A Tailored Phosphoaspartate Probe Unravels CprR as a Response Regulator in Pseudomonas Aeruginosa Interkingdom Signaling

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Pseudomonas aeruginosa is a difficult-to-treat Gram-negative bacterial pathogen causing life-threatening infections. Adaptive resistance (AR) to cationic peptide antibiotics such as polymyxin B impairs the therapeutic success. This self-protection is mediated by two component systems (TCS) consisting of a membrane-bound histidine kinase and an intracellular response regulator (RR). As phosphorylation of the key RR aspartate residue is transient during signaling and hydrolytically unstable, the study of these systems is challenging. Therefore, we applied a tailored reverse polarity chemical proteomic strategy to capture this transient modification and read-out RR phosphorylation in complex proteomes using a nucleophilic probe. An ideal trapping methodology was developed with a recombinant RR demonstrating the importance of fine-tuned acidic pH values to facilitate the attack on the aspartate carbonyl C-atom and prevent unproductive hydrolysis. Analysis of Bacillus subtilis and P. aeruginosa proteomes revealed the detection of multiple phosphoaspartate sites, which closely resembled the conserved RR sequence motif. With this validated strategy we dissected the signaling of dynorphin A, a human peptide stress hormone, which is sensed by P. aeruginosa to mediate AR. Intriguingly, our methodology identified CprR as an unprecedented RR in dynorphin A interkingdom signaling.

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A tailored phosphoaspartate probe unravels CprR as a response regulator in *Pseudomonas aeruginosa* interkingdom signaling

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Abstract

*Pseudomonas aeruginosa* is a difficult-to-treat Gram-negative bacterial pathogen causing life-threatening infections. Adaptive resistance (AR) to cationic peptide antibiotics such as polymyxin B impairs the therapeutic success. This self-protection is mediated by two component systems (TCS) consisting of a membrane-bound histidine kinase and an intracellular response regulator (RR). As phosphorylation of the key RR aspartate residue is transient during signaling and hydrolytically unstable, the study of these systems is challenging. Here, we apply a tailored reverse polarity chemical proteomic strategy to capture this transient modification and read-out RR phosphorylation in complex proteomes using a nucleophilic probe. In-depth mechanistic insights into an ideal trapping strategy were performed with a recombinant RR demonstrating the importance of fine-tuned acidic pH values to facilitate the attack on the aspartate carbonyl C-atom and prevent unproductive hydrolysis. Analysis of *Bacillus subtilis* and *P. aeruginosa* proteomes revealed the detection of multiple phosphoaspartate sites, which closely resembled the conserved RR sequence motif. With this validated strategy we dissected the signaling of dynorphin A, a human peptide stress hormone, which is sensed by *P. aeruginosa* to prepare AR. Intriguingly, our methodology identified CprR as an unprecedented RR in dynorphin A interkingdom signaling.

Introduction

*Pseudomonas aeruginosa*, a Gram-negative bacterial pathogen, is listed by the WHO as critical priority for the development of novel antibiotics to fight multiresistance. Its intricate pathogenesis mechanisms cause severe diseases such as pneumonia in cystic fibrosis patients. These mechanisms are controlled by elaborating signal transduction, which coordinate population density (quorum sensing), virulence and antibiotic defense.

*P. aeruginosa* was recently shown to sense the human peptide hormone dynorphin, which is secreted by eukaryotic cells upon stress e.g. induced by bacterial infections. While dynorphin only exhibits moderate antibiotic activity, other cationic antimicrobial peptides, produced in response to the infection, are able to destroy the intruding pathogen. Recently, chemical proteomics revealed that dynorphin A is sensed by the *P. aeruginosa* two-component system ParRS, which leads to upregulation of the antimicrobial peptide response mediated by the ArnBCADTEF system. This interkingdom signaling between a human stress hormone and *P. aeruginosa* confers a competitive advantage to the pathogen, which listens in on the human defense mechanism and thereby prepares itself for a forthcoming attack.

ParR and ParS belong to the prevalent group of two-component gene regulatory systems (TCS) with crucial roles for the physiology and pathogenicity of bacteria (Fig. 1a). They are composed of a membrane-bound histidine kinase (HK) and a response regulator (RR). Signal transduction occurs via sensing of external stimuli and signal relay to the kinase domain, which induces autophosphorylation of a histidine residue. In a second step the phosphate group is shuttled from the histidine to a conserved aspartate of the RR yielding phosphoaspartate (pAsp) and inducing further downstream signaling. TCSs facilitate rapid signaling events such as driving motility changes in chemotaxis, expression of virulence factors in quorum sensing and triggering adaptive resistance. In contrast to the chemically stable phosphorylation of serine, threonine and tyrosine in eukaryotic cells, the prokaryotic pAsp is a mixed carboxylic acid-phosphoric acid anhydride (acyl phosphate), which has limited half-life under neutral, acidic and alkaline conditions impeding its detection via common analytical methods such as immobilized metal affinity chromatography (IMAC) followed by liquid chromatography coupled to tandem MS (LC-MS/MS). Thus, a chemical methodology is required, which rapidly transforms the labile acyl phosphate into a stable modification that can be detected using standard proteomic workflows. Here, α-effect nucleophiles such as hydroxylamine capture the labile modification yielding stable *N*-hydroxy-asparagine derivatives (Fig. 1b). This reaction requires slightly acidic conditions in order to promote a selective attack at the carbonyl C-atom, while neutral conditions favor phosphate cleavage by attack on the P-atom. To expand the chemical proteomic toolbox for monitoring pAsp modifications, Chang et al. recently introduced a desthiobiotin containing hydroxylamine (DBHA) probe.
facilitating the profiling of pAsp in *Escherichia coli* proteomes under neutral pH (Fig. 1c). We here provide in-depth molecular insights into the preferred nucleophilic attack of hydroxylamine probes under different pH values and demonstrate the importance of acidic conditions along with solubilizing detergents for selective modification of the aspartate carboxylate during the reverse polarity activity-based profiling (RP-ABPP) approach. Moreover, our study accounts for the reactivity toward other electrophilic residues and elucidates their overall impact on model proteins. The tailored conditions of these model studies were integrated into an advanced chemical proteomic platform featuring a minimal clickable hydroxylamine alkyne probe (HA-yne) with access to sterically demanding protein pockets (Fig. 1c) as well as the application of isotopically labeled desthiobiotin azide (isoDTB) tags for the detection and quantification of pAsp sites. This methodology revealed 123 HA-yne modified aspartate sites with high fidelity, whose sequence motif closely resembles the known RR recognition sequence. These fine-tuned conditions enabled the surprising discovery of dynorphin A-mediated phosphorylation of the RR CprR, a so far unknown target in interkingdom signaling.

**Results**

**Acyl phosphates are trapped as stable hydroxamates under optimized acidic conditions.**

In this work, we aimed to decipher the role of dynorphin A in interkingdom signaling by unraveling its RRs in *P. aeruginosa*. Prior to these studies, we refined the nucleophilic profiling platform to become most efficient for this need. As previous work did not focus on the molecular basis of the nucleophile reaction with acyl phosphates within proteins, we initiated this study with a well-established model RR from *E. coli* termed PhoB. PhoB is a key regulator in *E. coli* phosphate metabolism, which is phosphorylated at Asp53 by PhoR to initiate transcription of its client genes. E. coli PhoB was recombinantly overexpressed, purified and phosphorylated at Asp53 in *vitro* with acetyl phosphate as a phosphor donor. Intact protein MS (IPMS) confirmed the addition of one phosphate group to PhoB, while a corresponding aspartate-to-asparagine (D53N) mutation prevented this modification demonstrating site specificity (Fig. 2b and Fig. S1†). To probe the conversion of phosphorylated D53 with a strong α-effect nucleophile, we treated phosphorylated PhoB with sterically hindered hydroxylamine at previously established pH = 7 (final concentration of NH$_2$OH·HCl = 0.5 M) in a time-dependent intact protein MS assay. However, this experiment resulted in a lack of hydroxylamine labeling in line with the favored attack at the phosphorous atom, leading to hydrolysis and loss of the modification (Fig. S2, ES1†). In order to foster an attack at the carbonyl C-atom, we conducted the same experiment at acidic pH (pH = 4) and a rapid modification to N4-hydroxyasparagine was observed within minutes with quantitative conversion after 30 min (Fig. 2b and Fig. S3a, ES1†). As expected, unphosphorylated PhoB did not show any mass change (Fig. S4, ES1†). We thus conclude that acidic pH is crucial for productive acyl phosphate trapping and maximizing labeling...
efficiency.

Prior to the analysis of pAsp modifications in complex biological samples, the sensitivity of detection needed to be enhanced by application of an enrichment strategy. In order to minimize undesired steric clashes within protein pockets, we synthesized a minimal hydroxylamine probe linked to an alkyne handle (HA-yn), which was previously used for oxime ligations (Fig. 1c).24 Once its reaction with pAsp is complete, desthiobiotin azide can be clicked to the alkyne moiety facilitating subsequent affinity enrichment. Furthermore, the modular nature of the alkyne handle has the advantage that it allows attachment of various labels for different detection methods like fluorophores for gel-based analysis or isoDTB tags18 for quantitative proteomics. Similar to hydroxylamine, the HA-yn probe led to rapid conversion of phosphorylated PhoB under the set conditions of pH = 4 (Fig. 2c and Fig. S3b, ESI†). For the application of the probe in whole proteomes, it is crucial to trap the transient acyl phosphate modifications rapidly upon cell lysis. We thus added the probe directly to the lysis buffer (pH = 4). Labeling of B. subtilis revealed an almost quantitative protein precipitation attributed to the high probe concentration at acidic labeling conditions (Fig. S5, ESI†). A screen of diverse detergents demonstrated that the addition of 1% (w/v) lauryldimethylamine oxide (LDAO) maintains protein solubility (Fig. S6, ESI†). Labeling under these conditions, acidic pH and LDAO, resulted in the detection of strong fluorescent bands upon clicking the probe to rhodamine azide and fluorescent SDS-PAGE analysis. Sufficient labeling intensity was observed at high concentrations starting at 125 mM HA-yn (Fig. S7, ESI†), which we set as ideal parameters for subsequent LC-MS/MS studies. We next spiked HA-yn labeled PhoB into the labeled proteome, clicked to commercially available desthiobiotin azide and upon streptavidin bead enrichment, proteolytic digestion and elution from the beads, the peptides were subjected to LC-MS/MS analysis. Our chemoproteomic workflow successfully identified the HA-yn modification at the expected residue D53 of PhoB (Fig. 3).

A hydroxylamine-alkyne probe reveals modified aspartate sites at conserved response regulator motifs in bacterial proteomes

With optimized conditions for proteome labeling in hand, we commenced with the analysis of modification sites in B. subtilis and P. aeruginosa proteomes. In brief, following HA-yn treatment during cell lysis, the proteome was clicked to desthiobiotin azide, digested and peptides were enriched on streptavidin beads followed by LC-MS/MS analysis (Fig. 4a and Fig. 3).
Fig. S8, ESI†. Overall, we detected 141 and 198 modified Asp sites with high fidelity in *B. subtilis* and *P. aeruginosa*, respectively, which includes 15% (6/41) and 19% (18/93) of known pAsp sites from UniProt (Fig. 4b, Fig. S9a, and Table S1, ESI†). Moreover, within pAsp annotated proteins the expected pAsp residue was modified almost exclusively (24/25). Within this dataset, so far only poorly characterized proteins, like the probable response regulator PA2798 and PA1243, were detected with modification at the expected residues confirming these pAsp sites. Hence, our method verifies many analogous conditions and residues for its detection. We thus performed a quantitative analysis on test proteins, α-Casein, BSA and phosphorylated PhoB, which we treated with the probe under labeling conditions and determined the extent of probe addition on each protein. Interestingly, while phosphorylated D53 on PhoB was labelled further investigated if the applied labeling conditions could be responsible for this effect. It is known that Asn and Gln containing peptides can form succinimide as well as glutarimide intermediates which could be attacked by nucleophiles resulting in ring opening and covalent bond formation (Fig. S12, ESI†). Although this deamination reaction may only occur to a minor extent, the MS workflow would still be sensitive enough for its detection. We only performed a quantitative analysis on test proteins, α-Casein, BSA and phosphorylated PhoB, which we treated with the probe under labeling conditions and determined the extent of probe addition on each protein. Interestingly, while phosphorylated D53 on PhoB was labelled with near quantitative conversion, modification of unphosphorylated proteins was not detected (Fig. S13, ESI†).

As we cannot *a priori* exclude the capture of other electrophilic modifications such as ADP-ribosylation,27 glutamate methylation28 and transamidation29 we extended our analysis to all amino acids as possible modification sites. To our surprise we obtained significant labeling at Glu, Asn and Gln which cumulated in about 6000 additional sites, foremost on Gln, in both proteomes (Fig. S11 and Table S1, ESI†). As previous work did not account for modification sites other than Asp, we further investigated if the applied labeling conditions could be responsible for this effect. It is known that Asn and Gln containing peptides can form succinimide as well as glutarimide intermediates which could be attacked by nucleophiles resulting in ring opening and covalent bond formation (Fig. S12, ESI†). Although this deamination reaction may only occur to a minor extent, the MS workflow would still be sensitive enough for its detection. We thus performed a quantitative analysis on test proteins, α-Casein, BSA and phosphorylated PhoB, which we treated with the probe under labeling conditions and determined the extent of probe addition on each protein. Interestingly, while phosphorylated D53 on PhoB was labelled with near quantitative conversion, modification of unphosphorylated proteins was not detected (Fig. S13, ESI†).

![Diagram](attachment:diagram.png)

**Fig. 4** RP-ABPP workflow and binding site analysis. (a) Bacterial cells of *P. aeruginosa* and *B. subtilis* were lysed in a buffer containing the nucleophilic HA-yne probe (125 mM) at pH = 4 and clicked to desthiobiotin-azide (DTB-PEG₅-N₃). Modified proteins were tryptically digested, enriched on streptavidin beads, eluted from the beads and subjected to LC-MS/MS analysis. (b) Table of HA-yne modified sites in *P. aeruginosa*, that also have UniProt annotated pAsp sites. Additionally, the corresponding genes, TCSs and their implications are listed. For the complete list of modified sites see Table S1 (ESI†). The analogous table for *B. subtilis* is shown in Fig. S9a (ESI†). Only sites with an Andromeda24 localization probability exceeding 75% for the relative HA-yne modified residue were included in the analysis using MaxQuant software.24 (c) Comparison of pAsp annotated and HA-yne modified sequence motifs in *P. aeruginosa* using pLogo.30 Residues at positions ranging from -10 to +10 next to the phosphorylation/modification site were included in the analysis. pAsp annotated and HA-yne modified sequences (fg) were compared with the complete proteomic background (bg) in *P. aeruginosa* from the UniProt database. Orange horizontal bars indicate the Bonferroni-corrected statistical significance (p = 0.05).
While we cannot exclude some contribution from electrophilic modifications on Asn and Gln, the vast number of detected sites in the proteomic context suggests an unspecific background modification, which occurs to a minor extent. This assumption is encouraged by the fact, that modified sites of predominantly high abundant proteins are detected (Fig. S14, ESI†). Since RRs are commonly low abundant, the ability to successfully identify many known pAsp sites in a complex background underscores our efficient labeling and enrichment methodology. In line with our initial goal, we thus recommend utilizing this strategy for detection of Asp phosphorylation.

Dynorphin A exposure uncovers CprR as a response regulator in P. aeruginosa interkingdom signaling
The refined and validated workflow was finally utilized for the investigation of downstream dynorphin A signaling in P. aeruginosa. Cells were treated with either dynorphin A or DMSO for either 1, 5 or 15 min followed by HA-ynE addition during lysis. Proteomes were clicked to desthiobiotin azide, digested and peptides enriched on streptavidin beads followed by LC-MS/MS analysis to unravel their identity and site of modification. Overall, 103 vs. 113 pAsp sites were detected in dynorphin A treated and untreated samples, respectively (Table S1). Importantly, among the annotated pAsp sites only one residue, D53 in the protein assigned as cationic peptide response regulator (CprR), was solely present in the dynorphin A treated sample at all time points. CprR and its cognate sensor kinase CprS belong to a family of TCSs, which sense environmental signals such as antibiotics to activate the expression of the arn lipopolysaccharide (LPS) modification operon. The corresponding modification of negatively charged LPS with positively charged arabinosamine reduces cationic peptide binding and facilitates adaptive resistance. Similar to the ParRS mediated arn activation via dynorphin A sensing, CprRS has been shown to respond to cationic peptide antibiotics such as polymyxin B.

To further boost sensitivity and enable direct quantification of sites, we applied the recently introduced isoDTB tags, utilizing heavy and light isotopes incorporated in the linker of the desthiobiocin azide. Their use allows a direct, quantitative comparison of pooled samples treated with dynorphin A or the control, respectively (Fig. 5a). Intriguingly, this experiment revealed D53 of CprR with the highest intensity ratio (dynorphin A treated vs. untreated = 16.2) among all detected modification sites (Fig. 5b and Fig. S15, Table S1, ESI†).

ParR, the cognate RR of the previously identified dynorphin A

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**Fig. 5** RP-isoDTB workflow for quantitative MS1 and MS2 (PRM) analysis. (a) Intact cells of P. aeruginosa were treated with DMSO or 10 µM dynorphin A (Dyn), lysed in a buffer containing the nucleophilic HA-ynE probe (125 mM) at pH 4 and clicked to the isoDTB tags. Modified proteins were combined, trypically digested, enriched on streptavadin beads, eluted from the beads and subjected to LC-MS/MS analysis. (b) Waterfall plot representing the ratio between dynorphin A (light) and DMSO (heavy) treated HA-ynE modified Asp and Glu residues. Red dots indicate sites, that are also annotated as pAsp sites in Uniprot. (c) PRM transitions (Dyn/light vs. DMSO/heavy) of pAsp annotated and HA-ynE modified peptides of response regulators CprR and ParR. Data was analyzed using the Skyline software. MS2 ratios of 20.8 and 2.0 were obtained for CprR and ParR, respectively, unraveling CprR as the only protein with highly enhanced pAsp modification.
Conclusions

Given the impressive success of phosphoproteomics to study signaling networks in eukaryotic cells, the exploration of prokaryotic phosphorylation signals lacks behind. One reason is the prevalent but transient modification of aspartate resulting in a labile acyl phosphate moiety, which escapes detection by conventional methods. The capture of this modification by strong nucleophiles is a promising approach, however, care has to be taken in the selection of appropriate labeling conditions in order to maximize the yield and fidelity. Our study revealed that the addition of a minimal hydroxylamine-alkyne probe at pH = 4 supplemented with a solubilizing detergent favors the desired attack on the aspartate carbonyl C-atom and maximizes the readout of conserved RR sites in whole proteomes. This approach was validated by the detection of about 20% of all known RRs with modification at the known pAsp sites but also a large number of so far unknown sites. Interestingly, an alignment of all detected aspartate phosphorylated peptides closely resembled the conserved RR consensus motif suggesting that the method not only reports known but also putative unknown signaling systems. Given the prevalence of open reading frames encoding regulator proteins in _P. aeruginosa_, these findings will be subject to further functional studies. The tailored alkyn probe (HA-ynε) is readily accessible and the reported procedures should allow straightforward implementation for the detection of this important post-translational modification in other laboratories. HA-ynε for the first time allows phosphoaspartate monitoring through downstream application of click chemistry, which largely increases the flexibility of the approach. Besides the possibility of attaching desthiobiotin azide for enrichment, this modular nature of the technology enabled straightforward implementation of fluorescence gel-based assays using a fluorescent azide and application of the probe in the isoDBTB-ABPP platform, which allowed quantitatively understanding of TCS signaling processes.

The main goal of implementing this refined prokaryotic phosphoproteomic platform was the application in deciphering molecular details of _P. aeruginosa_ cationic peptide signaling.

While ParS has been detected as a direct target of dynorphin A previously via a complementary affinity-based protein profiling (AfBPP) approach to identify sensor histidine kinases, we here show that under the selected conditions its cognate RR ParR is only slightly stronger phosphorylated in response to dynorphin A treatment. Unexpectedly, we here identify CprR as a phosphorylated RR, whose native histidine kinase CprS was not discovered by the previous AfBPP method. Thus, the two approaches (AfBPP and pAsp-trapping) unravel a high degree of complementarity with useful applications in deciphering bacterial signaling.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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Supplementary Information

A tailored phosphoaspartate probe unravels CprR as a response regulator in *Pseudomonas aeruginosa* interkingdom signaling

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Fig. S1 Confirmation of site-specific in vitro phosphorylation of wt *E. coli* PhoB at D53. IPMS analysis of PhoB D53N mutant before (a) and after (b) treatment with acetyl phosphate (AcP). No phosphorylation was observed for PhoB D53N.

Fig. S2 Hydrolysis of phosphorylated wt PhoB without and with hydroxylamine treatment at pH 7. IPMS analysis of phosphorylated wt PhoB incubated without (a) and with 500 mM hydroxylamine (b).
Fig. S3 Kinetics of the reaction of phosphorylated wt PhoB with 500 mM Hydroxylamine (a) or HA-yne (b) at pH = 4.

Fig. S4 Control reaction, that unphosphorylated wt PhoB does not react with hydroxylamine. IPMS analysis of unphosphorylated wt PhoB before (a) and after (b) treatment with 500 mM Hydroxylamine. No formation of N-hydroxyasparagine was observed.

Fig. S5 Optimization of labeling conditions by pH-dependent labeling. Exponentially growing B. subtilis were lyzed in the presence of 500 mM HA-yne at different pH-values and labeled proteins were clicked to rhodamine azide (ctrl: no HA-yne, 20 mM HEPES, pH = 7). SDS-PAGE analysis was performed by in-gel fluorescence scanning and staining using Coomassie Brilliant Blue. The labeling pattern and protein solubility turned out to be strongly pH dependent.
Fig. S6 Optimization of labeling conditions by assessment of detergents. Exponentially growing *B. subtilis* were lysed in the presence of 500 mM HA-yne at pH = 4 in labeling buffer containing different detergents. Labeled proteins were clicked to rhodamine azide and SDS-PAGE analysis was performed by in-gel fluorescence scanning and staining using Coomassie Brilliant Blue. Addition of 1% (w/v) LDAO revealed the most efficient solubilization of the proteome and the most pronounced labeling pattern.

![Fluorescence](image1.png)

![Coomassie](image2.png)

**Fig. S6** Optimization of labeling conditions by assessment of detergents. Exponentially growing *B. subtilis* were lysed in the presence of 500 mM HA-yne at pH = 4 in labeling buffer containing different detergents. Labeled proteins were clicked to rhodamine azide and SDS-PAGE analysis was performed by in-gel fluorescence scanning and staining using Coomassie Brilliant Blue. Addition of 1% (w/v) LDAO revealed the most efficient solubilization of the proteome and the most pronounced labeling pattern.

Fig. S7 Optimization of labeling conditions by dose-dependent labeling. Exponentially growing *B. subtilis* were lysed in the presence of different concentrations of HA-yne at pH = 4 in HEPES buffer containing 1% (w/v) LDAO. Labeled proteins were clicked to rhodamine azide and SDS-PAGE analysis was performed by in-gel fluorescence scanning and staining using Coomassie Brilliant Blue. A probe concentration of 125 mM was chosen for RP-ABPP experiments.

![Fluorescence](image3.png)

![Coomassie](image4.png)

**Fig. S7** Optimization of labeling conditions by dose-dependent labeling. Exponentially growing *B. subtilis* were lysed in the presence of different concentrations of HA-yne at pH = 4 in HEPES buffer containing 1% (w/v) LDAO. Labeled proteins were clicked to rhodamine azide and SDS-PAGE analysis was performed by in-gel fluorescence scanning and staining using Coomassie Brilliant Blue. A probe concentration of 125 mM was chosen for RP-ABPP experiments.
Fig. S8 Labeling of bacterial lysates under optimized conditions. Exponentially growing *P. aeruginosa* (a) and *B. subtilis* (b) were lyzed in the presence of 125 mM HA-yne at pH = 4 and labeled proteins were clicked to rhodamine azide. SDS-PAGE analysis was performed by in-gel fluorescence scanning and staining using Coomassie Brilliant Blue.

| Gene | Ann. Site | Id. Site | TCS | Implication |
|------|-----------|----------|-----|-------------|
| ComA | D55       | D55      | ComA/ComP | Regulation of genetic competence and quorum sensing |
| PhoP | D53       | D53      | PhoP/PhoR | Regulation of phosphate metabolism |
| ResD | D57       | D57      | ResD/ResE | Aerobic and anaerobic respiration |
| SpoE | D54       | D54      | SpoE/SpoB | Spore formation |
| YerH | D55       | D55      | YerH/YerG | Regulation of cell surface maintenance |
| ZmaA | D337      | D337     | / | Oxidative stress protection |

Fig. S9 Binding site analysis of HA-yne treated lysates of *B. subtilis*. (a) Table of HA-yne modified sites in *B. subtilis* (found sites), that also have UniProt annotated pAsp sites. Additionally, the corresponding genes, TCSs and their implications are listed. For the complete list of modified sites see Table S1 (ESI†). Only sites with an Andromeda localization probability exceeding 75% for the relative HA-yne modified residue were included in the analysis. (b) Comparison of pAsp annotated and HA-yne modified sequence motifs using pLogo. Residues at positions ranging from -10 to +10 next to the phosphorylation/modification site were included in the analysis. pAsp annotated and HA-yne modified sequences (fg) were compared with the complete proteomic background (bg) in *B. subtilis* from the UniProt database. Orange horizontal bars indicate the Bonferroni-corrected statistical significance (p = 0.05).
**Fig. S10** Enriched protein domains among HA-yne modified proteins in *P. aeruginosa* using DAVID\(^4,5\). Statistically most significant categories are shown. Phosphoaspartate related protein domains are indicated in red.

**Fig. S11** Distribution of HA-yne modified residues. Analysis of the amino acid specificity of HA-yne labeled proteomes was performed using MaxQuant software\(^6\) allowing the modification to be either on Asp/Glu (+ 509.33 Da) or Asn/Gln (+ 510.32 Da) residues, respectively. Peptides were only included in the analysis if the Andromeda localization probability for a specific residue exceeded 75%.
Fig. S12 Asn (a) and Gln (b) deamidation, isomerization and possible electrophilic sites for nucleophilic attack by hydroxylamines. The Figure was adapted from Geiger and Clarke.⁷
Fig. S13 Analysis of background probe reactivity by IPMS analysis of phosphorylated PhoB, α-Casein and BSA with 125 mM HA-yne at pH = 4. (a) PhoB was \textit{in vitro} phosphorylated with acetyl phosphate and converted with HA-yne. α-Casein (b) and BSA (c) were treated with HA-yne without prior phosphorylation. No background reactions could be observed under the applied conditions.
Fig. S14 Abundance of HA-yne modified proteins in proteomes of *P. aeruginosa* (a-d) and *B. subtilis* (e-h). Proteins were ranked according to their abundance in the relative organism and grouped in 10% steps. The number of HA-yne modified peptides (Table S1, ESI†) was assigned to the relative category. Modification of mainly high abundant proteins suggests a high background reactivity. Protein abundance data were obtained using the PaxDb database. 

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| Protein | No. of sites | Protein abundance (top x%) |
|---------|-------------|-----------------------------|
| Asp     | 150         | 0-10, 10-20, 20-30, 30-40, 40-50, 50-60, 60-70, 70-80, 80-90, 90-100, N/A |
| Glu     | 400         | 0-10, 10-20, 20-30, 30-40, 40-50, 50-60, 60-70, 70-80, 80-90, 90-100, N/A |
| Asn     | 1000        | 0-10, 10-20, 20-30, 30-40, 40-50, 50-60, 60-70, 70-80, 80-90, 90-100, N/A |
| Gln     | 1500        | 0-10, 10-20, 20-30, 30-40, 40-50, 50-60, 60-70, 70-80, 80-90, 90-100, N/A |
| Asp     | 150         | 0-10, 10-20, 20-30, 30-40, 40-50, 50-60, 60-70, 70-80, 80-90, 90-100, N/A |
| Glu     | 400         | 0-10, 10-20, 20-30, 30-40, 40-50, 50-60, 60-70, 70-80, 80-90, 90-100, N/A |
| Asn     | 1000        | 0-10, 10-20, 20-30, 30-40, 40-50, 50-60, 60-70, 70-80, 80-90, 90-100, N/A |
| Gln     | 1500        | 0-10, 10-20, 20-30, 30-40, 40-50, 50-60, 60-70, 70-80, 80-90, 90-100, N/A |
Fig. S15 Volcano plot of the isoDTB-ABPP experiment comparing the HA-yne modified sites of DMSO (light) and dynorphin (Dyn, heavy) pretreated samples. Plots show the log₂-fold enrichment of the ratio between light and heavy labeled samples and the probability in a one-sample t-test that the ratio is equal to one (-log₁₀(p)). Red and gray indicate proteins annotated as “phosphoaspartate” in UniProt and all other proteins, respectively. Data were visualized using Perseus software.⁹
Supplementary Tables

**Table S1.** RP-ABPP data (DDA) for the evaluation of the selectivity and quantification of the sites of modification with HA-ynε and either DTB or the isoDTB tags using MaxQuant software.⁶ The data can be found as an additional data file accompanying the manuscript.

**Table S2.** Precursor information for the establishment of the PRM method and RP-ABPP data (PRM) for the analysis of PROCAL peptides and the quantification of the sites of modification with HA-ynε and the isoDTB tags using Skyline.¹⁰ The data can be found as an additional data file accompanying the manuscript.

**Table S3.** Assignment of all raw files to the corresponding RP-ABPP experiment samples in this study. All Data files will be deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository¹¹ upon final publication of the manuscript.

| Description of the RP-ABPP sample | Raw file name |
|----------------------------------|---------------|
| B.subtilis_spike_in_E.coli_PhoB  |               |
| soluble + insoluble fraction     | 181103_SMH_181031_PA3 |
| B.subtilis_DTB                   |               |
| biol. repl. 1, techn. repl. 1, soluble fraction | 190421_PA_190411_1 |
| biol. repl. 1, techn. repl. 2, insoluble fraction | 190421_PA_190411_2 |
| biol. repl. 1, techn. repl. 3, soluble + insoluble fraction | 190505_PA_190503_1 |
| biol. repl. 2, techn. repl. 4, soluble fraction | 190421_PA_190411_3 |
| biol. repl. 2, techn. repl. 5, insoluble fraction | 190421_PA_190411_4 |
| biol. repl. 2, techn. repl. 6, soluble + insoluble fraction | 190505_PA_190503_2 |
| PAO1_DTB                         |               |
| biol. repl. 1, techn. repl. 1, soluble fraction | 190521_PA_190520_1 |
| biol. repl. 1, techn. repl. 2, insoluble fraction | 190523_PA_190520_1 |
| biol. repl. 2, techn. repl. 3, soluble fraction | 190523_PA_190520_2 |
| biol. repl. 2, techn. repl. 4, insoluble fraction | 190523_PA_190520_3 |
| PAO1_DynA_DTB                    |               |
| 1 min DynA, biol. repl. 1, techn. repl. 1, soluble fraction | 190826_PA_190826_1_DL1 |
| 1 min DynA, biol. repl. 1, techn. repl. 2, insoluble fraction | 190826_PA_190826_3_DL2 |
| 5 min DynA, biol. repl. 2, techn. repl. 3, soluble fraction | 190826_PA_190826_9_DL3 |
| 5 min DynA, biol. repl. 2, techn. repl. 4, insoluble fraction | 190826_PA_190826_11_DL4 |
| 15 min DynA, biol. repl. 2, techn. repl. 5, soluble fraction | 190826_PA_190826_17_DL5 |
| 15 min DynA, biol. repl. 2, techn. repl. 6, insoluble fraction | 190826_PA_190826_19_DL6 |
| 1 min DMSO, biol. repl. 1, techn. repl. 1, soluble fraction | 190826_PA_190826_5_L1 |
| 1 min DMSO, biol. repl. 1, techn. repl. 2, insoluble fraction | 190826_PA_190826_7_L2 |
| 5 min DMSO, biol. repl. 2, techn. repl. 3, soluble fraction | 190826_PA_190826_13_L3 |
| 5 min DMSO, biol. repl. 2, techn. repl. 4, insoluble fraction | 190826_PA_190826_15_L4 |
15 min DMSO, biol. repl. 2, techn. repl. 5, soluble fraction  |  190826_PA_190826_21_L5
15 min DMSO, biol. repl. 2, techn. repl. 6, insoluble fr. |  190826_PA_190826_23_L6

**PAO1_DynA_isoDTB and PAO1_DynA_isoDTB_DDA_quant**

| Sample Description                                                                 | Code               |
|-----------------------------------------------------------------------------------|--------------------|
| biol. repl. 1, techn. repl. 1, soluble fraction                                   | 200409_PA_200318_3 |
| biol. repl. 1, techn. repl. 2, insoluble fraction                                | 200409_PA_200318_6 |
| biol. repl. 2, techn. repl. 3, soluble fraction                                  | 200409_PA_200318_9 |
| biol. repl. 2, techn. repl. 4, insoluble fraction                                | 200409_PA_200318_12|
| biol. repl. 3, techn. repl. 5, soluble fraction                                  | 200303_PA_200224_3_HC1|
| biol. repl. 3, techn. repl. 6, insoluble fraction                                | 200303_PA_200224_6_HM1|
| biol. repl. 4, techn. repl. 7, soluble fraction                                  | 200303_PA_200224_9_HC2|
| biol. repl. 4, techn. repl. 8, insoluble fraction                                | 200303_PA_200224_12_HM2|

**PAO1_DynA_isoDTB_PROCAL_PRM_quant**

| Sample Description                                                                 | Code               |
|-----------------------------------------------------------------------------------|--------------------|
| biol. repl. 1, techn. repl. 1, soluble fraction                                   | 200731_PA_200318_3_PA1|
| biol. repl. 1, techn. repl. 2, insoluble fraction                                | 200731_PA_200318_6_PA2|
| biol. repl. 2, techn. repl. 3, soluble fraction                                  | 200731_PA_200318_9_PA3|
| biol. repl. 2, techn. repl. 4, insoluble fraction                                | 200731_PA_200318_12_PA4|
| biol. repl. 3, techn. repl. 5, soluble fraction                                  | 200731_PA_200224_HC1_PA5|
| biol. repl. 3, techn. repl. 6, insoluble fraction                                | 200731_PA_200224_HM1_PA6|
| biol. repl. 4, techn. repl. 7, soluble fraction                                  | 200731_PA_200224_HC2_PA7|
| biol. repl. 4, techn. repl. 8, insoluble fraction                                | 200731_PA_200224_HM2_PA8|
Experimental Procedures

General Remarks
All reactions sensitive to air and moisture were carried out under argon atmosphere in oven-dried flasks. Chemicals were purchased from Acros Organics, Alfa Aesar, Fisher Chemical and Sigma-Aldrich and were used without further purification. Solvents for column chromatography were distilled prior to use. Analytical thin layer chromatography was carried out on silica-coated aluminum plates (Silica gel 60 F254, Merck) with detection by UV-absorption (λ = 254 and/or 366 nm) and/or by coloration using a potassium permanganate (KMnO4) staining solution with subsequent heat treatment. Flash column chromatography was performed on silica gel (40-63 μM, VWR) with solvent compositions reported as volume/volume (v/v) ratios. 1H- and 13C-NMR spectra were recorded on Bruker Avance III HD (400 MHz and 500 MHz) instruments and referenced to the residual solvent signal (δH = 7.26 ppm and δC = 77.16 ppm for CDCl3; δH = 2.50 ppm and δC = 39.52 ppm for DMSO-d6). Signal assignment was reported using following abbreviations: s - singlet, d - doublet, t - triplet, q - quartet, m - multiplet. High-resolution mass spectrometry (HR-MS) spectra were recorded in the ESI mode on an LTQ-FT Ultra (Thermo Fisher Scientific) coupled to an UltiMate 3000 HPLC system (Thermo Fisher Scientific). DNA and Protein concentrations were determined in duplicates with a NanoQuant plate on an Infinite F200 PRO reader (Tecan) by measuring the absorbance at λ = 260 nm or 280 nm, respectively. Primers were purchased as custom synthesized and lyophilized solids (Eurofins). Dynorphin A (1-13) was obtained from Bachem.

Synthetic Procedures

2-(Hexyloxy)isoindoline-1,3-dione (1):
5-Hexyn-1-ol (3.07 g, 30.0 mmol, 1.00 eq.) was dissolved in dry THF (120 mL) and N-hydroxyphthalimide (6.36 g, 39.0 mmol, 1.30 eq.) and triphenylphosphine (PPh3) (11.8 g, 45.0 mmol, 1.50 eq.) were added. Upon cooling to 0 °C, a solution of diisopropyl azodicarboxylate (DIAD) (8.83 mL, 45.0 mmol, 1.50 eq.) in dry THF (30 mL) was added over 30 min at 0 °C. The mixture was stirred overnight at room temperature. After all volatiles were removed under reduced pressure, the residue was filtered and washed with hexane (3 × 15 mL). Purification by column chromatography (dry loading) (hexane/EtOA 4:1) yielded 1 as a white solid (6.23 g, 25.2 mmol, 84%).
$^1$H-NMR (400 MHz, CDCl$_3$, 298 K): δ [ppm] = 7.86-7.79 (m, 2H, H$_{Ar}$), 7.77-7.70 (m, 2H, H$_{Ar}$), 4.22 (t, $^3$J = 6.3 Hz, 2H, OCH$_2$), 2.37-2.22 (m, 2H, CCH$_2$), 1.95 (t, $^4$J = 2.7 Hz, 1H, CH), 1.94-1.86 (m, 2H, OCH$_2$CH$_2$), 1.84-1.67 (m, 2H, CCH$_2$CH$_2$).

$^{13}$C-NMR (101 MHz, CDCl$_3$, 300 K): δ [ppm] = 163.73 (s, 2C), 134.58 (s, 2C), 129.09 (s, 2C), 123.62 (s, 2C), 83.97 (s, 1C), 77.94 (s, 1C), 68.93 (s, 1C), 27.24 (s, 1C), 24.62 (s, 1C), 18.12 (s, 1C).

The analytical data obtained are in agreement with those reported in the literature.\textsuperscript{12}

O-(Hex-5-yn-1-yl)hydroxylamine (2):

1 (2.47 g, 10.0 mmol, 1.00 eq.) was dissolved in DCM/MeOH (2:1) and hydrazine monohydrate (509 µL, 10.5 mmol, 1.05 eq.) was added dropwise. The solution was stirred overnight at room temperature and completion was indicated by TLC. After solvent removal under reduced pressure, the residue was resuspended in H$_2$O and the pH adjusted to 12. The mixture was extracted with DCM (3 × 20 mL) and the combined organic layers were washed with brine (20 mL), dried over Na$_2$SO$_4$ and filtered. The solvent was removed under reduced pressure and the residue was dissolved in Et$_2$O, followed by addition of HCl in Et$_2$O (2 M). The formed precipitate was cooled to -20 °C overnight, filtered and dried under high vacuum to yield 2 as a light yellowish solid (962 mg, 6.43 mmol, 64%).

$^1$H-NMR (500 MHz, DMSO-d$_6$, 298 K): δ [ppm] = 10.84 (s, 3H, NH$_3$), 3.99 (t, $^3$J = 6.3 Hz, 2H, OCH$_2$), 2.80 (t, $^4$J = 2.7 Hz, 1H, CH), 2.20 (td, $^3$J = 7.1, $^4$J = 2.6 Hz, 2H, CCH$_2$), 1.71-1.61 (m, 2H, OCH$_2$CH$_2$), 1.54-1.45 (m, 2H, CCH$_2$CH$_2$).

$^{13}$C-NMR (101 MHz, DMSO-d$_6$, 300 K): δ [ppm] = 84.09 (s, 1C), 73.41 (s, 1C), 71.55 (s, 1C), 26.23 (s, 1C), 24.24 (s, 1C), 17.27 (s, 1C).

HRMS-ESI (m/z): calc. (C$_6$H$_{12}$NO [M+H]$^+$): 114.0919; found: 114.0914.
Bacterial Strains and Media
Unless stated otherwise, *E. coli* BL21 (DE3), *B. subtilis* 168 and *P. aeruginosa* (PAO1) were cultivated in LB medium (10 g/L peptone, 5 g/L NaCl, 5 g/L yeast extract, pH 7.5). For the growth of *E. coli* BL21 (DE3) bearing the pET300 expression vector, LB medium was supplemented with ampicillin (100 mg/L). Overnight cultures of bacteria were inoculated with a pipette tip of the corresponding glycerol stock in 5 mL of the corresponding medium and cells were grown overnight at 37 °C with shaking at 220 rpm.

Cloning, Expression and Purification of *E. coli* PhoB
*N*-terminal His6-tagged *E. coli* PhoB with a TEV-cleavage site between the His6-tag and the protein sequence was cloned in a pET300 vector in *E. coli* BL21 (DE3) competent cells via Gateway cloning (*Life Technologies*). For PhoB expression, LB medium was inoculated (1:100) with *E. coli* overnight cultures (37 °C, 220 rpm) and incubated at 37 °C, 220 rpm. After induction at OD600 = 0.6 with 0.5 mM IPTG, PhoB was expressed overnight at 25 °C with shaking at 220 rpm. Cells were harvested by centrifugation (6,000 g, 4 °C, 10 min), washed with PBS, resuspended in lysis buffer (20 mM Trizma, pH 8.0, 150 mM NaCl, 2 mM β-mercaptoethanol, 10 mM imidazole, 0.4% (v/v) NP-40) and lysed by sonication (2 x (7 min, 30% int.; 3 min, 80% int.); *Bandelin* Sonopuls HD 2070) under constant cooling with ice. The lysate was centrifuged (38,000 g, 4 °C, 30 min) to remove cellular debris and by using an ÄKTAPurifier 10 system (*GE Healthcare*), the supernatant was loaded on a 5 mL HisTrap HP column (*GE Healthcare*) equilibrated with wash buffer 1 (20 mM Trizma, pH 8.0, 150 mM NaCl, 2 mM β-mercaptoethanol, 10 mM imidazole). The column was washed with wash buffer 1 (8 CV), wash buffer 2 (20 mM Trizma, pH 8.0, 1 M NaCl, 2 mM β-mercaptoethanol, 10 mM imidazole; 8 CV) and wash buffer 3 (20 mM Trizma, pH 8.0, 150 mM NaCl, 2 mM β-mercaptoethanol, 40 mM imidazole; 8 CV). Elution was performed with elution buffer (20 mM Trizma, pH 8.0, 1 M NaCl, 2 mM β-mercaptoethanol, 500 mM imidazole; 4 CV) and PhoB containing fractions were pooled, concentrated using a 50 kDa MWCO centrifugal filter (Merck) and purified by size-exclusion chromatography with a HiLoad 16/60 Superdex 76 pg column (*GE Healthcare*) equilibrated in PhoB storage buffer (20 mM HEPES, pH 7.0, 150 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT). Fractions containing PhoB were pooled, concentrated and stored at -80 °C after addition of 10% (v/v) glycerol. Purity of the protein was verified by SDS-PAGE and intact-protein mass spectrometry (IPMS).

A point mutant of PhoB (D53N) was generated using the Quickchange Site-Directed Mutagenesis Kit (*Stratagene*) with the pET300 PhoB expression vector as template. Sequences of PhoB and the D53N point mutant were verified by Sanger sequencing (*GATC Biotech AG*). Expression and purification of the mutant was performed as described above.

Unless stated otherwise, tagged PhoB was used for further experiments since it behaved identical to tag-free PhoB. For the generation of tag-free PhoB, the protein was dialyzed after His-affinity purification in PhoB storage buffer at 4 °C overnight. PhoB was incubated with 1:3 (w/w) TEV protease at 10 °C overnight without shaking and complete cleavage was verified by IPMS. Tag-free PhoB was concentrated and purified by size-exclusion chromatography as described above.
Intact Protein Mass Spectrometry

High-resolution IPMS measurements were performed on an UltiMate 3000 HPLC system (Thermo Fisher Scientific) coupled to an LTQ-FT Ultra (Thermo Fisher Scientific) mass spectrometer with an electrospray ionization source (spray voltage 4.0 kV, tube lens 110 V, capillary voltage 48 V, sheath gas 60 a.u., aux gas 10 a.u., sweep gas 0.2 a.u.). Protein samples (1-10 pmol) were desalted on-line with a Massprep desalting cartridge (Waters) prior to measurement. The mass spectrometer was operated in positive ion mode and full scans were recorded at high resolution (200,000) in a range of m/z = 600-2000 Th. Protein spectra were deconvoluted using the Xcalibur Xtract algorithm (Thermo Fisher Scientific).

In vitro phosphorylation and phosphoaspartate conversion of PhoB

In vitro phosphorylation of PhoB was initiated by addition of MgCl2 and lithium potassium acetyl phosphate to PhoB in reaction buffer (20 mM HEPES, pH 7.0, 0.1 mM DTT; final concentrations: 12.5 μM PhoB, 10 μM MgCl2 and 20 μM lithium potassium acetyl phosphate). The reaction mixture was incubated for 1 h at 37 °C without shaking. Meanwhile, Bio-Spin 6 Columns (Bio-Rad) for gel filtration were equilibrated four times by addition of 500 μL reaction buffer, centrifugation (1,000 g, 1 min, 4 °C) and removal of the supernatant. Acetyl phosphate was then removed from the sample by application of the sample to the column and centrifugation (1,000 g, 4 min, 4 °C). An aliquot of the sample was taken and 1-10 pmol protein were subjected to IPMS analysis to assess the degree of phosphorylation. The remaining sample was treated with either hydroxylamine or HA-yneline at the indicated concentrations. Both preceding steps (IPMS and nucleophile addition) were conducted immediately in order to minimize loss of phosphorylation. Unless stated otherwise, the reaction proceeded at pH = 4 and was checked with pH-indicator strips (Merck). The reaction was allowed to stand at room temperature for 1 h before IPMS measurement and assessment of phosphoaspartate conversion. For time-course experiments, aliquots of the samples were subjected to IPMS analysis at the indicated time points and performed in triplicates.

Several control experiments were conducted in order to prove the selectivity of the conversion with hydroxylamine and HA-yneline at the optimized conditions with exclusively phosphoaspartate modified proteins. Control experiments were conducted analogously to the procedure described above. For reactions at different pH values, the pH value of the solution containing the nucleophile was adjusted with 0.5 M KOH or HCl prior to the reaction with the indicated protein. Proteins α-Casein and BSA were used as 200 μg/mL solutions and treated with HA-yneline without prior phosphorylation.

Gel-based RP-ABPP Experiments for HA-yneline Labeling Optimization

For the development of an RP-ABPP workflow, exponentially growing B. subtilis were labeled with HA-yneline on analytical scale. LB medium was inoculated (1:100) with B. subtilis overnight cultures (37 °C, 220 rpm) and incubated at 37 °C with shaking at 220 rpm. At OD600 = 0.5-0.6, cells were harvested by centrifugation (6,000 g, 4 °C, 10 min), washed with ice-cold PBS and resuspended to OD600 = 40 in 100 μL lysis buffer (basis: 20 mM HEPES, pH 7.0, 500 mM HA-yneline; pH value, detergent content and HA-yneline were adjusted as indicated) for each condition to be tested. Cells were lysed by sonication (3 x 15 s, 80% int.) under constant cooling with ice and fractions were separated by centrifugation (21,000 g, 4 °C, 30 min). The
insoluble fraction was washed twice with 100 µL ice-cold PBS and stored at -20 °C until subjection to click chemistry. To remove excess HA-yn, the soluble fraction was precipitated in 400 µL of cold acetone (-80 °C) and stored at -20 °C. The precipitate was centrifuged (9,000 g, 4 °C, 10 min) and washed twice by resuspension in 100 µL MeOH (-80 °C) by sonication (10 s, 10% int.), centrifugation (9,000 g, 4 °C, 10 min) and removal of the supernatant.

Soluble and insoluble fractions were resuspended in 100 µL 0.8% SDS in PBS by sonication (10 s, 10% int.) and clicked to rhodamine azide by addition of 6 µL TBTA ligand (0.9 mL/mL in 4:1 tBuOH/DMSO), 2 µL rhodamine azide (5 mM stock in DMSO; final concentration: 100 µM), 2 µL TCEP (13 mg/mL in water) and 2 µL CuSO4 (12.5 mg/mL in water). The click reaction was incubated for 1 h at room temperature in the dark, quenched by addition of 112 µL 2× Laemmli buffer and analyzed by SDS-PAGE. Rhodamine azide modified proteins were detected by in-gel fluorescence scanning and protein loading was visualized by Coomassie Brilliant Blue staining.

E. coli PhoB spike-in RP-ABPP Experiments in B. subtilis
For PhoB spike-in experiments, PhoB was phosphorylated and converted with 500 mM HA-yn as described in section “in vitro phosphorylation and phosphoaspartate conversion of PhoB.” 5 µg of HA-yn modified PhoB was precipitated in 400 µL of cold acetone (-80 °C) and stored at -20 °C until further processing.

LB medium was inoculated (1:100) with a B. subtilis overnight culture (37 °C, 220 rpm) and incubated at 37 °C with shaking at 220 rpm. At OD600 = 0.5-0.6, cells were harvested by centrifugation (6,000 g, 4 °C, 10 min), washed with ice-cold PBS and resuspended to OD600 = 40 in 1 mL of HA-yn buffer (20 mM HEPES, pH 4.0, 125 mM HA-yn, 1% (w/v) LDAO). Cells were lysed by sonication (4 x 15 s, 80% int.) under constant cooling with ice. The reaction proceeded for 1 h at 37 °C without shaking. Fractions were separated by centrifugation (21,000 g, 4 °C, 30 min). The insoluble fraction was washed twice with 1 mL ice-cold PBS and stored at -20 °C until subjection to click chemistry. The soluble fraction was precipitated in 4 mL of cold acetone (-80 °C) and incubated overnight at -20 °C. The precipitate was centrifuged (9,000 g, 4 °C, 10 min) and washed twice by resuspension in 1 mL MeOH (-80 °C) by sonication (10 s, 10% int.), centrifugation (9,000 g, 4 °C, 10 min) and removal of the supernatant.

The pellet of PhoB, the soluble and insoluble fraction were resuspended in 0.8% SDS in PBS by sonication (10 s, 10% int.) and combined (final volume: 1 mL). 1 mL of each sample was clicked to desthiobiotin azide (Jena Bioscience) by addition of 60 µL TBTA ligand (0.9 mL/mL in 4:1 tBuOH/DMSO), 20 µL desthiobiotin azide (5 mM stock in DMSO, final concentration: 100 µM), 20 µL TCEP (13 mg/mL in water) and 20 µL CuSO4 (12.5 mg/mL in water). The click reaction was incubated for 1 h at room temperature and quenched by addition of 4 mL of cold acetone (-80 °C) and stored overnight at -20 °C.

RP-ABPP Experiments in B. subtilis and P. aeruginosa
LB medium was inoculated (1:100) with B. subtilis or P. aeruginosa overnight cultures (37 °C, 220 rpm) and incubated at 37 °C with shaking at 220 rpm. At OD600 = 0.5-0.6, cells were
harvested by centrifugation (6,000 g, 4 °C, 10 min), washed with ice-cold PBS and resuspended to OD_{600} = 40 in 1 mL of HA-yne buffer (20 mM HEPES, pH 4.0, 125 mM HA-yne, 1% (w/v) LDAO). Cells were lysed by sonication (4 x 15 s, 80% int.) under constant cooling with ice. The reaction proceeded for 1 h at 37 °C without shaking. Fractions were separated by centrifugation (21,000 g, 4 °C, 30 min). The insoluble fraction was washed twice with 1 mL ice-cold PBS and stored at -20 °C until subjecting to click chemistry. The soluble fraction was precipitated in 4 mL of cold acetone (-80 °C) and incubated overnight at -20 °C. The precipitate was centrifuged (9,000 g, 4 °C, 10 min) and washed twice by respension in 1 mL MeOH (-80 °C) by sonication (10 s, 10% int.), centrifugation (9,000 g, 4 °C, 10 min) and removal of the supernatant. Soluble and insoluble fractions were resuspended in 1 mL 0.8% SDS in PBS by sonication (10 s, 10% int.) and protein concentration of both fractions was determined using a bicinchoninic acid (BCA) assay and adjusted to 1 mg/mL with 0.8% SDS in PBS. 1 mL of each sample was clicked to desthiobiotin azide by addition of 60 µL TBTA ligand (0.9 mL/mL in 4:1 tBuOH/DMSO), 20 µL desthiobiotin azide (5 mM in DMSO), 20 µL TCEP (13 mg/mL in water) and 20 µL CuSO₄ (12.5 mg/mL in water). The click reaction was incubated for 1 h at room temperature and quenched by addition of 4 mL of cold acetone (-80 °C) and stored overnight at -20 °C.

For gel-based analysis, 100 µL of all samples (1 mg/mL) were additionally clicked to rhodamine azide and visualized as described in the previous section.

Moreover, one additional sample was prepared for *B. subtilis* replicates, for which the soluble and insoluble fraction (500 µL each) of the lysate was combined before the click reaction and further processing.

**RP-ABPP Experiments in dynorphin A treated *P. aeruginosa***

MOPS medium (50 mM MOPS, pH 7.2, 20 mM NaCl, 20 mM di-sodium succinate, 1 mM MgSO₄, 10 mM KCl, 4 mM K$_2$HPO$_4$ and 3.5 µM FeSO₄) was inoculated (1:100) with a *P. aeruginosa* overnight culture (37 °C, 220 rpm) in LB medium and incubated at 37 °C with shaking at 220 rpm. At OD$_{600}$ = 0.8-1.0, cells were harvested by centrifugation (6,000 g, 4 °C, 10 min) and washed with ice-cold PBS. Cells were resuspended to OD$_{600}$ = 1 in 40 mL PBS and incubated at 37 °C with shaking at 220 rpm. 4 µL of Dynorphin A (1-13) (100 mM stock in DMSO; final concentration: 10 µM) or DMSO were added and incubated for either 1, 5 or 15 min at 37 °C with shaking at 220 rpm. The cells were harvested by centrifugation (6,000 g, 4 °C, 10 min), washed with ice-cold PBS and resuspended to OD$_{600} = 40$ in 1 mL of HA-yne buffer (20 mM HEPES, pH 4.0, 125 mM HA-yne, 1% (w/v) LDAO). The samples were lysed, separated and washed as described above. Soluble and insoluble fractions were resuspended in 1 mL 0.8% SDS in PBS by sonication (10 s, 10% int.) and protein concentration of both fractions was determined by BCA assay and adjusted to 0.5 mg/mL with 0.8% SDS in PBS. 1 mL of each sample was clicked to desthiobiotin azide by addition of 60 µL TBTA ligand (0.9 mL/mL in 4:1 tBuOH/DMSO), 20 µL desthiobiotin azide (5 mM in DMSO), 20 µL TCEP (13 mg/mL in water) and 20 µL CuSO₄ (12.5 mg/mL in water). The click reaction was incubated for 1 h at room temperature and quenched by addition of 4 mL of cold acetone (-80 °C) and stored overnight at -20 °C.
isoDTB-ABPP Experiments in dynorphin A treated *P. aeruginosa*

MOPS medium (50 mM MOPS, pH 7.2, 20 mM NH₄Cl, 20 mM di-sodium succinate, 1 mM MgSO₄, 10 mM KCl, 4 mM K₂HPO₄ and 3.5 μM FeSO₄) was inoculated (1:100) with a *P. aeruginosa* overnight culture (37 °C, 220 rpm) in LB medium and incubated at 37 °C with shaking at 220 rpm. At OD₆₀₀ = 0.8-1.0, cells were harvested by centrifugation (6,000 g, 4 °C, 10 min) and washed with ice-cold PBS. Cells were resuspended to OD₆₀₀ = 1 in 40 mL PBS and incubated at 37 °C with shaking at 220 rpm. After reduction of disulfides by addition of 15 µL dithiothreitol (DTT) to a Protein LoBind tube, centrifugation (9,000 g, 10 min) and the supernatant was removed. The beads were resuspended in 600 µL 0.1% NP-40 alternative in PBS (4 × 1 mL), centrifugation (400 rpm, 2 min) and removal of the supernatant, the beads were resuspended in 300 µL 8 M urea in 0.1 M TEAB. After transfer to a Protein LoBind tube, centrifugation (1,000 g, 2 min) and removal of the supernatant, the beads were resuspended in 600 µL 0.1% NP-40 alternative in PBS and transferred to a centrifuge column (11894131, Fischer Scientific). The beads were washed with 0.1% NP-40 alternative (2 × 600 µL), PBS (3 × 600 µL) and ddH₂O (3 × 600 µL) and then resuspended in 600 µL 8 M urea in 0.1 M TEAB. After transfer to a Protein LoBind tube, centrifugation (1,000 g, 2 min) and removal of the supernatant, the beads were resuspended in 300 µL 8 M urea in 0.1 M TEAB.

To remove unbound proteins, the beads were centrifuged (1,000 g, 2 min) and the supernatant was removed. The beads were resuspended in 600 µL 0.1% NP-40 alternative in PBS and transferred to a centrifuge column (11894131, Fischer Scientific). The beads were washed with 0.1% NP-40 alternative (2 × 600 µL), PBS (3 × 600 µL) and ddH₂O (3 × 600 µL) and then resuspended in 600 µL 8 M urea in 0.1 M TEAB. After transfer to a Protein LoBind tube, centrifugation (1,000 g, 2 min) and removal of the supernatant, the beads were resuspended in 300 µL 8 M urea in 0.1 M TEAB.

After reduction of disulfides by addition of 15 µL dithiothreitol (DTT; 31 mg/mL) and incubation at 37 °C with shaking at 850 rpm for 45 min, free thiols were alkylated by adding 15 µL iodoacetamide (IAA; 74 mg/mL) and incubation in the dark at 25 °C with shaking at 850 rpm for 30 min. Remaining IAA was quenched by addition of 15 µL DTT (31 mg/mL) and...
incubation at 25 °C with shaking at 850 rpm for 30 min. 900 µL 0.1 M TEAB were added to obtain a urea concentration of 2 M for trypsin digestion. 2 µL of 0.5 mg/mL sequencing grade modified trypsin (1 µg; Promega) were added and samples were incubated at 37 °C with shaking at 220 rpm overnight. After centrifugation (400 rpm, 2 min) and removal of the supernatant, the beads were washed three times by addition of 50 µL Tris-HCl buffer (50 mM, pH 7.5), centrifugation (1,000 g, 2 min) and removal of the supernatant. The beads were resuspended in 100 µL Tris-HCl buffer, followed by addition of 16 µL of 0.04 mg/mL sequencing grade AspN (0.64 µg; Promega) and incubation at 37 °C with shaking at 220 rpm for 7 h.

The beads were resuspended in 500 µL 0.1% NP-40 alternative in PBS and transferred to a centrifuge column (11894131, Fischer Scientific). The beads were washed with 0.1% NP-40 alternative (2 x 600 µL), PBS (3 x 600 µL) and ddH2O (3 x 600 µL). The peptides were eluted by addition of 200 µL elution buffer (0.1% formic acid (FA) in 1:1 acetonitrile (ACN)/H2O) and two more elution steps with 100 µL elution buffer, followed by centrifugation (5,000 g, 3 min). The solvent was removed using a vacuum centrifuge and samples were stored at -80°C until further processing. The samples were dissolved by addition of 30 µL 1% FA in H2O and sonication for 3 min. The samples were filtered through pre-equilibrated 0.22 µm PVDF filters (UVC30GVNB, Merck) and transferred into MS vials for LC-MS/MS analysis.

MS Sample Preparation for RP-ABPP and isoDTB-ABPP Experiments (Peptide Enrichment)
Precipitates were centrifuged (9,000 g, 4 °C, 10 min) and washed twice by resuspension in 1 mL MeOH (-80 °C) by sonication (10 s, 10% int.), centrifugation (9,000 g, 4 °C, 10 min) and removal of the supernatant. Pellets were dissolved in 300 µL 8 M urea in 0.1 M triethylammonium bicarbonate (TEAB) by sonication (10 s, 10% int.). After reduction of disulfides by addition of 15 µL dithiothreitol (DTT; 31 mg/mL) and incubation at 37 °C with shaking at 850 rpm for 45 min, free thiols were alkylated by adding 15 µL iodoacetamide (IAA; 74 mg/mL) and incubation in the dark at 25 °C with shaking at 850 rpm for 30 min. Remaining IAA was quenched by addition of 15 µL DTT (31 mg/mL) and incubation at 25 °C with shaking at 850 rpm for 30 min. 900 µL 0.1 M TEAB were added to obtain a urea concentration of 2 M for trypsin digestion. 20 µL of 0.5 mg/mL sequencing grade modified trypsin (10 µg; Promega) were added and samples were incubated at 37 °C with shaking at 220 rpm overnight. This solution was added to 1.2 mL of washed streptavidin agarose beads (50 µL initial slurry; A9207, Sigma Aldrich) in 0.2% nonyl phenoxypolyethoxylethanol (NP-40 alternative), which were previously washed by addition of 0.2% NP-40 alternative in PBS (4 x 1 mL), centrifugation (400 rpm, 2 min) and removal of the supernatant. The samples were incubated by rotation at room temperature for 1 h.

To remove unbound peptides, the beads were centrifuged (1,000 g, 2 min) and the supernatant was removed. The beads were resuspended in 600 µL 0.1% NP-40 alternative in PBS and transferred to a centrifuge column (11894131, Fischer Scientific). The beads were washed with 0.1% NP-40 alternative (2 x 600 µL), PBS (3 x 600 µL) and ddH2O (3 x 600 µL). The peptides were eluted by addition of 200 µL elution buffer (0.1% formic acid (FA) in 1:1 ACN/H2O) and two more elution steps with 100 µL elution buffer, followed by centrifugation (5,000 g, 3 min). The solvent was removed using a vacuum centrifuge and samples were stored
at -80°C until further processing. The samples were dissolved by addition of 30 µL 1% FA in H₂O and sonication for 3 min. The samples were filtered through pre-equilibrated 0.22 µm PVDF filters (UVC30GVNB, Merck) and transferred into MS vials for LC-MS/MS analysis.

**LC-MS/MS Analysis**

Samples (injection volume: 5 µL) were analyzed with an Ultimate 3000 nano HPLC system (Dionex) coupled to a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific). Samples were loaded on an Acclai m C18 PepMap100 trap column (75 µm ID x 2 cm), washed with 0.1% TFA and separated on an Acclaim C18PepMapRSLC column (75 µm ID x 50 cm) with a flow of 300 nL/min using buffer A (0.1% FA in H₂O) and buffer B (0.1% FA in ACN): 5% B for 7 min, 5-40% B in 105 min, 40-60% B in 10 min, 60-90% B in 10 min, 90% B for 10 min, 90-5% B in 0.1 min, 5% B for 9.9 min. The Q Exactive Plus mass spectrometer was operated in a TOP10 data dependent acquisition mode (DDA). Full MS (MS1) scans were acquired at a resolution of 70,000, a scan range of m/z = 300-1500 Th, an automatic gain control (AGC) target of 3e6, and a maximum injection time of 80 ms. The ten most intense precursors (Top10) were selected for MS2 scan acquisition at a resolution of 17,500, an AGC target of 1e5, and a maximum injection time of 100 ms. Precursors with unassigned charge or a charge of +1 were excluded and dynamic exclusion was set to 60 s. Quadrupole isolation of the precursor was set to a window of 1.6 Th. Fragment ions were generated using higher-energy dissociation (HCD) with a normalized collision energy (NCE) of 27% and detected in the orbitrap.

**RP-ABPP Data Analysis**

MS raw data were analyzed using MaxQuant software (version 1.6.2.10). Standard settings were used with the following changes and additions: The normal FASTA databases without manual changes were downloaded from UniProt (B. subtilis 168 taxon identifier: 224308, date of download: 20.09.2018; P. aeruginosa PAO1 taxon identifier: 208964, date of download: 22.05.2019). No labels were used. The proteolytic enzyme was set to Trypsin/P with up to three missed cleavages. Variable modifications with HA-yne and desthiobiotin azide were allowed on Asp, Glu; Asn and Gln:

- **HA-yne** and desthiobiotin azide on Asp or Glu: C₂₄H₄₄N₇O₅ (509.3326 Da)
- **HA-yne** and desthiobiotin azide on Asn or Gln: C₂₄H₄₂N₆O₆ (510.3166 Da)

N-terminal acetylation and oxidation of methionine were selected as further variable modifications and carbamidomethylated cysteine as fixed modification. The maximum number of modifications per peptide was 5. The “Re-quantify” option was enabled. Contaminants were included. Peptides were searched with a minimum peptide length of 6 and a maximum peptide mass of 4,600 Da. “Second peptides” was enabled and “Dependent peptides” was disabled. “Match between runs” was enabled with a Match time window of 0.7 min and an alignment window of 20 min. A minimal Andromeda score¹ of 40 and a delta score of 6 was set for modified peptides. An FDR of 0.01 was used for Protein FDR, PSM FDR and XPSM FDR. Technical replicates were analyzed in the same MaxQuant analysis.

The “DTB-PEG-N3-Sites.txt” files generated by MaxQuant analysis for modification with HA-yne and the desthiobiotin azide tag were used for further analysis. All peptides for “reverse” sequences and “potential contamination” were removed. The data were filtered to
only include peptides with a localization probability of at least 75% for a single amino acid residue. Within these peptides, for each potentially electrophilic amino acid, the number of sequences was counted that is modified on this amino acid and reported as sum of all different modified sites from biologically independent replicates.

**isoDTB Data Analysis**

MS raw data were analyzed using MaxQuant software (version 1.6.2.10). Standard settings were used with the following changes and additions: The normal FASTA databases without manual changes were downloaded from UniProt (P. aeruginosa PAO1 taxon identifier: 208964, date of download: 22.05.2019). No labels were used. The proteolytic enzyme was set to Trypsin/P with up to three missed cleavages. Variable modifications with HA-yne and either the light or heavy isoDTB tag were allowed on Asp and Glu:

- **HA-yne** and light isoDTB tag on Asp or Glu: C$_{26}$H$_{44}$N$_{10}$O$_{5}$ (576.3496 Da)
- **HA-yne** and heavy isoDTB tag on Asp or Glu: C$_{22}$C$_{13}$H$_{44}$N$_{8}$O$_{5}$ (582.3571 Da)

N-terminal acetylation and oxidation of methionine were selected as further variable modifications and carbamidomethylated cysteine as fixed modification. The maximum number of modifications per peptide was 5. The “Re-quantify” option was enabled. Contaminants were included. Peptides were searched with a minimum peptide length of 7 and a maximum peptide mass of 4,600 Da. “Second peptides” was enabled and “Dependent peptides” was disabled. “Match between runs” was enabled with a Match time window of 0.7 min and an alignment window of 20 min. A minimal Andromeda score of 40 and a delta score of 6 was set for modified peptides. An FDR of 0.01 was used for Protein FDR, PSM FDR and XPSM FDR. Technical replicates were analyzed in the same MaxQuant analysis.

The “isoDTB light HA-yne (DE)Sites.txt” and “isoDTB heavy HA-yne (DE)Sites.txt” files generated by MaxQuant analysis for modification with **HA-yne** and isoDTB tags were used for further analysis. All peptides for “reverse” sequences and “potential contamination” were removed. The data were filtered to only include peptides with a localization probability of at least 75% for a single amino acid residue. Within these peptides, for each potentially electrophilic amino acid, the number of sequences was counted that is modified on this amino acid and reported as sum of all different modified sites from biologically independent replicates.

**Adjustment of FASTA Databases for quantitative isoDTB-ABPP Data Analysis**

The challenge for the quantification of modified aspartates and glutamates in this project was to quantify the relative abundance of peptides modified at one residue with **HA-yne** and the light and heavy isoDTB tags, respectively. We have previously reported this procedure for addressing a single modified amino acid, cysteine, and also applied it for two modified amino acids, aspartate and glutamate. To the best of our knowledge, relative quantification of two “variable modifications” relative to one another is not possible using MaxQuant software at this point. Therefore, we set out to use the “label” function in MaxQuant for quantification. Nevertheless, while this function allows very reliable relative quantification of light- and
heavy-labeled peptides, this function assumes every amino acid of a certain type (e.g., aspartate and glutamate) to be modified with the label. Therefore, peptides with two or more of these residues are only detected and quantified, if all of these residues have reacted with the probe and the isoDTB tags. However, the peptides that are modified at one residue with the probe and the isoDTB tags but are unmodified at the others are not detectable. For this reason, we utilized our workaround as described previously, in order to achieve this quantification. We utilized “U” respectively “O”, which normally stand for selenocysteine and pyrrolysine, as a placeholder amino acid for the modified residue (“U” used for glutamates and “O” used for aspartates). To do so, we deleted all selenocysteine- or pyrrolysine-containing proteins from the FASTA database, which were very few or nonexistent, respectively. We then individually replaced each glutamate in the FASTA database with a “U” and additionally every aspartate individually with an “O” generating n different sequences with a single “U” or “O” for a protein with n aspartates and glutamates. For each individual replacement, we created an entry in the FASTA database, which was named in the format “UniProt code”_“E”“number of the glutamate” respectively “UniProt code”_“D”“number of the aspartate”. The unmodified sequence was deleted from the FASTA database, except if the protein did not contain any aspartate or glutamate, in which case the unmodified entry was renamed to “UniProt Code”_“0” and kept in the database. In this way, for each aspartate and glutamate in the database, we created a unique sequence, in which it is marked as the modified residue (by being replaced by the placeholder “U” or “O”) and all other aspartates and glutamates are marked as unmodified (are remaining “D” or “E” in the database). Therefore, we were able to make sure that there is always only one modified residue in each peptide to be detected and quantified. Therefore, this allows us to detect and quantify all peptides that contain several aspartates and glutamates but are only modified with the probe and the isoDTB tags at one of them. During MaxQuant analysis, we define the labels in a way to not only add the modification with the tag but also to transfer the placeholder “U” or “O” back to a glutamate or aspartate. During downstream data analysis, the “U” or “O” in the sequence is changed back to the indicator for a modified glutamate (“E*”) or aspartate (“D*”).

**isoDTB-ABPP Data Analysis for quantification**

MS raw data were analyzed using MaxQuant software (version 1.6.2.10). Standard settings were used with the following changes and additions analogous to our previous study: The modified FASTA database with individual substitutions of aspartates and glutamates with the placeholder “O” or “U” was used (“PA8_DO_EU.fasta”). Labels were set on the placeholder amino acids “O” and “U” for the light isoDTB tag as light label and the heavy isoDTB tag as heavy label. The following labels were used:

- **HA-yne** and light isoDTB tag on “O” as placeholder for D: C_{18}H_{30}N_{8}O_{6}
- **HA-yne** and heavy isoDTB tag on “O” as placeholder for D: C_{14}^{13}C_{4}H_{30}N_{6}^{15}N_{2}O_{6}
- **HA-yne** and light isoDTB tag on “U” as placeholder for E: C_{28}H_{48}N_{10}O_{7}Se_{1}
- **HA-yne** and heavy isoDTB tag on “U” as placeholder for E: C_{24}^{13}C_{4}H_{48}N_{8}^{15}N_{2}O_{7}Se_{1}

Multiplicity of 2 and maximum number of labeled amino acids of 1 was set. The proteolytic enzyme was set to Trypsin/P with up to three missed cleavages. The “Re-quantify” option was
enabled. N-terminal acetylation and oxidation of methionine were selected as further variable modifications and carbamidomethylated cysteine as fixed modification. Contaminants were included. Peptides were searched with a minimum peptide length of 7 and a maximum peptide mass of 4,600 Da. “Second peptides” was enabled and “Dependent peptides” was disabled. “Match between runs” was enabled with a Match time window of 0.7 min and an alignment window of 20 min. A minimal Andromeda score\(^1\) of 40 and a delta score of 6 was set for modified peptides. An FDR of 0.01 was used for Protein FDR, PSM FDR and XPSM FDR. Technical replicates and all competitive data were analyzed in the same MaxQuant analysis.

The “peptides.txt” file of the MaxQuant analysis was used for further analysis. All peptide sequences without a modified aspartate or glutamate (placeholder “O” or “U”) and with an Andromeda Score\(^1\) below 40 were deleted. Also all peptides for “reverse” sequences and “potential contamination” were removed. Only the columns “Sequence”, “Leading Razor Protein”, “Start Position” and the columns for “Ratio H/L” for all experiments were kept. The “Leading Razor Protein” was renamed to the UniProt Code without the indicator for the number of the aspartate or glutamate. All individual ratios were filtered out if they were “NaN”, and all other values were transformed into the log2-scale. For each peptide, the data were filtered out, if there were not at least two data points for individual technical replicates or if the standard deviation between the technical replicates exceeded a value of 1.41. For each peptide, an identifier was generated in the form “UniProt Code”~“D”~residue number of the modified aspartate” or “UniProt Code”~“E”~residue number of the modified glutamate”. The data for all peptides with the same identifier, and therefore the same modified aspartate or glutamate, were combined. Here, the median of the data was used. The data were filtered out if the standard deviation exceeded a value of 1.41. Each modified aspartate or glutamate was kept in the dataset once with the shortest peptide sequence as the reported sequence. For each modified residue, all values of replicates were combined, but the individual values are also reported. The values were combined as the median and the data were filtered out, if there were not at least two data points or if the standard deviation exceeded a value of 1.41. These are the final ratios \(\log_2(\text{ratio L/H})\) that are reported. For all comparisons between different MaxQuant runs, the data were combined into one table based on the modified residue.

All individual values (4 biological replicates: soluble and insoluble) for each modified residue were loaded into Perseus (version 1.6.5.0)\(^9\) and analyzed using a one-sample \(t\)-test against a value of \(\log_2(\text{ratio L/H}) = 0\). Sites were considered as significantly regulated, if the statistical significance was \(p < 0.05\) and the median ratio was \(\log_2(\text{ratio L/H}) > 2\).

Ratios and \(p\)-values of the modified peptides were matched with the corresponding UniProt data (\(P.\ aeruginosa\) PAO1 taxon identifier: 208964) and the corresponding categorized protein abundance data obtained from PaxDb\(^8\) and listed in Table S1.

**PRM method development**

Based on the results of the quantitative data dependent acquisition (DDA) isoDTB experiments, the most interesting HA-yné modified peptides with the highest light to heavy MS1 ratios (L/H) were chosen for PRM measurements. The corresponding peptides from the response regulators CprR and ParR were selected for fragmentation, showing the highest or a
so far uncharacterized MS1 ratio (light to heavy), respectively. Additionally, two peptides (from response regulators GacA and PhoP) with an MS1 ratio of roughly one, were chosen as controls. Precursors for fragmentation were selected based on their respective most intense charge state from the DDA measurements analyzed by MS1 Filtering using Skyline (version 20.2.1.286).10 Experimental spectral libraries were built within Skyline using DDA and PRM isoDTB data processed with MaxQuant and will be available for download from Panorama Public15 upon final publication of the manuscript. For retention time comparison, PROCAL retention time peptides (JPT Peptide Technologies) were used, consisting of 40 non-naturally occurring peptides. PROCAL peptides were spiked into the samples (final quantity: 100 fmol/peptide). For 34 PROCAL peptides only MS1-chromatogram information was acquired in PRM mode, while five PROCAL peptides were also selected for fragmentation. For further information see Table S2 and Panorama Public15 upon final publication of the manuscript.

**PRM LC-MS/MS Analysis**

For PRM measurements, the same samples from the isoDTB experiments were used. Additionally, PROCAL retention time peptides were spiked into the samples (v/v 1:6) directly before measurement. 6 µL of sample were injected in order to obtain similar intensities as in previous DDA measurements and 100 fmol/peptide of the PROCAL retention time peptides.

PRM measurements were performed using the same instruments and LC-setup as described in section “LC-MS/MS Analysis”, but the Q Exactive Plus (Thermo Fisher Scientific) was operated in PRM mode. Full MS (MS1) scans were acquired at a resolution of 70,000, a scan range of m/z = 300-1500 Th, an automatic gain control (AGC) target of 3e6, and a maximum injection time of 80 ms. Targeted MS2 scans were acquired at a resolution of 17,500, an AGC target of 1e5, and a maximum injection time of 100 ms. The number of targeted precursors was adjusted to maintain a maximum cycle time of 2 s for at least 8 points across the peak in a non-scheduled PRM measurement. In total, 4 different HA-yne-modified peptides from proteins CprR, ParR, GacA and PhoP (light/heavy isoDTB version) and 5 PROCAL peptides were targeted (Table S2). Quadrupole isolation of the precursor was set to a window of 1.6 Th. Fragment ions were generated using higher-energy dissociation (HCD) with a normalized collision energy (NCE) of 27% and detected in the orbitrap.

**PRM Data Analysis**

PRM data analysis was performed using the Skyline-daily (64-bit) software (version 20.2.1.286).10 For all target peptides, the 6 most intense fragment ions (top6) were automatically picked by Skyline using the generated experimental spectral library. Raw PRM data were also processed by MaxQuant in order to visualize in Skyline the exact time point of successful peptide identification for any given MS2 spectrum. Peak picking, peak integration and transition interferences were reviewed and integration boundaries were adjusted manually in Skyline, if necessary. Mass accuracy information (“average mass error [ppm]”), correlation of fragment ion intensities between the detected light and heavy peptides (“dot product L/H”) and correlation of fragment ion intensities between the detected peptides measured by PRM and the experimental library spectrum from Skyline (“library dot product” separately for light and heavy) were exported from Skyline. Peptide identifications with a dot
product L/H > 0.9 and a library dot product > 0.85 were included for the overall ratio (L/H) calculation. The ratio of the respective MS2 peak areas (“total area fragment” L/H) was used for the ratio (L/H) calculation. For further information see Table S2 and Panorama Public15 upon final publication of the manuscript.

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2-(Hexyloxy)isoindoline-1,3-dione (1) ($^1$H, 400 MHz, DMSO-d$_6$):

2-(Hexyloxy)isoindoline-1,3-dione (1) ($^{13}$C, 101 MHz, CDCl$_3$):
**O-(Hex-5-yn-1-yl)hydroxylamine (2)** ($^1$H, 500 MHz, DMSO-$d_6$):

![Chemical Structure](image)

**O-(Hex-5-yn-1-yl)hydroxylamine (2)** ($^{13}$C, 101 MHz, DMSO-$d_6$):

![Chemical Structure](image)
| File Name                        | Size          | Action          |
|---------------------------------|---------------|-----------------|
| Allihn_et_al_Table_S1.xlsx      | 1.15 MiB      | download file   |
| Allihn_et_al_Table_S2.xlsx      | 51.64 KiB     | download file   |