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A Photocrosslinking Probe to Capture the Substrates of Caseinolytic Protease P

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Abstract:
Protein homeostasis in bacteria is regulated by proteases such as the tetradecameric caseinolytic protease P (ClpP). Although substrates of ClpP have been successfully deciphered in genetically engineered cells, methods which directly trap processed proteins within native cells remain elusive. Here, we introduce an *in situ* trapping strategy which utilizes trifunctional probes that bind to the active site serine of ClpP and capture adjacent substrates with an attached photocrosslinking moiety. After enrichment using an alkyne handle, substrate deconvolution by mass spectrometry (MS) is performed. We show that our two traps bind substoichiometrically to ClpP, retain protease activity, exhibit unprecedented selectivity for *Staphylococcus aureus* ClpP in living cells and capture numerous known and novel substrates. The exemplary validation of trapped hits using a targeted proteomics approach confirmed the fidelity of this technology. In conclusion, we provide a novel chemical platform suited for the discovery of serine protease substrates beyond genetic engineering.

Introduction:
Caseinolytic protease P (ClpP) plays a major role in cellular homeostasis by removing damaged or misfolded proteins with the help of AAA+ ATPases such as ClpX or ClpC.[1] [2] Additionally, in pathogenic bacteria, ClpXP is essential for the regulation of virulence and its genetic knockout or chemical inhibition leads to a global attenuation of toxin secretion.[3] [4] Substrate degradation is initiated by the chaperone, which recognizes the substrate protein, unfolds it under ATP consumption, and threads it into the tetradecameric barrel of ClpP. Within the core, catalytically active serine-histidine-aspartate triads cleave peptide bonds resulting in fragments of 6 to 13 amino acid residues depending on the organism.[1] [5] [6] When in complex with the chaperone, ClpP exhibits a rather broad substrate specificity enabling the rapid removal of client proteins.[7] The recognition of substrates by ClpXP is best understood for proteins stalled at the ribosome. Here, a SsrA peptide degradation tag is co-translationally attached to truncated polypeptides by its RNA sequence, leading to rapid degradation.[8] Since this seminal discovery, the SsrA tag remains one of the few and best-characterized peptide recognition sequences of ClpXP proteolysis.[9] Recently, post-translational phosphorylation of arginine residues by the kinase system McsBA was discovered to function as a recognition tag for ClpCP-mediated proteolysis, suggesting that other, still undiscovered post-translational modifications (PTMs) may serve as degrons.[10] Despite these examples, a vast number of proteins affected by ClpP proteolysis still lack a firm consensus for degradation. In the absence of such a cleavage sequence, bioinformatic predictions fall short of deciphering putative substrates.
Thus, the search for ClpXP substrates rather relies on proteomic approaches. For example, whole proteome analysis of *Staphylococcus aureus* wildtype (wt) versus ClpP knockout cells revealed numerous proteins accumulating in the absence of active ClpP and were thus stated as putative substrates.[11] [12] This finding was further complemented by a comparison to a chemical knockout of ClpP achieved by selective inhibition with beta-lactones.[13] Furthermore, an elegant method utilized inactive serine-to-alanine mutants of ClpP, which retain the proteolytic complex formation *in situ* and promote chaperone-mediated recognition and translocation of substrate proteins into the proteolytic core. However, due to the lack of an active site serine, substrates are not degraded and were identified after affinity enrichment via LC-MS.[9] [14] With this methodology, about 70 putative substrate proteins could be identified by Feng *et al*. in *S. aureus*, of which one-third were reported previously to be unstable in the presence of ClpP or actual substrates in other organisms.[14] Selected putative substrates have been validated for ClpP proteolysis by cellular studies, e.g., by comparing their protein levels in wildtype or ClpP knockout cells, respectively.
Recently, we introduced an additional capture method for human, mitochondrial ClpP, which is based on the incorporation of diazirine photoactivatable amino acids at strategic positions within the ClpP

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barrel. UV-light crosslinked cognate substrates *in situ* within the active complex and thereby provided unique and complementary substrates to the previous methods.[15] However, a major limitation of all these approaches is the need for engineered cells which may artificially alter biological processes. Despite the urgent demand to better understand the role of ClpP for cellular growth and virulence, a direct capture method for wildtype cells is elusive.

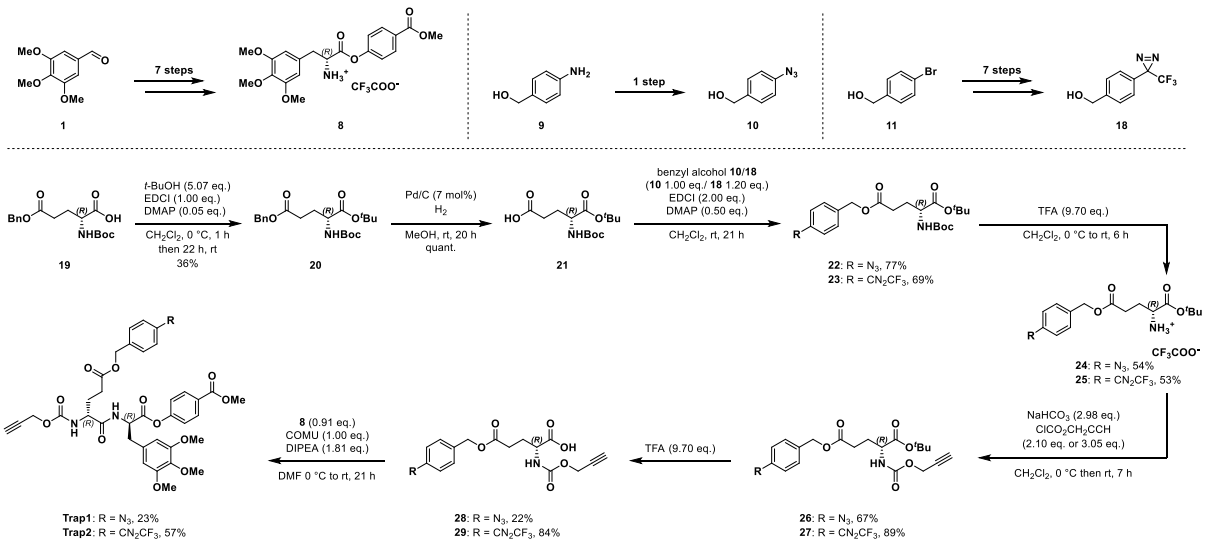
Here, we introduce trifunctional active site traps that selectively recognize the ClpP active site serine in whole cells, maintain turnover of the unoccupied subunits within the complex by substoichiometric binding, and capture substrates while being processed by ClpP via a photocrosslinker moiety in the active site traps. The identity of captured substrates is revealed after enrichment through an alkyne handle attached to the trap, followed by click chemistry and mass spectrometric (MS) analysis. The trapping experiments provide unprecedented insights into processed proteins during cellular stress with roles in cell division and virulence. This novel methodology eases the access for elucidating protease substrates of wildtype cells with putative applications also beyond ClpP.

Results:

Design and synthesis of active site traps

In the search for ClpP active site binders, which do not fully occupy all 14 sites of the proteolytic barrel and thus still facilitate proteolysis, we focused on beta-lactone and phenylester compounds that were previously discovered by our group. Both molecular classes address the active site serine by a covalent ester formation.[3] [16] Although both achieve the desired substoichiometric binding, a new generation of phenlyesters, hallmarked by trimethoxy phenyl as well as benzylester moieties, was shown to additionally enhance protease turnover.[17] Based on these desired properties, we investigated suitable sites for introducing a photoactivatable crosslinking unit and an alkyne handle for substrate capture and enrichment, respectively. Previous structure-activity relationship studies guided the design of two traps in which the tert-butyl protecting group of the parent compound was replaced by a propargyl moiety and the benzylester functionalized as aryl azide (**trap1**) or trifluoromethyl diazirine (**trap2**).[16] [17] We deliberately selected two photocrosslinking units of different reactivity and selectivity (forming either a nitrene or carbene upon UV-irradiation)[18], to enhance the capture of diverse proteins.

Synthesis of **trap1** and **trap2** started with the preparation of precursors **8**, **10,** and **18** according to published synthesis routes (Scheme S1).[17] [19] [20] Next, esterification of *N*-Boc-L-glutamic acid 5-benzyl ester (**19**) with *t*-butanol resulted in **20** (Scheme 1). Palladium-on-carbon-catalyzed hydrogenative deprotection of the benzyl group gave *N*-Boc-1-tert-butyl-L-glutamate (**21**) as starting point for esterification with alcohols **10** and **18** resulting in **22** or **23**. The alkyne handle was introduced after *N*-deprotection with trifluoroacetic acid by reaction with propargyl chloroformate yielding compounds **26** or **27**. Release of the carboxylic acid by acidic cleavage of the ester resulted in fragments **28** and **29,** which were coupled to fragment **8** to obtain desired photoprobes **trap1** and **trap2**.



Scheme 1: Synthesis scheme of **trap1** and **trap2**. Precursors **8**, **10**, and **18** were prepared according to published procedures.[17] [19] [20] Starting from *N*-Boc-L-glutamic acid 5-benzyl ester (**19**), the desired traps were synthesized in 7 steps. EDCI = 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide, DMAP = 4-Dimethylaminopyridine, TFA = Trifluoroacetic acid, COMU = 1-[1-(Cyano-2-ethoxy-2-oxoethylideneaminooxy)-dimethylamino-morpholino]-uronium hexafluorophosphate, DIPEA = Diisopropylethylamine, DMF = Dimethylformamide.

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Chemical traps substoichiometrically bind ClpP, retain its proteolytic activity and selectively address the protease in whole cells

With two trifunctional traps at hand, we first validated if these compounds are 1) binding to the ClpP active site, 2) retain its proteolytic activity, and 3) are selective for ClpP in living bacterial cells, all crucial prerequisites for the use as versatile trapping probes. We initiated these studies by incubation of recombinantly expressed and purified *S. aureus* ClpP with the two traps at 1:1, 1:10, and 1:100 molar ratios followed by intact protein MS to decipher the extent of binding. **Trap1** achieved a modification ranging up to 28% (lowest 9%) and **trap2** up to 6% (lowest 3%), which is in the substoichiometric range as desired (Figure 1A). With the majority of active sites still available for substrate processing, we next evaluated the ClpP peptidase and ClpXP protease substrate turnover in the presence of the traps (Figure S1 and Figure 1B). Both compounds retained peptide and protein turnover of ClpP and ClpXP, respectively, with **trap1** even slightly enhancing the protease activity to 124 % at 50 µM, which demonstrates its descent from the parent activator scaffold. **Trap2** reduced protease activity to 56 % at the highest concentration, demonstrating that even minor modifications in the scaffold (photocrosslinker moiety) have notable effects on the overall performance. However, they still exhibit sufficient turnover for substrate capture. A previously established phenylester inhibitor (**AV170**)[16] served as a negative control in these studies. We thus reasoned that moderately activating and inhibiting traps would exhibit complementary utility for comprehensive substrate coverage.

Finally, we evaluated the selectivity of the probes for ClpP target engagement within living cells. Both trapping probes were incubated with intact *S. aureus* cells, lysed, and clicked to rhodamine azide or biotin azide for target visualization via fluorescent SDS-gel analysis or enrichment for LC-MS/MS, respectively (Figure 1C). Gel-based analysis revealed concentration-dependent labeling of a protein with a molecular mass corresponding to ClpP with both traps (Figure 1D). Interestingly, the protein was selectively labeled at probe concentrations below 1 µM and the band remained even visible down to 30 nM suggesting high affinity binding. This labeling occurred independently of UV-light confirming the covalent binding to the active site serine via the phenylester moiety (Figure S2). To obtain a comprehensive overview of ClpP selectivity, we performed quantitative MS with both probes at 10 µM each. After incubation with living cells and lysis, the probes were clicked to biotin azide, enriched on streptavidin beads, peptides released by tryptic digest, and analyzed with LC-MS/MS with label-free quantification (LFQ).[21] Proteins enriched with $\log_2 = 1$ (2-fold enrichment) and a p-value < 0.05 compared to DMSO-treated samples were regarded as hits. Importantly, ClpP protruded as one of the most significant hits, with both **trap1** and **trap2** enriching only one and two additional proteins (Figure 1E), that are not known to digest proteins.[22] This emphasizes the desired probe specificity for ClpP in *S. aureus* cells.

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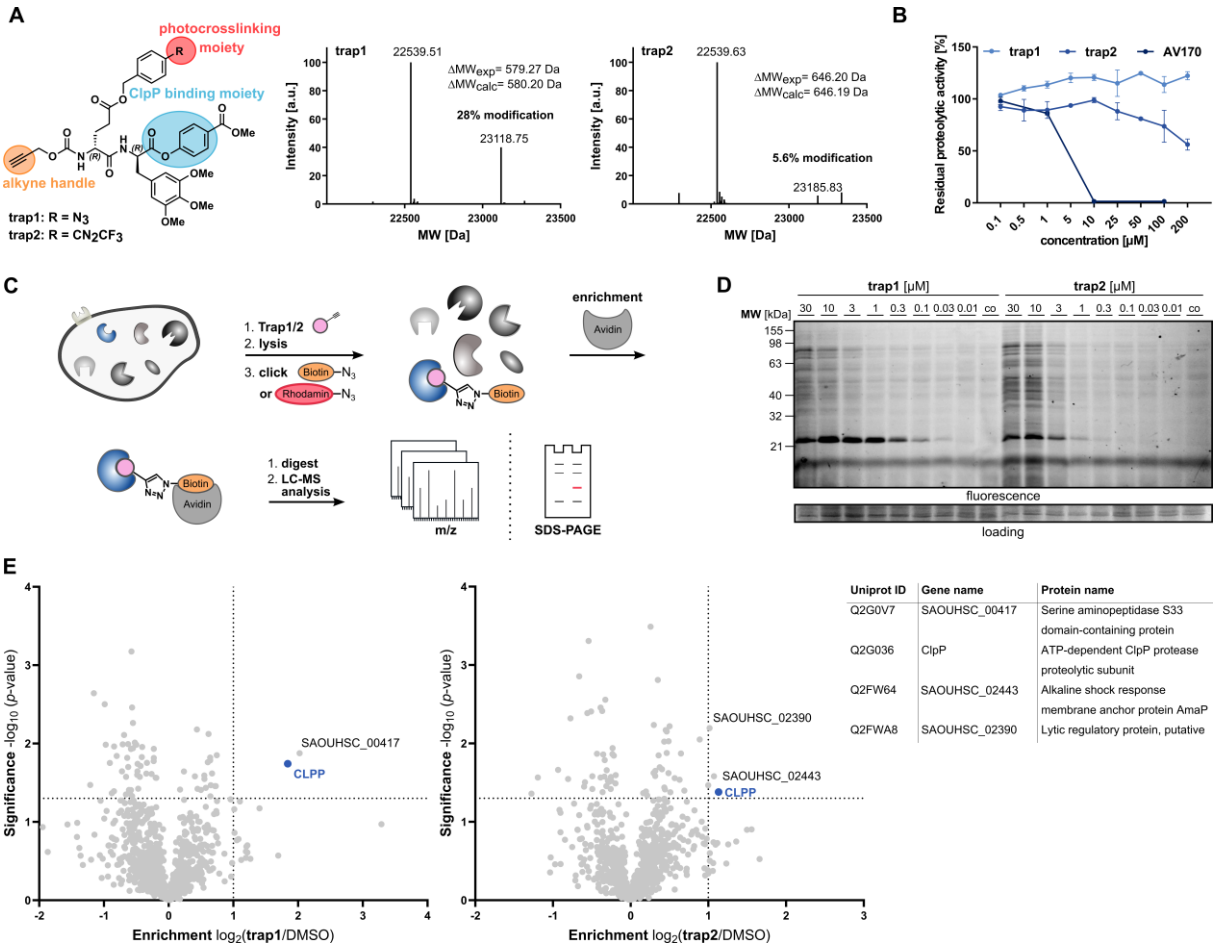

Figure 1: **Trap1** and **trap2** bind to ClpP and retain its proteolytic activity. **A**: Chemical structure of **trap1** and **trap2** and degree of ClpP modification upon incubation with the traps at 100:1 molar ratio (trap:ClpP). **B**: Residual ClpXP proteolytic activity after incubation with **trap1**, **trap2,** or ClpP inhibitor **AV170**[16] at different compound concentrations measured by the rate of GFP-ssrA degradation. Experiments were carried out in triplicates. Results are in mean ± SD. **C**: Schematic representation of evaluation of trap selectivity by SDS-PAGE or LC-MS/MS. **D**: Gel-based labelling of *S. aureus* intact cells with **trap1** and **trap2** shown by fluorescence sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The uncropped Coomassie-stained gel (loading control) is shown in **Figure S7**. **E**: Volcano plots of whole cell trap selectivity evaluation via LC-MS/MS in *S. aureus.* Threshold lines represent a $\log_2$ enrichment of 1 or greater and a $-\log_{10}$(*p*-value) of 1.3 (two-sided two-sample *t*-test, $n = 3$ replicates per group).

ClpP substrates are trapped in the stationary and exponential growth phase

To focus the analysis solely on trapped proteins, we treated intact *S. aureus* cells with the probes and divided these samples into two fractions which were either irradiated with UV-light for 15 min or remained in the dark (Figure 2A). In this way, analysis of the corresponding enriched proteins of both fractions should only differ in the capture of proteins under UV irradiation.

First, we performed the trapping experiment with cells treated in the stationary phase.[13] Here, plotting the proteins selectively enriched upon UV-treatment of both traps revealed only a few ClpP trapped proteins (Figure S3), including the known substrates IsaA[11] (probable transglycosylase) and Sbi[11] [12] (immunoglobulin-binding protein) as well as 5 additional candidates which were not previously linked to ClpP digestion. Among those are protein DltD which is involved in D-alanylation of teichoic acids on the cell surface,[23] GcvH (glycine cleavage system H protein) involved in lipoic acid synthesis[24] and the virulence factor Hly (alpha-hemolysin).[25] Of note, GCSH (mitochondrial glycine cleavage system H protein), a homolog of GcvH, was previously found as a substrate of eukaryotic ClpP, suggesting a conserved specificity throughout species.[15]

Next, in order to investigate the substrate scope of ClpP under exponential growth, we repeated the experiment and plotted proteins of both traps in an identical format (Figure 2B). Both traps combined significantly enriched 134 proteins emphasizing a more pronounced substrate processing under exponential growth conditions. Pathway analysis of captured proteins revealed their pronounced role in ribosomal processes (Figure S4 and Figure S5).

Interestingly, **trap2** significantly captured 83 proteins, of which 14 are known annotated ClpP substrates, followed by **trap1** which enriched 59 proteins, with 5 known substrates (8 proteins were identified by

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both traps) (Figure 2C).[11] [12] [14] This overall performance suggests especially for **trap2** a good balance between the identification of new protein hits as well as of previously confirmed substrates validating the fidelity of this novel methodology. The differences in protein coverage by chemical traps vs. previous genetic methods are not surprising. For example, the chemical approach does not require cell engineering and protein overexpression, thus keeping the cells in their native state. On the other side, the small molecule covalently binds proteins via photocrosslinking which could give bias towards proteins with hydrophobic patches. Thus, both approaches are complementary facilitating the most comprehensive substrate identification.

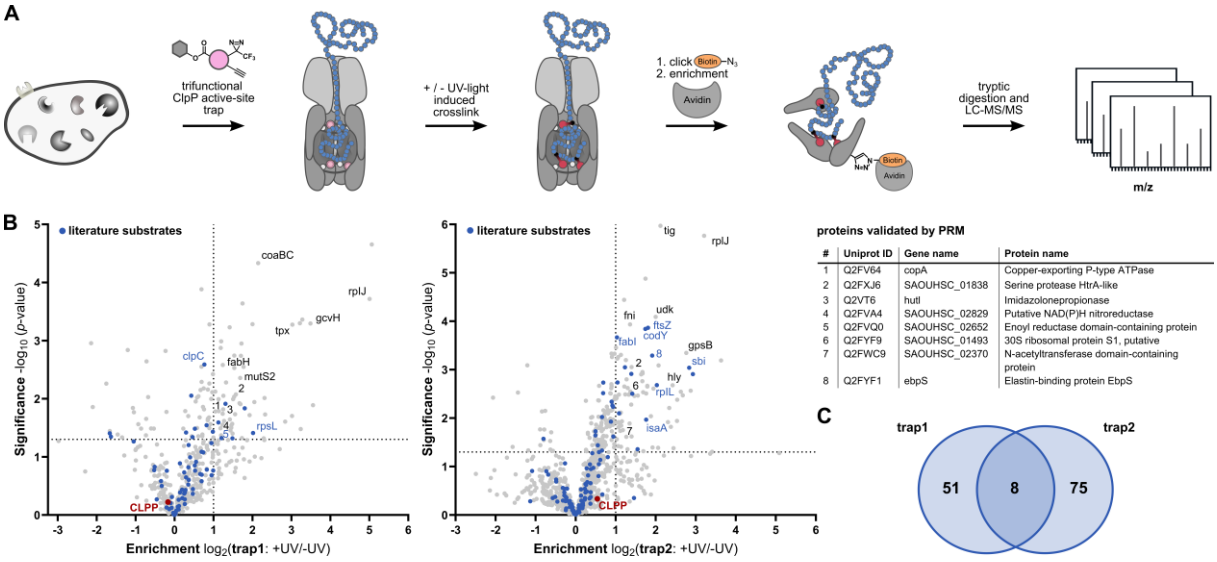

Figure 2: Substrate scope of ClpP active site traps during exponential growth phase. **A**: Schematic representation of identification of *S. aureus* ClpP substrates via LC-MS/MS using **trap1** and **trap2**. **B**: Volcano plots of enriched proteins after treatment of *S. aureus* cells with **trap1** (left) or **trap2** (right) in exponential growth phase. Blue dots represent literature reported substrates.[11] [12] [14] Threshold lines represent a $\log_2$ enrichment of 1 or greater and a $-\log_{10}$(*p*-value) of 1.3 (two-sided two-sample *t*-test, $n = 3$ replicates per group). Proteins listed in table were validated using PRM (Figure 4). **C**: Venn-diagram comparing the substrate scope of **trap1** and **trap2**.

Distinct proteins were captured under heat shock

As ClpP is crucial for the removal of damaged proteins, we performed a final trapping experiment under cellular heat stress to investigate a putatively altered substrate scope under these conditions. *S. aureus* cells were heated to 42°C, treated with the probes, and cells analogously processed as described above. In total, **trap1** and **trap2** significantly enriched 67 proteins (31 solely by **trap1** and 17 solely by **trap2**) of which 35 were unique hits of the heat stress conditions (Figure 3A and Figure 3B). Proteins trapped during heat shock are involved in cell division (e.g. cell division protein FtsZ, trigger factor (tig), cell cycle protein GpsB)[26] [27] [28] or virulence (e.g. Sbi (immunoglobulin-binding protein), alpha-hemolysin (hly) or HlgC (gamma-hemolysin component C (hlgC)).[29] [30] [31] Of note, about 20% of these hits have been previously assigned as ClpP substrates [11] [12] [14] further highlighting the fidelity of the traps.

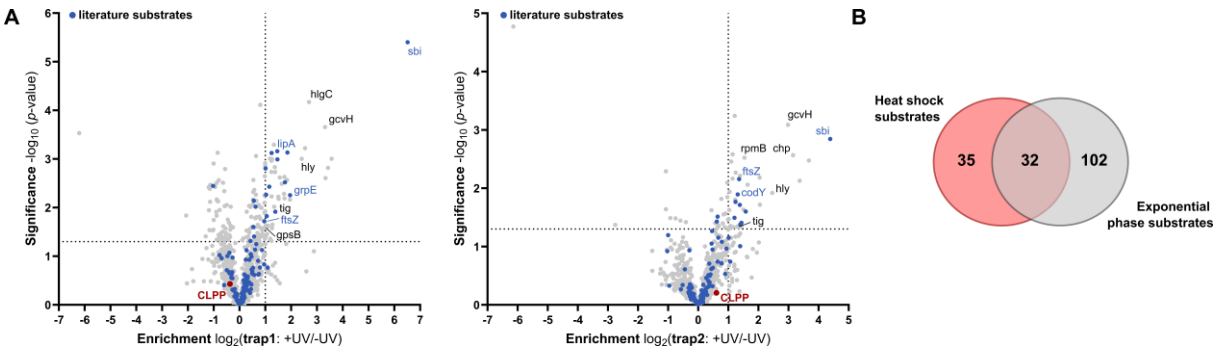

Figure 3: Substrate scope of ClpP active site traps in exponential phase during heat shock response. **A**: Volcano plots of enriched proteins after treatment of *S. aureus* cells with **trap1** (left) or **trap2** (right) in the exponential phase during heat shock response. Threshold lines represent a $\log_2$ enrichment of 1 or greater and a $-\log_{10}$(*p*-value) of 1.3 (two-sided two-sample *t*-test, $n = 3$ replicates per group). **B**: Venn-diagram of overlap between ClpP heat shock and non-heat shock substrates.

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Captured signature proteins are validated as substrates of ClpP

The direct validation of ClpP substrates via *in vitro* assays is challenging due to the lack of knowledge about putative PTM degrons or the help of chaperones and adaptors needed for recognition and delivery. Therefore, validation of protease substrates is usually performed by analyzing protein abundance in wt vs. ClpP knockout cells, e.g., using western blots.[14] Here, ClpP substrates are believed to be more abundant in the protease knockout strain as they are not proteolytically digested. Thus, we performed a whole proteome MS analysis of exponentially grown *S. aureus* wt and the proteolytically inactive ClpP S98A mutant for validation of our identified substrates.[11] Here, cells were treated with chloramphenicol to inhibit protein synthesis[14] to gain a global insight into protein regulation under these conditions. In fact, mapping all substrates that were postulated in literature[11] [12] [14] into the corresponding full proteome plot revealed that under the given conditions a large number of these substrates was not significantly regulated (Figure S6A). However, literature substrates clearly stabilized in the ClpP mutant cells included the chaperone protein ClpB, the adapter protein MecA, the transcriptional regulator CtsR, and the staphylococcal secretory antigen ssaA2, which were previously proposed to be ClpP substrates using a trapping approach and thus regarded as high confidence hits.[11] [12] [14] Next, we mapped the substrates identified in this study into the same full proteome volcano plot (Figure S6B). Proteins that were identified using our trapping approach and were additionally stabilized in the *S. aureus* ClpP S98A strain compared to the wt strain included known and unknown hits.

Next, we selected eight identified ClpP substrates that were stabilized in the *S. aureus* ClpP S98A mutant for a more accurate quantification of abundance using parallel reaction monitoring (PRM) (Figure S6B). In comparison to the classical western-blot analysis of ClpP substrates, we here chose targeted proteomics via PRM with the advantage of being independent on the availability of specific antibodies and at the same time displaying highest precision by focusing on signature peptides. Of the eight chosen substrates for quantification using PRM, two were previously reported to be processed by ClpP (EbpS and enoyl reductase (ER) domain-containing protein as control)[11] [12] and six were so-far unknown substrates of ClpP. Applying PRM, we accurately quantified protein abundances between samples by selection of signature peptides for each protein (Figure 4A) by their MS2 fragment ion peak areas (Figure 4B). Log-transformed fragment ion peak areas for each peptide were combined and compared for *S. aureus wt* and *S. aureus* ClpP S98A (Figure 4C) to analyze the overall protein abundance. Satisfyingly, using PRM, we could confirm all eight proteins as ClpP substrates. This successful validation not only demonstrates the power of targeted proteomics in monitoring proteolysis of proteins but also demonstrates the fidelity of our approach to unravel novel ClpP substrates.

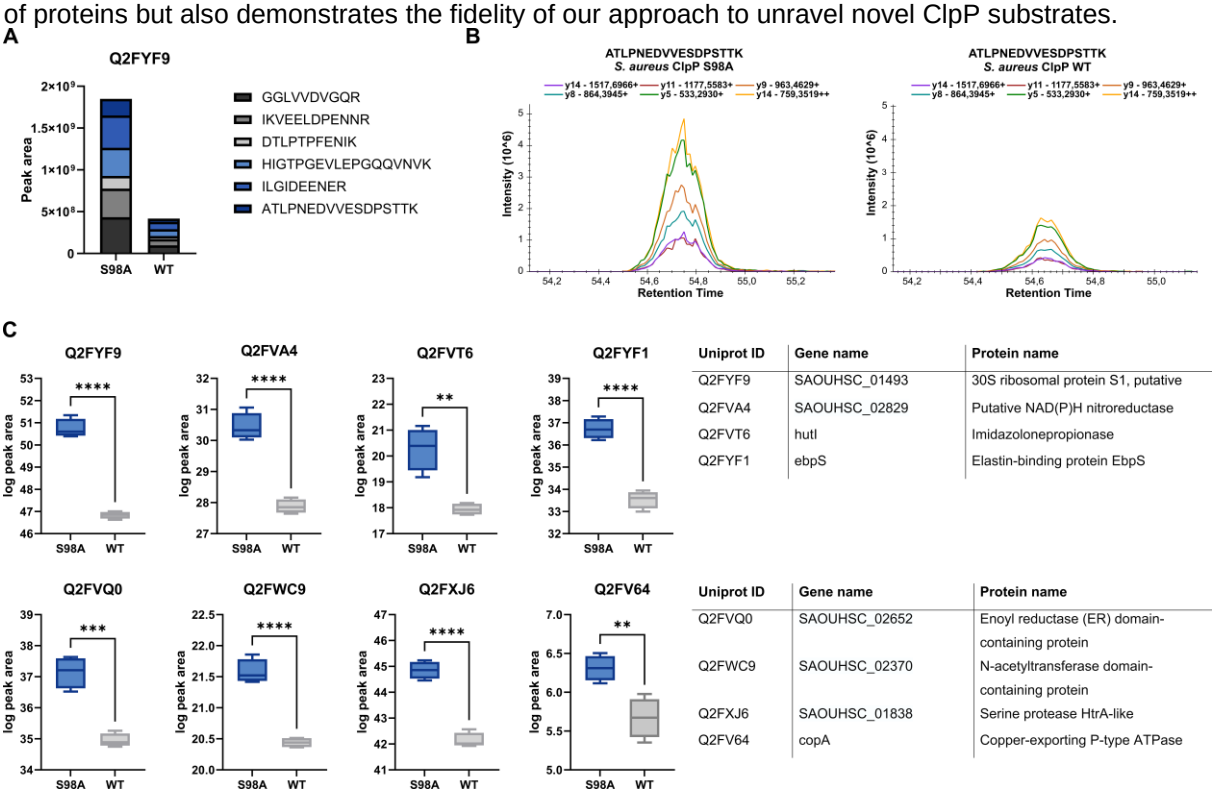

Figure 4: PRM experiment for validation of selected ClpP substrates by comparison of protein abundance in *S. aureus* WT cells and the inactive mutant ClpP S98A. **A**: Schematic representation of protein quantification based

on six unique peptides shown exemplary for ClpP substrate Q2FYF9. **B**: Schematic representation of peptide quantification based on specific fragment ion peak areas shown exemplarily for peptide ATLPNEDVVESDPSTTK from Q2FYF9. **C**: Protein quantification of selected ClpP substrates in *S. aureus* WT and *S. aureus* ClpP S98A using a targeted proteomic approach (PRM). Protein abundance is visualized in box plots based on the sum of log-transformed peak areas for each signature peptide per protein. ** Represents *p*-value ≤ 0.01 determined by Student's *t*-test, *** represents *p*-value ≤ 0.001 determined by Student's *t*-test, **** represents *p*-value ≤ 0.0001 determined by Student's *t*-test, *n* = 4 replicates per group.

Conclusion:

Multiple methods for the trapping of protease substrates have been successfully applied most relying on engineered cells or studies in lysates. While these procedures not only require genetic manipulations, they also alter cellular physiology, e.g., by knockdown of target proteases or overexpression of trap proteins. The capture of proteins directly in the active site within wildtype cells enhances the reliability of identified target proteins. Therefore, we introduced a complementary method that directly accesses the ClpP active site within intact wildtype cells, retains its activity, and captures substrates *in situ*. We showed the utility of this strategy by capturing predominantly unprecedented protein substrates and exemplarily validated these using a targeted proteomic approach. The conceptual differences between this chemical strategy and the previous biochemical approaches emphasize a complementarity of both methodologies with individual strengths and weaknesses. For example, the chemical trapping approach requires specific binders to a single protease, as shown for ClpP, to ensure the needed selectivity across the proteome. In addition, although independent of cellular engineering, the strategy may fall short in capturing substrates that kinetically escape photocrosslinking or do not sufficiently accumulate in the barrel to reach high concentrations needed for efficient photo-trapping, issues which could be resolved by further refining the trapping unit. Thus, while the full complement of substrates could be even larger, this novel methodology demonstrates its utility in identifying unprecedented substrates of the ClpP serine protease. As an outlook, the technology bears the potential to be expanded as a general plug-and-play tool for capturing serine protease substrates by tailoring the core scaffold for individual enzymes of interest.

Supporting Information

The authors have cited additional references within the Supporting Information.[34-42]

Keywords

Chemical biology, bacterial proteases, Caseinolytic protease P (ClpP), substrate traps, chemical proteomics.

Data availability

All mass spectrometric raw files, as well as the MaxQuant output files, have been deposited to the ProteomeXchange Consortium via the PRIDE[32] partner repository and can be accessed using the identifier PXD047274 (reviewer account username: reviewer_pxd047274@ebi.ac.uk, wCRK03mP). The mass spectrometric raw files measured in PRM mode and analyzed with the Skyline software have been deposited to Panorama Public[33] and to the ProteomeXchange Consortium (identifier PXD047469) and can be accessed via https://panoramaweb.org/ClpP-Trap-PRM.url (reviewer account email: panorama+reviewer228@proteinms.net; password: DASkebii).

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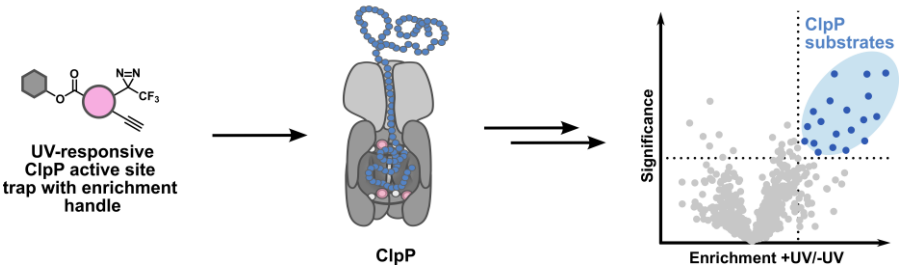
Literature:

[1] T. A. Baker, R. T. Sauer, *Biochim. Biophys. Acta* **2012**, *1823*, 15-28.
[2] V. Bhandari, K. S. Wong, J. L. Zhou, M. F. Mabanglo, R. A. Batey, W. A. Houry, *ACS Chem. Biol.* **2018**, *13*, 1413-1425.
[3] T. Böttcher, S. A. Sieber, *J. Am. Chem. Soc.* **2008**, *130*, 14400-14401.
[4] D. Frees, S. N. A. Qazi, P. J. Hill, H. Ingmer, *Mol. Microbiol.* **2003**, *48*, 1565-1578.
[5] C. Y. Tremblay, R. H. Vass, R. W. Vachet, P. Chien, *Biochemistry* **2020**, *59*, 4294-4301.
[6] K.-H. Choi, S. Licht, *Biochemistry* **2005**, *44*, 13921-13931.
[7] M. Gersch, M. Stahl, M. Poreba, M. Dahmen, A. Dziedzic, M. Drag, S. A. Sieber, *ACS Chem. Biol.* **2016**, *11*, 389-399.
[8] A. W. Karzai, E. D. Roche, R. T. Sauer, *Nat. Struct. Biol.* **2000**, *7*, 449-455.
[9] J. M. Flynn, S. B. Neher, Y.-I. Kim, R. T. Sauer, T. A. Baker, *Mol. Cell* **2003**, *11*, 671-683.
[10] D. B. Trentini, M. J. Suskiewicz, A. Heuck, R. Kurzbauer, L. Deszcz, K. Mechtler, T. Clausen, *Nature* **2016**, *539*, 48-53.
[11] V. C. Kirsch, C. Fetzer, S. A. Sieber, *J. Proteome Res.* **2021**, *20*, 867-879.
[12] D. Frees, J. H. Andersen, L. Hemmingsen, K. Koskenniemi, K. T. Bæk, M. K. Muhammed, D. D. Gudeta, T. A. Nyman, A. Sukura, P. Varmanen, K. Savijoki, *J. Proteome Res.* **2012**, *11*, 95-108.
[13] J. Krysiak, M. Stahl, J. Vomacka, C. Fetzer, M. Lakemeyer, A. Fux, S. A. Sieber, *J. Proteome Res.* **2017**, *16*, 1180-1192.
[14] J. Feng, S. Michalik, A. N. Varming, J. H. Andersen, D. Albrecht, L. Jelsbak, S. Krieger, K. Ohlsen, M. Hecker, U. Gerth, H. Ingmer, D. Frees, *J. Proteome Res.* **2013**, *12*, 547-558.
[15] T.-A. Nguyen, T. F. Gronauer, T. Nast-Kolb, S. A. Sieber, K. Lang, *Angew. Chem. Int. Ed.* **2022**, *61*, 202111085.
[16] M. W. Hackl, M. Lakemeyer, M. Dahmen, M. Glaser, A. Pahl, K. Lorenz-Baath, T. Menzel, S. Sievers, T. Böttcher, I. Antes, H. Waldmann, S. A. Sieber, *J. Am. Chem. Soc.* **2015**, *137*, 8475-8483.
[17] M. Lakemeyer, E. Bertosin, F. Möller, D. Balogh, R. Strasser, H. Dietz, S. A. Sieber, *Angew. Chem. Int. Ed* **2019**, *58*, 7127-7132.
[18] M. Hashimoto, Y. Hatanaka, *Eur. J. Org. Chem.* **2008**, *2008*, 2513-2523.
[19] B. Chen, P. Wang, Q. Jin, X. Tang, *Org. Biomol. Chem.* **2014**, *12*, 5629-5633.
[20] D. P. Smith, J. Anderson, J. Plante, A. E. Ashcroft, S. E. Radford, A. J. Wilson, M. J. Parker, *Chem. Commun.* **2008**, 5728-5730.
[21] J. Cox, M. Y. Hein, C. A. Luber, I. Paron, N. Nagaraj, M. Mann, *Mol. Cell. Proteom.* **2014**, *13*, 2513-2526.
[22] *Nucleic Acids Res.* **2022**, *51*, D523-D531.
[23] B. M. Wood, J. P. Santa Maria, Jr., L. M. Matano, C. R. Vickery, S. Walker, *J. biol. chem.* **2018**, *293*, 17985-17996.
[24] Q. H. Christensen, J. E. Cronan, *Biochemistry* **2010**, *49*, 10024-10036.
[25] R. J. O'Callaghan, M. C. Callegan, J. M. Moreau, L. C. Green, T. J. Foster, O. M. Hartford, L. S. Engel, J. M. Hill, *Infect Immun.* **1997**, *65*, 1571-1578.
[26] J. Lutkenhaus, *Mol. Microbiol.* **1993**, *9*, 403-409.
[27] H. Veiga, A. Jousselin, S. Schäper, B. M. Saraiva, L. B. Marques, P. Reed, J. Wilton, P. M. Pereira, S. R. Filipe, M. G. Pinho, *EMBO J.* **2023**, *42*, 112140.
[28] M. D. Barbuti, I. S. Myrbråten, D. Morales Angeles, M. Kjos, *Microbiologyopen* **2023**, *12*, 1338.
[29] E. J. Smith, L. Visai, S. W. Kerrigan, P. Speziale, T. J. Foster, *Infect. Immun.* **2011**, *79*, 3801-3809.
[30] M. C. Callegan, L. S. Engel, J. M. Hill, R. J. O'Callaghan, *Infect. Immun.* **1994**, *62*, 2478-2482.
[31] M. Pivard, I. Caldelari, V. Brun, D. Croisier, M. Jaquinod, N. Anzala, B. Gilquin, C. Teixeira, Y. Benito, F. Couzon, P. Romby, K. Moreau, F. Vandenesch, *Microbiol Spectr* **2023**, *11*, 01073-23.
[32] Y. Perez-Riverol, J. Bai, C. Bandla, D. García-Seisdedos, S. Hewapathirana, S. Kamatchinathan, Deepti J. Kundu, A. Prakash, A. Frericks-Zipper, M. Eisenacher, M. Walzer, S. Wang, A. Brazma, Juan A. Vizcaíno, *Nucleic Acids Res.* **2021**, *50*, D543-D552.
[33] V. Sharma, J. Eckels, B. Schilling, C. Ludwig, J. D. Jaffe, M. J. MacCoss, B. MacLean, *Mol. Cell. Proteom.* **2018**, *17*, 1239-1244.
[34] J. T. Spletstoser, P. T. Flaherty, R. H. Himes, G. I. Georg, *J. Med. Chem.* **2004**, *47*, 6459-6465.
[35] K. Nakamoto, Y. Ueno, *J. Org. Chem.* **2014**, *79*, 2463-2472.

[36] M. Gersch, K. Famulla, M. Dahmen, C. Göbl, I. Malik, K. Richter, V. S. Korotkov, P. Sass, H. Rübsamen-Schaeff, T. Madl, H. Brötz-Oesterhelt, S. A. Sieber, *Nat. Commun* **2015**, *6*, 6320.
[37] D. Szklarczyk, A. Franceschini, S. Wyder, K. Forslund, D. Heller, J. Huerta-Cepas, M. Simonovic, A. Roth, A. Santos, K. P. Tsafou, M. Kuhn, P. Bork, L. J. Jensen, C. von Mering, *Nucleic Acids Res* **2015**, *43*, D447-452.
[38] P. Shannon, A. Markiel, O. Ozier, N. S. Baliga, J. T. Wang, D. Ramage, N. Amin, B. Schwikowski, T. Ideker, *Genome Res.* **2003**, *13*, 2498-2504.
[39] S. Tyanova, T. Temu, J. Cox, *Nat. Protoc* **2016**, *11*, 2301-2319.
[40] S. Tyanova, T. Temu, P. Sinitcyn, A. Carlson, M. Y. Hein, T. Geiger, M. Mann, J. Cox, *Nat. Methods* **2016**, *13*, 731-740.
[41] F. Coscia, S. Doll, J. M. Bech, L. Schweizer, A. Mund, E. Lengyel, J. Lindebjerg, G. I. Madsen, J. M. Moreira, M. Mann, *J. Pathol.* **2020**, *251*, 100-112.
[42] L. K. Pino, B. C. Searle, J. G. Bollinger, B. Nunn, B. MacLean, M. J. MacCoss, *Mass Spectrom. Rev.* **2020**, *39*, 229-244.

Entry for the Table of Contents

A new tool for profiling of bacterial protease substrates



Here, we introduce an *in situ* trapping strategy using trifunctional probes that bind to the active site serine of the tetradecameric caseinolytic protease P (ClpP) and capture substrates within the barrel via a photocrosslinking moiety upon UV irradiation. The probes retain protease activity and capture numerous known and novel substrates of ClpP, allowing the discovery of serine protease substrates beyond genetic engineering.