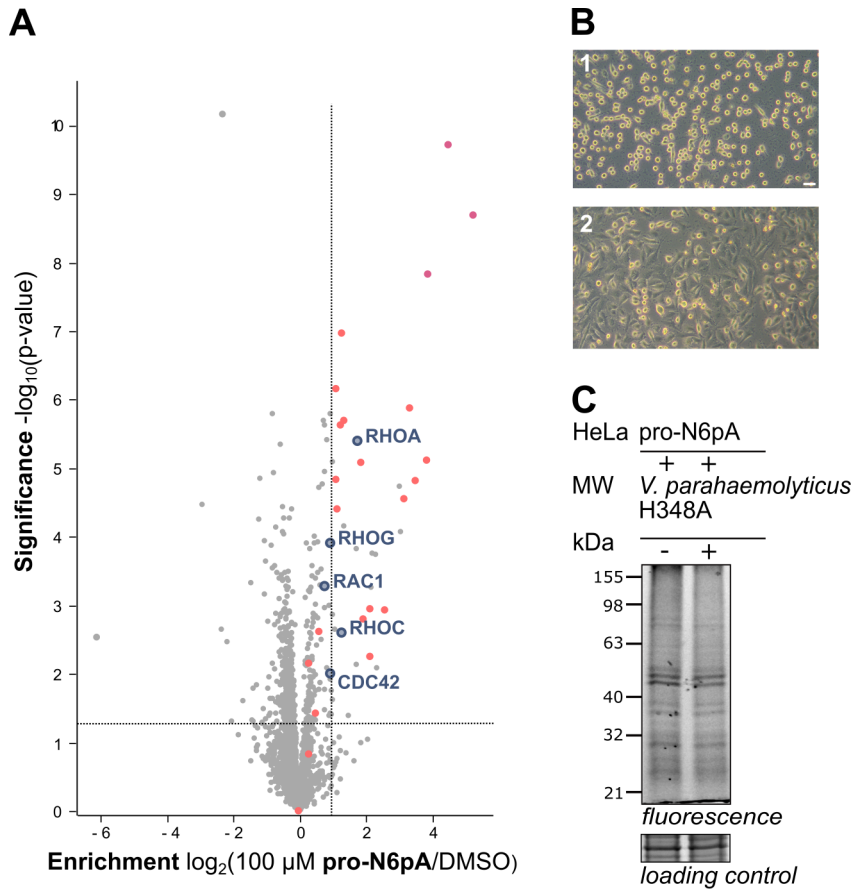

Figure 3. Identification of protein targets of VopS by combining an infection workflow with a LC-MS/MS based proteomic profiling workflow. (A) Volcano plot of *in situ* proteomics approach with HeLa cells (100 μM **pro-N6pA**/DMSO) infected with *V. parahaemolyticus* wild type [n = 5, $\log_2$(enrichment) > 1.0, p-value < 0.05]. Protein targets of VopS are shown in blue while proteins included in the non-virulence associated reference list (Table S1) are marked in red. Proteins that showed no clear indication of being modified with AMP in the MS analyses are depicted in light gray. (B) Differences in phenotypic appearance when infecting HeLa cells with *V. parahaemolyticus* wild type or mutant H348A; (1) HeLa cells, 100 μM **pro-N6pA** (16 h), *V. parahaemolyticus* wild type (MOI = 10, 92 min) or (2) HeLa cells, 100 μM **pro-N6pA** (16 h), *V. parahaemolyticus* mutant H348A (MOI = 10, 96 min). The scale bar represents 100 μm. (C) Gel-based analysis of *V. parahaemolyticus* mutant H348A infected as well as noninfected HeLa cells treated with 100 μM **pro-N6pA**.

performed full proteome analysis with the residual SILAC lysate (Figure S3A). With this setup, we compared infected *vs* uninfected HeLa cells and confirmed AMPylation of the known targets Cdc42, RhoA, and Rac1 (Figure S3B) which were not upregulated in the full proteome. However, given the predicted existence of additional AMPylated human proteins by VopS,[20,25] we switched to LFQ analysis with a slightly modified workflow (Figure 2A). Here, all HeLa cells were infected with bacteria and treated for 1.5 h with 100 μM **pro-N6pA** or DMSO. Afterward, the samples were processed according to a standard proteomic profiling workflow (Figure 2B). This methodological setup directly excludes bacteria-prone changes on the proteome level and LFQ provides accurate sample analysis even in case of impaired peptide measurement.[31] Besides several intrinsically AMPylated proteins, 100 μM **pro-N6pA** treatment revealed not only Cdc42, RhoA, and Rac1 but also the Rho GTPases RhoC and RhoG as significantly enriched targets of VopS under physiological conditions (Figure 3A).

Vice versa, applying the methodology to *Pseudomonas aeruginosa* PAO1 as well as *Escherichia coli* CFT073, both lacking AMPylators with high similarity to VopS (alignment $\text{AMPylator}_{\text{PAO1/CFT073}}$ to VopS < 15% similarity), did not show any significant and reproducible host protein AMPylation by fluorescent SDS-PAGE and LC-MS/MS analysis (Figure S4–S6), which is in line with recent literature.[14,35] Interestingly, as both strains encode proteins with putative AMPylation domains, we prepared or bought the corresponding (transposon) mutants; however, a lack of consistent protein targets throughout our proteomic analysis strongly suggests that these proteins are not involved in bacterial virulence *via* AMPylation. Overall, the results highlight the utility of our versatile chemoproteomic platform for the precise prediction and *in situ* monitoring of bacterial host AMPylation.

Validation of *in Situ* AMPylation Targets by Using a *V. parahaemolyticus* VopS Inactive Mutant (H348A) and Tracking of the Wild Type Infection Progress. AMPylation of host proteins by *V. parahaemolyticus* is associated with its Fic domain containing effector protein VopS.[5] The Fic-domain consists of a highly conserved motif comprising nine amino acids, with histidine being responsible for the deprotonation of serine, tyrosine or threonine hydroxyl groups of target proteins.[10] Replacing the essential histidine (His348) within this motif with an alanine residue renders the Fic-domain inactive.[5] To validate the novel AMPylated protein hits as substrates of VopS, a *V. parahaemolyticus* mutant was generated that produces an inactive VopS variant by introducing a point mutation into the chromosome (des-

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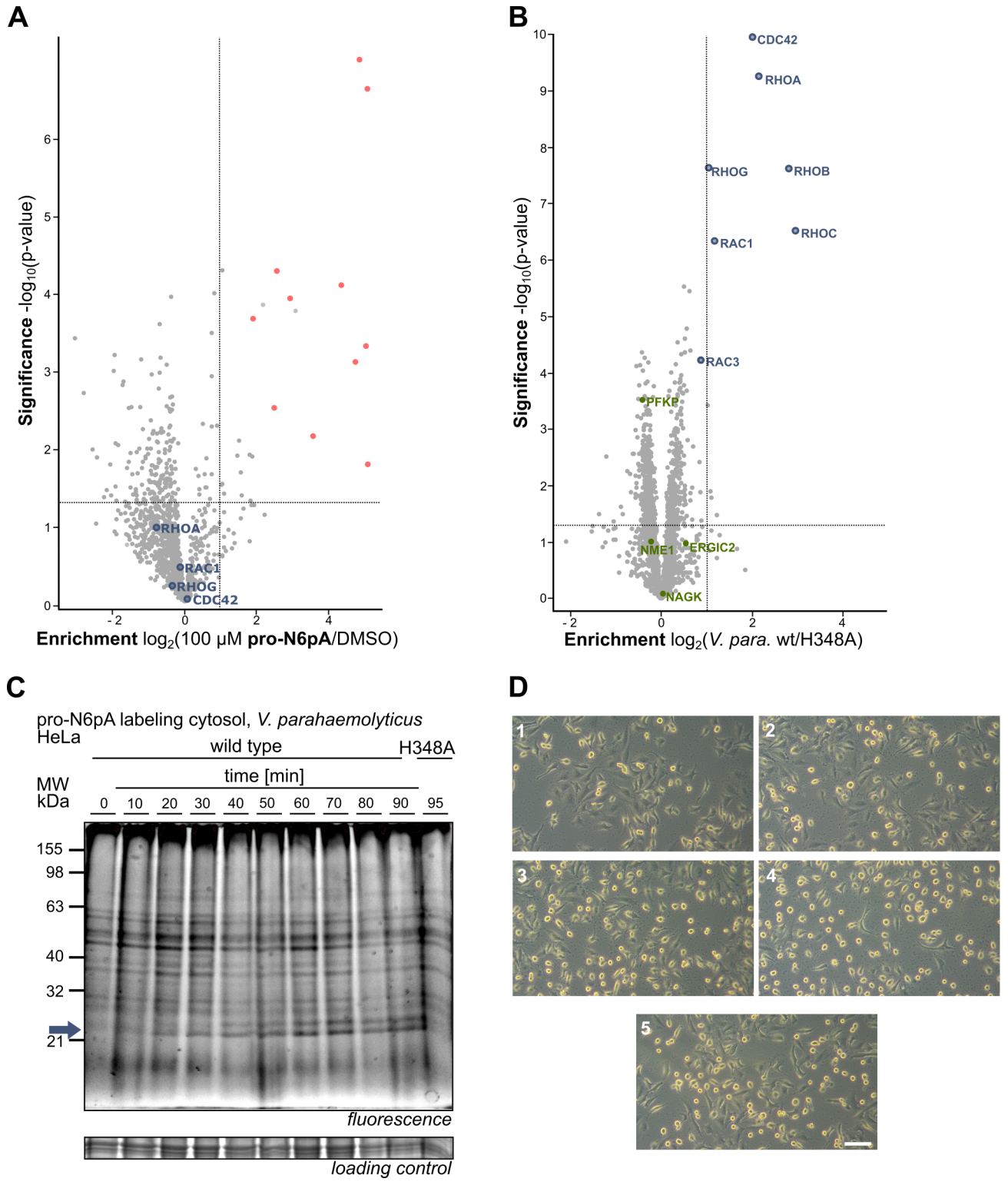

Figure 4. Validation of the infection workflow and following-up on AMPylation dynamics. (A) Volcano plot of *in situ* proteomics approach with HeLa cells (100 μM **pro-N6pA**/DMSO) infected with *V. parahaemolyticus* mutant H348A and (B) volcano plot of *in situ* proteomics approach with HeLa cells (100 μM **pro-N6pA**) infected with *V. parahaemolyticus* wild type or mutant H348A; A/B: $n = 5$, $\log_2$(enrichment) > 1.0, p-value < 0.05. Protein targets of VopS are shown in blue, while proteins included in the non-virulence associated reference list are marked in red. Proteins which according to literature should be AMPylated by VopS are represented in green. Proteins that showed no clear indication of being modified with AMP in the MS analyses are depicted in light gray. (C) Labeling of AMPylated proteins (100 μM **pro-N6pA**) with increasing infection times up to 90 min (*V. parahaemolyticus* wt) and as a control infection with the H348A mutant for 95 min. (D) Phenotypic appearance of HeLa cells infected with *V. parahaemolyticus* for different periods of time; 1 = wt, 60 min; 2 = wt, 70 min; 3 = wt, 80 min; 4 = wt, 90 min; 5 = H348A, 95 min. The scale bar represents 100 μm.

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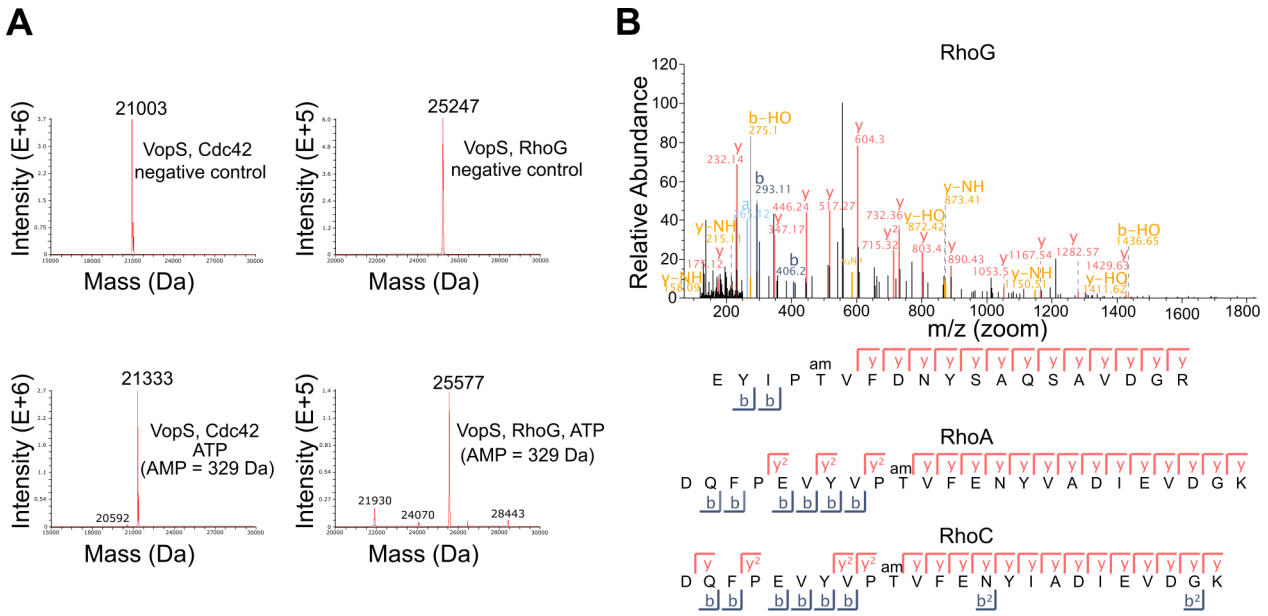

Figure 5. Elucidation of VopS AMPylation sites of RhoC and RhoG *in situ*. (A) IPMS measurements of unmodified (without addition of ATP) and modified recombinant Cdc42 and RhoG. Both proteins are AMPylated once. (B) *In situ* site identification of AMPylation sites of VopS on RhoG (T35), RhoA (T37), and RhoC (T37).

ignated as mutant H348A). This mutant was utilized for chemoproteomic analysis, and as expected,[5] the cell rounding of HeLa cells, which was observed after wild type infection, was abrogated in case of the H348A mutant demonstrating impaired virulence (Figure 3B). Correspondingly, fluorescent SDS-PAGE bands indicative of AMPylated host proteins were lacking upon infection with this strain (Figure 3C). Subsequent LC-MS/MS analysis *via* the LFQ workflow confirmed these results by a drastically diminished enrichment [$\log_2$(enrichment) $\leq$ 0] of all reference AMPylation targets (Rac1, RhoA, Cdc42) compared to the wild type plot (Figure 4A). In addition, RhoG also showed a significant down-shift validating it as AMPylation target of VopS.[25] Most of the significantly enriched proteins [$\log_2$(enrichment) > 2] were part of the compiled reference list of intrinsically AMPylated human proteins. In addition, HeLa cells were treated with probe followed by infection with *V. parahaemolyticus* wild type and mutant H348A (Figure 2A) to account only for proteins being AMPylated by VopS. Importantly, this setup not only confirmed the previously detected AMPylation targets but also unraveled RhoB and Rac3 as so far unknown targets of VopS under physiological conditions. In addition, some previously predicted substrate proteins could not be confirmed suggesting a possible difference between *in situ* labeling and *in vitro* assays (Figure 4B).

As AMPylation is a highly dynamic process, this *in situ* methodology could be beneficial for monitoring the propagation of protein modification during bacterial infection within the host and thereby help to correlate molecular alterations with phenotypic cell damage. In order to test this hypothesis, we infected **pro-N6pA** treated HeLa cells with a 10-fold excess of *V. parahaemolyticus* wt as well as the H348A mutant. Samples were drawn every 10 min, followed by cell lysis and click-chemistry to rhodamine azide. Following fluorescent band intensities of the two signature VopS AMPylated protein bands revealed that these modifications were already visible after short incubation times (20−40 min) (Figure 4C). Interestingly, phenotypic cell rounding lacked significantly behind (60−90 min) indicating that molecular dysregulation does not immediately cause cell damage (Figure 4D). Taken together, these results emphasize that our chemical proteomic method provides a direct connection between the virulence phenotype of cell rounding and molecular mechanism of toxicity by VopS mediated AMPylation of essential targets.

RhoC and RhoG GTPases Are AMPylated by VopS at Conserved Threonine Residues. With putative VopS substrates in hand, we commenced with their in-depth validation. First, to directly confirm AMPylation, we incubated recombinant RhoG with VopS in the presence of ATP or N6pATP. We then analyzed protein modification by intact-protein mass-spectrometry (IPMS) and fluorescent SDS-PAGE (data not shown), using recombinant Cdc42 as a positive control. Satisfyingly, AMPylation of Cdc42 and RhoG with ATP and N6pATP was clearly observed by IPMS as well as fluorescent gel analysis (Figure 5A).

Furthermore, LC-MS/MS analysis of recombinant RhoG upon incubation with VopS revealed threonine 35 as the AMPylation site, experimentally validating a previous prediction.[25] Next, we performed a consolidated site identification of AMPylated proteins in HeLa cells incubated with *V. parahaemolyticus*. An azide-TEV-cleavable-biotin linker was clicked to the probe treated proteome and after enrichment, cleaved peptides were analyzed *via* HCD (higher-energy collisional dissociation) and ETD (electron-transfer dissociation) fragmentation. This combined procedure was necessary to increase the chance of identifying AMPylated sites, since HCD by itself enhances group cleavage.[23] Although this task remains challenging due to the limited percentage of endogenously modified proteins,[28] we were able to confirm a known AMPylation site of RhoA on Thr37. Furthermore, we mapped the AMPylation sites of RhoG to Thr35 and RhoC to Thr37 (Figure 5B) again experimentally validating previous predictions for the first time.[25]

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CONCLUSION

Bacterial virulence is based on intricate mechanisms leading to cell damage. *V. parahaemolyticus* mutant H348A does not affect HeLa cell morphology indicating that AMPylation plays a predominant role in its pathogenesis. Thus, implementing a methodology for the *in situ* detection and monitoring of AMPylated host targets represents a significant advancement to consolidate previous reports on protein hits but also expand our knowledge by RhoB, RhoC, RhoG, and Rac3 as so far unrecognized VopS substrates. In addition to the validation of these hits, previously predicted but yet unconfirmed substrates of VopS such as PFKP, NME1, NAGK, and ERGIC2 were not enriched, suggesting that they may not be targets under *in situ* conditions.[20,25] Overall, besides the application as a monitoring tool for bacterial AMPylation, the pronucleotide probe would be an ideal tool to interrogate VopS activity, *e.g.*, by screening a small molecule library with huge potential for new anti-infective discoveries.

METHODS

General Remarks. For all proteomic experiments, if not stated otherwise, MS-grade solvents were used. All given percentages mean percentage by volume (v/v) for liquid substances and percentage by weight (w/v) for all solids.

Cell Culture. Human epithelioid cervix carcinoma HeLa cells were purchased from Sigma-Aldrich (93021013_1VL) and cultivated in a T175 culture flask (Sarstedt) containing high glucose Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich) and 2 mM L-glutamine (Sigma-Aldrich). Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere. The cells were routinely tested for mycoplasma contamination.

For SILAC experiments, HeLa cells were passaged at least six times in SILAC-DMEM (Sigma-Aldrich) supplemented with 10% dialyzed FBS and 2 mM L-glutamine as well as 214 μM [$^{13}C_6$, $^{15}N_4$] L-arginine HCl (Arg10) and 419 μM [$^{13}C_6$, $^{15}N_2$] L-lysine 2 HCl (Lys8) (Cambridge Isotope Laboratories) resulting in "heavy" cells or with 214 μM [$^{13}C_6$] L-arginine HCl (Arg6) and 419 μM [4,4,5,5-D4] L-lysine 2 HCl (Lys4) (Cambridge Isotope Laboratories) resulting in "light" cells instead.

Bacterial Strains and Media. The *Vibrio parahaemolyticus* strain RIMD 2210633 was a kind gift from Dr. Tetsuya Iida and Dr. Takeshi Honda (Research Institute for Microbial Diseases, Osaka University). The strain was routinely cultured in lysogeny broth (LB) medium (10 g/L casein peptone, 5 g/L NaCl, 5 g/L yeast extract, pH 7.5) + 3% NaCl at 30 °C with agitation at 200 rpm.

The strain *Pseudomonas aeruginosa* PAO1 was obtained from the Institute Pasteur in France and *Escherichia coli* CFT073 was obtained from the Urological Clinic in Munich (Dr. Giuseppe Magistro). The *P. aeruginosa* transposon mutants PW3486 genotype PA1366-A11::ISphoA/hah as well as PW2059 genotype PA0574-A11::ISlacZ/hah were obtained from the University of Washington Manoil Lab PAO1 transposon mutant library.[36] Transposon mutants were plated onto pure LB agar plates from glycerol stock dilutions and grown for 24 h at 37 °C. Subsequently, colonies were picked for each transposon mutant and grown in overnight cultures containing 20 μg/mL tetracycline. *Pseudomonas aeruginosa* PAO1 wt was routinely cultured in LB medium, whereas the transposon mutants were cultivated in LB medium containing 5 μg/mL tetracycline. The *E. coli* CFT073 deletion mutants c4136::Km and c4409::Km were grown in LB medium supplemented with 50 μg/mL kanamycin, whereas the *E. coli* wild type was cultured in pure LB medium. All of them were grown at 37 °C with agitation at 200 rpm.

The *V. parahaemolyticus* RIMD 2210633 mutant H348A in VopS was obtained by double homologous recombination using the suicide plasmid pNPTS138-R6KT-*VopS*-H348A. Briefly, two DNA fragments comprising 620 base pairs upstream and downstream of the H348A position of VopS were amplified by PCR using *V. parahaemolyticus* RIMD 2210633 genomic DNA as template and the primer pairs pNPTS_VopS_fwd and VopS_H348A_rev as well as VopS_H348A_fwd and VopS_pNPTS_rev (Table S2). Thereby, a nucleotide point mutation is introduced (cac to gcc) to obtain the amino acid exchange H348A in VopS. After purification of the PCR fragments, these fragments were assembled *via* Gibson assembly[37] into EcoRV-digested pNPTS138-R6KT plasmid,[38] resulting in the pNPTS138-R6KT-*VopS*-H348A plasmid. The resulting plasmid was introduced into *V. parahaemolyticus* RIMD 2210633 by conjugative mating using *E. coli* WM3064[39] as a donor on LB medium containing *meso*-diamino-pimelic acid (DAP) with a final concentration of 300 μM. Single-crossover integration mutants were selected on LB plates containing kanamycin but lacking DAP. Single colonies were then streaked out on LB plates containing 10% (wt/vol) sucrose to select for plasmid excision. Kanamycin-sensitive colonies were then checked for targeted exchange by colony PCR using primers (VopS_check_fwd and VopS_check_rev) bracketing the location of the nucleotide exchange and sequencing of the respective PCR fragment.

The *E. coli* in frame deletion mutants c4136::Km and c4409::Km were constructed by Red/ET recombination using the *E. coli* Quick and Easy gene deletion kit (Gene Bridges, Heidelberg, Germany). Briefly, primers for the deletion of c4136 and c4409 were designed according to the manual (Table S2). These primer pairs each target the surrounding of the genes c4136 and c4409 in order to amplify the FRT-PGK-gb2-neo-FRT template. A double stranded PCR fragment was introduced *via* electroporation into *E. coli* CFT073 according to the manual. Deletion of the c4136 and c4409 gene was verified by colony PCR and sequencing.

Overnight Culture. For overnight cultures, 5 mL of the appropriate medium were added to a sterile culture tube and inoculated with 5 μL of the respective glycerol stock (in case of the *P. aeruginosa* transposon mutants 20 μg/mL tetracycline and for the *E. coli* deletion strains 50 μg/mL kanamycin). The culture was then grown at the appropriate temperature with 200 rpm for 16 h. To exclude undesired contaminations, a sterile control (medium only) was always included.

Probe Treatment. Labeling of cells was performed as previously described.[28] Briefly, HeLa cells were either seeded in 6 cm dishes, 10 cm dishes, or 15 cm dishes and grown to 80−90% confluency. Then, the old culture medium was removed and new medium containing 100 μM **pro-N6pA** (stock 100 mM in DMSO) or 0.1% DMSO was added. Cell dishes were statically incubated at 37 °C and 5% CO_2 before further treatment.

Analytical *in Situ* Labeling. HeLa cells were seeded in 6 cm dishes and treated with **pro-N6pA** (stock solution 100 mM in DMSO) or DMSO with various concentrations and for different periods of time. Labeling was performed as previously

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mentioned.[29] In short, medium containing probe or DMSO was removed, and cells were washed twice with ice-cold PBS (2 × 1 mL). Next, cells were lysed by adding 150 μL lysis buffer (1% NP40, 1% sodium deoxycholate, 1 tablet protease inhibitor (cOmplete, Mini, EDTA-free protease inhibitor cocktail, Roche) in 10 mL PBS) and scrapping the cells off the plate. The lysis was performed at 4 °C for at least 15 min while rotating the samples. The insoluble fraction has been separated from the soluble fraction (15 min, 4 °C, 13 000 rpm) before the protein concentration of the latter has been determined by BCA assay (Roti Quant, Roth). As next step, protein concentration was adjusted to equal protein amounts and samples were filled up to a total volume of 200 μL using 0.2% SDS in PBS. Click reactions were performed as described previously,[40] for 1.5 h at 25 °C and shaking at 450 rpm using 0.096 mM rhodamine-azide (10 mM stock in DMSO, Rh−N_3, base click), 0.96 mM TCEP (100 mM stock in ddH_2O, Roth), 0.096 mM TBTA ligand (83.5 mM stock in DMSO, TCI), and 0.96 mM $CuSO_4$ (50 mM stock in ddH_2O). Finally, the proteins were precipitated by adding 1 mL acetone and storing the samples in upright position at −20 °C overnight. As next step, the proteins were harvested by centrifugation (15 min, 4 °C, 13 000 rpm) and the pelletized proteins were resuspended in 100 μL 2× SDS loading buffer (63 mM Tris-HCl, 10% glycerol, 139 mM sodium dodecyl sulfate (SDS), 0.0025% bromophenol blue, 5% 2-mercaptoethanol) by sonication (10% intensity, 10 s) before heating the sample for 5 min at 95 °C. Each sample was thoroughly mixed prior to applying 40 μL per gel-lane on a SDS-PAGE gel (12 pockets, 12.5% acrylamide). A Fujifilm LAS 4000 luminescent image analyzer equipped with a Fujinon VRF43LMD3 lens and a 575DF20 filter (both Fujifilm) were used to record the fluorescence image of the gel.

Analytical *in Situ* Labeling of AMPylated Proteins in HeLa Cells after Infection. For each analytical infection assay, HeLa cells were seeded in as much 6 cm dishes as needed for the planned number of samples plus two. All dishes were treated with **pro-N6pA** as described previously. Parallel to the probe treatment, an overnight culture of the desired bacterial strain and the respective medium was inoculated. The next morning, a fresh day culture of the desired bacterial strain was inoculated under the same conditions used to determine the CFU to OD_{600} correlation.

Next, the number of HeLa cells in the two additionally seeded dishes was counted. Therefore, cells were washed with 1 mL PBS, detached with 0.6 μL Accutase solution (Sigma-Aldrich), taken up in 1.4 mL DMEM and diluted 1:1 with 0.5% trypan blue solution. Subsequently, cells were counted using a Neubauer improved cell counting chamber. As soon as the HeLa cells, which were seeded for the infection assay, were exposed to probe treatment for 16 h, the OD_{600} of the previously inoculated bacterial day culture (already grown for at least 2.5 h) was determined. Having the number of HeLa cells per dish as well as the OD_{600} value of the bacterial culture in hand, the required volume of bacterial culture needed for a multiplicity of infection (MOI) of 10 was calculated and harvested (10 min, 4 °C, 6000*g*). The bacterial pellet was resuspended in DMEM solely supplemented with 2 mM L-glutamine and 10 μM **pro-N6pA**. In the meantime, HeLa cells were washed with 1 mL PBS. After that, the infection was initiated by adding 2 mL of DMEM containing probe and bacteria with a MOI of 10. Infected cells were incubated under static conditions at 37 °C and 5% CO_2.

At various time points, images of each dish were acquired at 10× magnification using the Zeiss microscope Primovert equipped with a Zeiss AxioCam ERc 5s. Then, cells were scraped off and pelletized for 10 min at 4 °C and 750*g*. The cells were washed with 1 mL PBS and resuspended in 150 μL lysis buffer. Further sample processing, containing click reaction, protein precipitation and in-gel analysis were performed according to the Analytical *in Situ* Labeling procedure.

Preparative *in Situ* Labeling—Label-Free Quantification (LFQ). As described above, cells (6 cm dishes) were treated with **pro-N6pA** probe and DMSO for 16 h. Afterward, cells were washed twice with 1.5 mL ice-cold PBS, while scratching them off during the second washing step. Next, intact cells were pelletized (15 min, 4 °C, 750*g*) and resuspended in 150 μL ice-cold lysis buffer consisting of 1% NP40, 1% sodium deoxycholate and 1 tablet protease inhibitor (cOmplete, Mini, EDTA-free protease inhibitor cocktail, Roche) in 10 mL PBS. The lysis was performed at 4 °C for at least 15 min while rotating the samples. The insoluble fraction has been separated from the soluble fraction (15 min, 4 °C, 13 000 rpm) before determining the protein concentration of the latter by BCA assay (Roti Quant, Roth). As next step, protein concentration was adjusted to equal protein amounts and samples were filled up to a total volume of 200 μL with PBS. Click chemistry was performed as described in Analytical *in Situ* Labeling using 0.096 mM azide-PEG_3-biotin conjugate instead of rhodamine-azide. Finally, the proteins were precipitated by adding at least 4-fold volume excess of acetone and storing the samples at −20 °C overnight. As next step, the proteins were harvested by centrifugation (15 min, 4 °C, 13 000 rpm) and washed twice with ice-cold MeOH. Therefore, 1 mL of MeOH was added to the protein pellet which was resuspended by sonication (10% intensity, 10 s, Sonopuls HD 2070 ultrasonic rod, Bandelin electronic GmbH) and harvested again as described previously followed by the enrichment procedure.

Prior to the enrichment, 50 μL of avidin-agarose beads per sample were washed trice with 1 mL 0.2% SDS in PBS. The protein pellets were resuspended in 0.2% SDS in PBS by sonication (10% intensity, 10 s) before separating the remaining insoluble part by centrifugation (5 min, 25 °C, max. speed). After that, the supernatant was transferred to the avidin-agarose beads and incubated under rotation for 1.5 h at 25 °C. Subsequently, the beads were washed with 0.2% SDS in PBS trice, twice with 6 M urea solution and finally three times with PBS (after each washing step collect beads by centrifugation for 3 min and 400*g*). The washed beads were resuspended in 200 μL digestion buffer (3.9 M urea, 1.1 M thiourea in 20 mM Hepes, pH 7.5) and the enriched proteins were first reduced (1 mM DTT, 45 min, 25 °C) and second alkylated (5.5 mM IAA, 30 min, 25 °C). Last, the alkylation reaction was quenched by adding 4 mM DTT (30 min, 25 °C). Proteins were predigested with 1 μL LysC (0.5 mg/mL, Wako) for 2 h at 25 °C, diluted in 600 μL 50 mM triethylammonium bicarbonate buffer (TEAB, 1 M) and digested with 1.5 μL trypsin (0.5 mg/mL, Promega) for 15 h at 37 °C. Then, trypsin digestion was stopped by adding 1% formic acid (FA) and in the following, peptides were desalted with 50 mg Sep-Pak C18 cartridges (Waters Corp.). Therefore, the C18 material of each column was equilibrated with 1 mL acetonitrile, 1 mL elution buffer (80% acetonitrile, 0.5% FA in H_2O) and 3 mL wash buffer (0.5% FA in H_2O). As

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soon as the peptides were loaded on the cartridges, they were washed with 3 mL wash buffer and eluted (2× 250 μL elution buffer). Afterward, peptides were freeze-dried using a speedvac centrifuge and reconstituted in 30 μL 1% FA. Finally, they were sonicated for 10 min, filtered through 0.22 μm Ultrafree-MC centrifugal filters (Merck, UFC30GVNB) and stored at −20 °C until MS/MS measurement. All enrichment experiments were measured on the Q Exactive instrument. Experiments were conducted with 4 to 12 replicates.

Preparative *in Situ* Labeling of AMPylated Proteins in HeLa Cells after Infection—LFQ. For the label-free quantification-based infection assays, HeLa cells were seeded either in 6 cm dishes (infection with *V. parahaemolyticus*) or in 10 cm dishes (infection with *V. parahaemolyticus* wt *vs* H348A, infections with *P. aeruginosa* or *E. coli*). The further procedure was performed as already mentioned in Analytical *in Situ* Labeling of AMPylated Proteins in HeLa Cells after Infection with some changes. In brief, the two dishes thought for cell counting were treated with **pro-N6pA**, whereas the other dishes were either treated with 100 μM **pro-N6pA** or 0.1% DMSO, depending on the experimental setup. An overnight culture of the bacterial strain was inoculated, with which a fresh day culture was started the next day. The two additional dishes of HeLa cells were counted (for 10 cm dish: washed with 2 mL PBS, added 1 mL Accutase, resuspended in 3 mL DMEM, diluted 1:1 with trypan blue), the bacterial OD_{600} value measured and the respective volume for MOI 10 was calculated. The bacteria were harvested and resuspended in DMEM (plus 2 mM L-glutamine) and either 10 μM **pro-N6pA** or 0.01% DMSO. To each 10 cm dish, 7 mL of this suspension were added (2 mL to 6 cm dish) before further incubation at 37 °C and 5% CO_2 for a previously defined period.

Once the desired infection time was completed, images of each dish were taken and the human cells were scraped off, washed in PBS, and lysed in 250 μL lysis buffer. Further sample handling was performed according to Preparative *in Situ* Labeling—Label-Free Quantification (LFQ). Equal protein amounts were adjusted prior to performing the click reaction with azide-PEG_3-biotin conjugate. The proteins were precipitated overnight, pelletized and washed twice with ice-cold MeOH. The pellet was resuspended in 1 mL 0.2% SDS in PBS and added to 50 μL prewashed (3× 1 mL 0.2% SDS in PBS) avidin-agarose beads. After incubation for 1.5 h at 25 °C, the beads were washed (3× 1 mL 0.2% SDS in PBS, 2× 1 mL 6 M urea, 3× 1 mL PBS) and the proteins reduced and alkylated (1 mM DTT, 45 min, 25 °C; 5.5 mM IAA, 30 min, 25 °C). The alkylation reaction was stopped (4 mM DTT, 30 min, 25 °C) and the proteins were predigested (1 μL LysC (0.5 mg/mL), 2 h, 25 °C) prior to being digested overnight (600 μL 50 mM TEAB, 1.5 μL trypsin (0.5 mg/mL), 15 h, 37 °C). Digestion was stopped (1% FA) and peptides were desalted as described previously. These peptides were freeze-dried and reconstituted in 30 μL 1% FA for MS/MS measurements on the Q Exactive instrument.

Preparative *in Situ* Labeling of AMPylated Proteins in HeLa Cells after Infection—SILAC—Enrichment. Both, "heavy" and "light" labeled HeLa cells were seeded in 10-cm dishes (2 additional dishes per label) and treated with **pro-N6pA**. Further procedure was performed according to Preparative *in Situ* Labeling of AMPylated Proteins in HeLa Cells after Infection—LFQ with minor modifications. Half of the dishes of "heavy" and "light" labeled HeLa cells were treated with bacteria (MOI 10), the rest without bacteria (MOI 0). Bacterial pellets were resuspended in SILAC-DMEM supplemented with 2 mM L-glutamine, 10 μM **pro-N6pA** and "heavy" or "light" lysine and arginine. After cell lysis, a BCA assay was performed, and the resulting cell lysate was divided into two portions for further analysis: protein enrichment (250 μg) and full proteome analysis (250 μg). For the enrichment, "light" lysate being treated with bacteria was combined with "heavy" lysate treated without bacteria and *vice versa*. All combined samples were adjusted to a total volume of 250 μL with 0.2% SDS in PBS.

Preparative *in Situ* Labeling of AMPylated Proteins in HeLa Cells after Infection—SILAC—Whole Proteome. For the whole proteome analysis, the same cell lysate was used as for the SILAC enrichment experiments. After successfully determining the lysate's protein concentration by BCA assay, 250 μg protein of the "heavy" lysate treated with bacteria was combined with 250 μg of the "light" lysate treated without bacteria and *vice versa*. The volume of the combined samples was adjusted to 250 μL using 0.2% SDS in PBS, and 0.96 mM $CuSO_4$ were added to each sample. They were further incubated for 1.5 h at 25 °C prior to being precipitated overnight using 1.1 mL acetone. Protein pellets were harvested by centrifugation (15 min, 4 °C, 13 000 rpm) and washed with ice-cold MeOH. Therefore, 1 mL of MeOH was added to the protein pellet which was resuspended by sonication (10% intensity, 10 s) and harvested again as described previously. The MeOH washing step was repeated once again, and the resulting protein pellet was dissolved in 200 μL digestion buffer (3.9 M urea, 1.1 M thiourea in 20 mM Hepes, pH 7.5). Reduction and alkylation of disulfides as well as further protein digestion was performed as described for Preparative *in Situ* Labeling of AMPylated Proteins in HeLa Cells after Infection—LFQ. The protein digestion was stopped by adding 1% FA and peptides were desalted with Sep-Pak C18 cartridges (Waters Corp.) as previously described. As last step, peptides were freeze-dried, reconstituted in 1% FA in H_2O to a final concentration of 2 μg/μL and filtered. Whole proteome samples were measured on the Orbitrap Fusion with a sample injection volume of 4 μL.

***In Situ* Site Identification of AMPylated Proteins during Infection with TEV-Cleavable Linker.** After performing an infection assay as described before with *V. parahaemolyticus* having **pro-N6pA** and bacteria (MOI 10) as positive sample and DMSO treated cells infected with bacteria (MOI 10) as negative control, cell lysate was adjusted to a protein concentration of 6 mg and a total volume of 2 mL. Click reaction was performed with all three samples (2× positive, 1× negative), for 1.5 h at 25 °C (0.096 mM azide-TEV-biotin (10 mM stock in DMSO), 0.51 mM TCEP (53 mM stock in ddH_2O), 0.1 mM TBTA (83.5 mM stock in DMSO), and 0.96 mM $CuSO_4$ (stock 50 mM in ddH_2O).[41] Subsequently, proteins were precipitated with at least 4-fold excess of acetone in an upright position overnight at −20 °C. MeOH washing of the pellets as well as avidin-agarose bead enrichment and on-beads digest was performed according to the procedure Preparative *in Situ* Labeling of AMPylated Proteins in HeLa Cells after Infection—LFQ with minor deviations. Namely, 100 μL of beads slurry were used per sample and the digestion buffer only contained urea (no thiourea). After digestion, the beads were transferred onto membrane filters (Ultrafree-MC centrifugal filters (Merck, UFC30GVNB)) which were washed beforehand (500 μL 1% FA in H_2O, 1 min with 1000*g*). The flow through was further

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processed according to the standard enrichment protocol described in Preparative *in Situ* Labeling of AMPylated Proteins in HeLa Cells after Infection—LFQ. The beads were washed with H_2O (2× 50 μL), PBS (3× 600 μL) and again H_2O (3× 600 μL). They were further resuspended in 150 μL TEV buffer (141 μL H_2O, 7.5 μL 20× TEV buffer (Invitrogen), 1.5 μL 100 μM DTT in H_2O) and transferred into 1.5 mL low-bind Eppendorf tubes. Next, they were centrifuged (2 min, 25 °C, 400*g*), the supernatant was removed and the remaining beads—once again—resuspended in 150 μL TEV buffer. 50 U of AcTEV protease (5 μL, 10 U/μL, Invitrogen) were added to each sample. TEV digestion was conducted overnight with agitation at 29 °C prior to transferring the beads onto a new membrane filter. As next step, the beads were spun down (1 min, 1000*g*) and washed with H_2O (2× 50 μL). The flow through was collected and acidified with 4 μL FA. Samples were desalted on stage tips (self-made pipet tips containing double C_{18} layer, Empore disc-C18, 47MM, Agilent Technologies) which were equilibrated and washed before (70 μL MeOH, 70 μL elution buffer (80% acetonitrile, 0.5% FA in H_2O) and 3× 70 μL 0.5% FA in H_2O; 1 min with 1000*g*). The peptides were transferred onto the membranes, spun down (5 min, 25 °C, 1000*g*) and washed thrice with 70 μL 0.5% FA in H_2O. Finally, the modified peptides were eluted (2× 30 μL elution buffer) and lyophilized prior to being reconstituted in 30 μL 1% FA and filtered. Samples were measured on the Fusion instrument.

Mass Spectrometry Analysis on Q Exactive. Enrichment samples were analyzed on a Q Exactive Plus instrument (Thermo Fisher) coupled to an UltiMate 3000 nano-HPLC (Dionex) equipped with an Acclaim C18 PepMap100 75 μm ID × 2 cm trap column (Thermo Fisher) and a 25 cm Aurora Series emitter column (25 cm × 75 μm ID, 1.6 μm FSC C18) (Ionoptics) in an EASY-spray setting. Both columns were heated to 40 °C during the measurement process. For analysis, 4 μL of each peptide sample were injected. The samples were first loaded on the trap column with 0.1% trifluoroacetic acid (TFA) applying a flow rate of 5 μL/min. Second, the samples were transferred to the separation column with a flow rate of 0.400 μL/min where peptides were separated with a 152 min gradient (buffer A: H_2O with 0.1% FA, buffer B: acetonitrile with 0.1% FA). The gradient consisted of the following steps: holding buffer B at 5% for 7 min, further increasing buffer B to 22% during 105 min, to 32% the next 10 min and to 90% within 10 min. Once buffer B reached a concentration of 90%, this solvent ratio was maintained for another 10 min before decreasing it to 5% in 0.1 min, at which level it stayed until the end of the run. Peptides were ionized at a capillary temperature of 275 °C and the instrument was operated in a Top12 data dependent mode. For full scan acquisition, the Orbitrap mass analyzer was set to a resolution of R = 140 000, an automatic gain control (AGC) target of 3×10^6, and a maximal injection time of 80 ms in a scan range of 300−1500 m/z. Precursors having a charge state of >1, a minimum AGC target of 1×10^3 and intensities higher than 1×10^4 were selected for fragmentation. Peptide fragments were generated by HCD (higher-energy collisional dissociation) with a normalized collision energy of 27% and recorded in the Orbitrap at a resolution of R = 17 500. Moreover, the AGC target was set to 1×10^5 with a maximum injection time of 100 ms. Dynamic exclusion duration was set to 60 s and isolation was performed in the quadrupole using a window of 1.6 m/z.

Mass Spectrometry Analysis on Orbitrap Fusion. Whole proteome samples were measured on an Orbitrap Fusion instrument (Thermo Fisher Scientific Inc.) being equipped with an UltiMate 3000 nano HPLC system (Dionex) and the following trap and separation columns (heated to 50 °C, both Thermo Fisher): Acclaim C18 PepMap 100 75 μm ID × 2 cm and Acclaim PepMap RSLC C18 75 μm ID × 50 cm. Samples were measured in an EASY-spray setting and loaded on the trap column with a flow rate of 5 μL/min using 0.1% TFA. Subsequently, samples were separated using a flow rate of 0.3 μL/min and a 152 min gradient (buffer A: H_2O with 0.1% FA, buffer B: acetonitrile with 0.1% FA, gradient: starting with 5% buffer B, holding it for 7 min, the next 105 min increasing buffer B from 5% to 22%, from 22% to 32% buffer B in 10 min, then to 90% buffer B in 10 min and holding it there for 10 min, decreasing it to buffer B in 0.1 min and holding it there until the end of the run). The instrument was operated in a 3 s top speed data dependent mode. Peptides were ionized with a capillary temperature of 275 °C and MS full scans were performed with a resolution of R = 120 000 in the Orbitrap. Further settings were an AGC ion target value of 2×10^5 and a scan range between 300 to 1500 m/z having a maximal injection time of 50 s. For MS^2 scans, precursors having charge states between 2 and 7 and intensities higher than 5×10^3 were further selected for fragmentation. The fragmentation was conducted with an HCD collision energy of 30% and the isolation was performed in the quadrupole using a window of 1.6 m/z. The AGC target was set to 1×10^4, the maximum injection time to 35 ms and the function "inject ions for all available parallelizable time" was enabled. Furthermore, the dynamic exclusion time was set to 60 s with 10 ppm low and high mass tolerance.

The site-ID experiments applying the TEV cleavable linker were analyzed on the Orbitrap Fusion with slightly modified parameters. After HCD fragmentation, peptides having the following targeted masses of m/z 558.3, 279.65, or 186.77 (adenine-TEV, single, double, or triple charged), m/z of 672.33, 336.67, or 224.78 (adenosine-TEV, single, double, or triple charged) or m/z of 770.31, 385.66, or 257.44 (phosphoadenosine-TEV, single, double, or triple charged) were further selected for electron-transfer dissociation (ETD) fragmentation while prioritizing the highest charge states. ETD fragmentation scans were acquired in the Orbitrap with a resolution of R = 30 000, a maximum infection time of 40 ms and an AGC target value of 5×10^4.

Bioinformatics and Statistics. For peptide and protein identification, MS data were processed with MaxQuant (version 1.6.2.10) having Andromeda as search engine.[42] Searches were performed against the UniProt database for *Homo sapiens* (taxon identifier: 9606, canonical version, reviewed and unreviewed proteome, not older than three months prior to MS measurements). For infection assays, all proteins in the UniProt database of the respective bacteria (*Vibrio parahaemolyticus* serotype O3:K6, strain RIMD 2210633, taxon identifier: 223926; *Pseudomonas aeruginosa* PAO1, taxon identifier: 208964, and *Escherichia coli* CFT073, taxon identifier: 199310; canonical versions, reviewed and unreviewed proteomes, not older than three months prior to MS measurements) were added to the MaxQuant contaminants file. As search parameters, mostly default settings were applied (trypsin/P as digest enzyme, max. 2 missed cleavages, oxidation (M) and acetylation (protein N-term) as variable modifications, carbamido-methylation (C) as fixed modifica-

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tion, label-free quantification LFQ with a minimal ratio count of 2, min peptide length 7, 20 ppm for precursor mass tolerance (FTMS MS/MS match tolerance) and 0.5 Da for fragment mass tolerance (ITMS MS/MS match tolerance)). Furthermore, proteins were identified using PSM FDR 0.01, protein FDR 0.01, min peptides 2, min razor + unique peptides 2, min unique peptides 2, razor protein FDR enabled, second peptides enabled. Additionally, the match between run function with its settings match time window of 0.7 min and alignment time window of 20 min was also enabled.

Multiplicity was set to 2 for SILAC experiments. Then, parameter groups were defined and in the setting group-specific parameters, labels were selected. For samples containing bacterial treated (MOI 10) "heavy" lysate and bacteria free (MOI 0) "light" lysate, Arg10 and Lys8 were selected for the first and Arg6 and Lys4 for the latter. For samples containing bacterial treated (MOI 10) "light" lysate and bacteria free (MOI 0) "heavy" lysate, Arg 10 and Lys8 were selected for the first and Arg6 and Lys 4 for the latter. The requantify option was enabled for SILAC samples.

Peptides of the site-ID experiments were searched for the fixed carbamidomethyl (C) modification (57.02146) as well as for the variable modification of the "light" TEV linker coupled to AMP (751.2915) on serine, tyrosine or threonine residues. Furthermore, the options min peptides, min razor + unique peptides as well as min unique peptides were used with default settings (1 − 1 − 0).

For further statistical data analysis, the Perseus software (version 1.6.2.3) was used. Therefore, the *protein groups* table in the *txt* folder resulting from the MaxQuant analysis was uploaded into the program. LFQ intensities or SILAC ratios were $\log_2$ transformed and putative contaminants as well as reverse hits were removed. Next, all samples were categorical annotated according to their treatment conditions. Then, the resulting matrix was filtered against 75% of valid values in at least one group. Missing values were imported from normal distribution (width 0.3, down shift 1.8, total matrix) and *p*-values were obtained by a two-sided two sample *t* test over replicates with a Benjamini−Hochberg false discovery rate correction (FDR 0.05). Data were visualized by generating scatter plots [Student's *t* test difference (treated/control) against −log student's *t* test *p*-value (treated/control)].

For SILAC experiments, rows were filtered for 75% of valid values in total and a one-sample Student's *t* test was performed.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium[43] *via* the PRIDE[44] partner repository with the data set identifier PXD022078.

CFU Assays. For each bacterial strain, the CFU (colony forming units) *vs* OD_{600} assay was performed at least twice. Therefore, an overnight culture of the desired bacterial strain was diluted 1:100 into fresh media and cultivated under standard condition. At various time points, the OD_{600} value was measured and 5 μL of the day culture were further diluted. Each dilution was plated on pure LB agar plates in triplicates and incubated for 24 h at 37 °C. Each plate, having between 12 and 120 colonies per plated sample, was counted. Having various data points connecting each OD_{600} value to a certain CFU amount, different mathematical functions were applied to represent the OD_{600} to CFU ratio in the area of interest.

AMPylation Assay. The *in vitro* AMPylation assay was performed as described previously.[19] Briefly, the purified AMPylator VopS (AA 31−378, 1 μM) was incubated with either 100 μM ATP or 100 μM N^6pATP and the AMPylation targets Cdc42 (AA 1−188, 50 μM) or RhoG (full protein, 5 μM) with 2 μL 10× AMPylation buffer (1× buffer: 20 mM Hepes pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mg/mL BSA and 1 mM DTT). Samples were diluted to a final volume of 20 μL. In case of negative control experiments, the volume of the missing ingredient was compensated by water. The AMPylation reaction was performed for 1.5 h at 30 °C before being further analyzed.

Intact Protein MS Measurement (IPMS). Intact protein MS of recombinant protein or proteins modified *via* the AMPylation assay were performed as described previously.[45] The samples were measured with an UltiMate 3000 HPLC system (Dionex) being equipped with a Massprep online desalting cartridge (Waters) and coupled to a Finnigan LTQ-FT Ultra mass spectrometer (Thermo Fisher Scientific). Ions were generated by electrospray ionization (capillary temperature 275 °C, spray voltage 4.0 kV, tube lens 110 V, capillary voltage 48 V, sheath gas 60 arb, aux gas 10 arb, sweep gas 0.2 arb) and separated with a flow rate of 0.4 mL/min and the following gradient: (buffer A: 0.1% FA in H_2O, buffer B: 0.1% FA, 9.9% H_2O in acetonitrile, 1 min hold 6% buffer B, in 1.5 min increase buffer B to 95%, hold at 95% buffer B for 2 min, decrease to 6% buffer B in 0.2 min, stay at 6% buffer B for further 0.3 min). The instrumented was operated with a resolution of R = 200 000 and a mass range of m/z from 600 to 2000 while acquiring full scans. The ProMass software for Xcalibur (Version 2.8) was used for deconvolution (input m/z range: 600−2000; output mass range: 15 000−30 000 Da).

Site-ID of Recombinant Protein. Once the successful AMPylation reaction was confirmed by IPMS, the samples were diluted in 150 μL digestion buffer (3.9 M urea, 1.1 M thiourea in 20 mM Hepes, pH 7.5) and treated with DTT (1.2 mM, 1 h, 25 °C), IAA (6.5 mM, 1.5 h, 25 °C), and DTT (4.7 mM, 45 min, 25 °C). Next, the samples were predigested with LysC (1 μL LysC, 0.5 mg/mL, 2 h, 25 °C) before diluting them in 600 μL 50 mM TEAB and digesting them with trypsin (1.5 μL, 0.5 mg/mL, 15 h, 37 °C). After digestion, samples were acidified (1% FA in H_2O) and desalted using stage-tips. The membranes were washed and equilibrated (70 μL acetonitrile, 70 μL elution buffer (80% acetonitrile, 0.5% FA in H_2O), 3× 70 μL 0.5% FA in H_2O) before being loaded with samples. Afterward, membranes were washed again (3× 70 μL 0.5% FA in H_2O) and peptides were eluted with elution buffer (2× 30 μL). The freeze-dried peptides were reconstituted in 25 μL 1% FA in H_2O and filtered (0.22 μm Ultrafree-MC centrifugal filters (Merck, UFC30GVNB)). The samples (5 μL) were injected on the Q Exactive instrument for further analysis.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsinfecdis.0c00740.

Figure S1: Volcano plot of pro-N6pA treated HeLa cells; Figure S2: SDS-PAGE of HeLa cells treated with **pro-N6pA** and infected with various amounts of bacteria; Figure S3: Volcano plots of SILAC based experiments: Figure S4: SDS-PAGE of HeLa cells time-dependently infected with *P. aeruginosa* PAO1 or *E. coli* CFT073; Figure S5: Volcano plots of HeLa cells treated with **pro-N6pA** and infected with *P. aeruginosa* PAO1; Figure S6:

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Volcano plots of HeLa cells treated with **pro-N6pA** and infected with *E. coli* CFT073; Table S2: All primer sequences (PDF)

Table S1: All final Perseus output tables (XLSX)

AUTHOR INFORMATION

Corresponding Author

Stephan A. Sieber − *Department of Chemistry, Chair of Organic Chemistry II, Center for Functional Protein Assemblies (CPA), Technische Universität München, 85748 Garching, Germany;* orcid.org/0000-0002-9400-906X; Email: stephan.sieber@tum.de

Authors

Theresa Rauh − *Department of Chemistry, Chair of Organic Chemistry II, Center for Functional Protein Assemblies (CPA), Technische Universität München, 85748 Garching, Germany*

Sophie Brameyer − *Department of Biology I, Microbiology, Ludwig-Maximilians-Universität München, 82152 Martinsried, Germany*

Pavel Kielkowski − *Department of Chemistry, Ludwig-Maximilians-Universität München, 81377 München, Germany*

Kirsten Jung − *Department of Biology I, Microbiology, Ludwig-Maximilians-Universität München, 82152 Martinsried, Germany*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsinfecdis.0c00740

Author Contributions

T.R. and S.A.S. designed and conceived the project. T.R. synthesized the probe and designed as well as executed proteomic (infection) experiments including site-ID measurements. Data analysis and interpretations were done by T.R. with contributions from P.K. Mutants were created with the assistance of S.B. and K.J. The first draft of the manuscript was written by T.R. and S.A.S. and was further reviewed by all authors. All authors have read and given approval to the final version.

Notes

The authors declare no competing financial interest.

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