

Article

**Chemical probes
unravel an antimicrobial
defense response
triggered by binding
of the human opioid
dynorphin to a
bacterial sensor kinase**

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Fetzer, and Stephan A. Sieber



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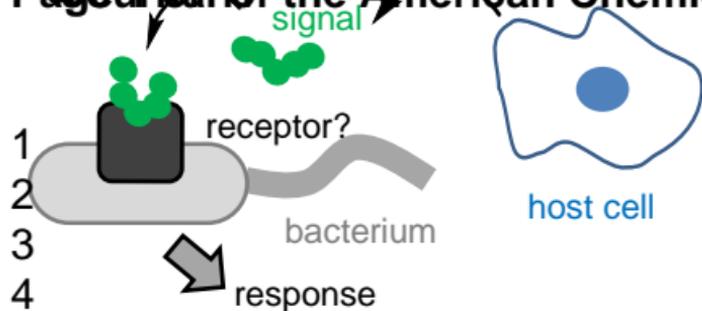


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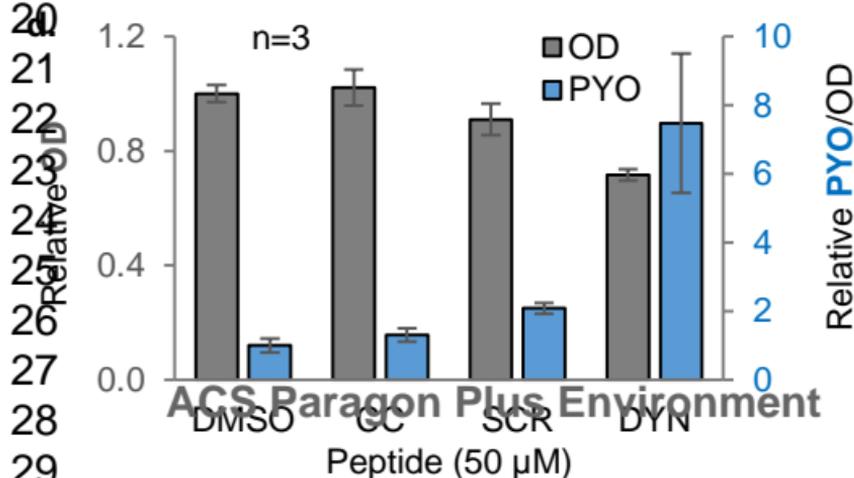
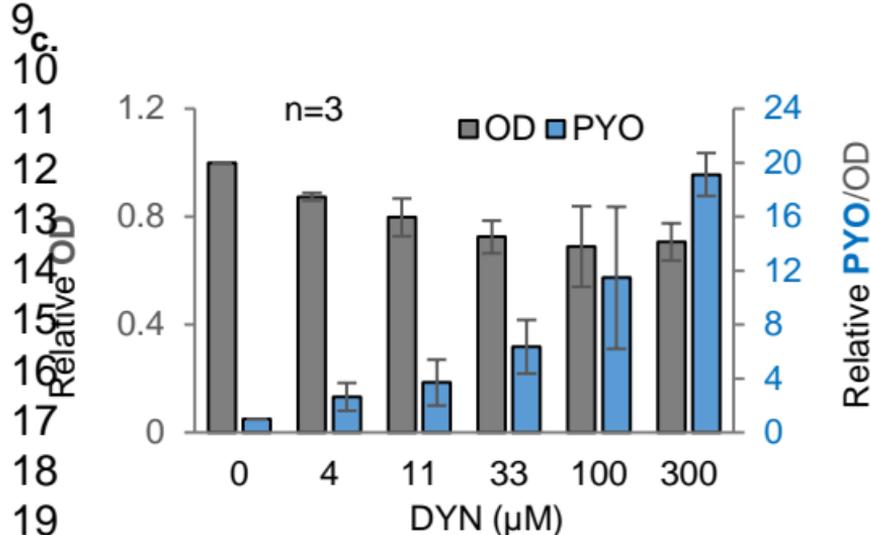
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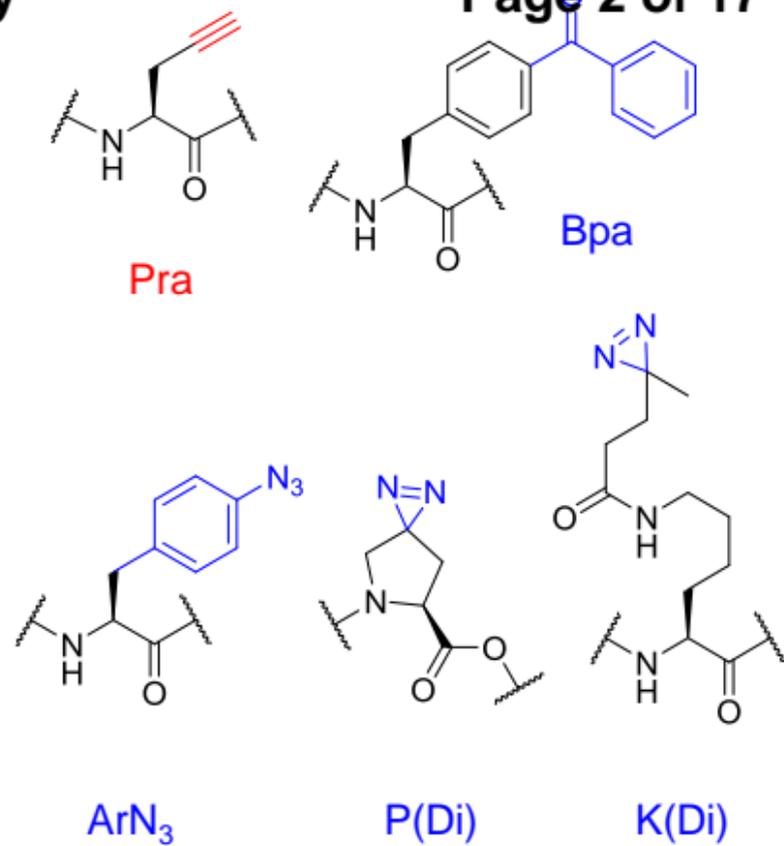
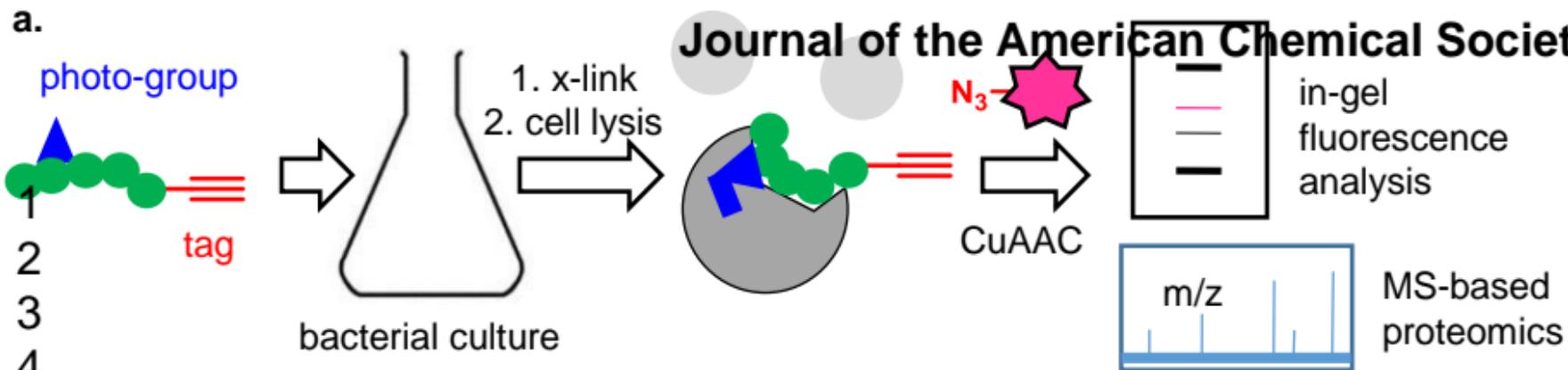
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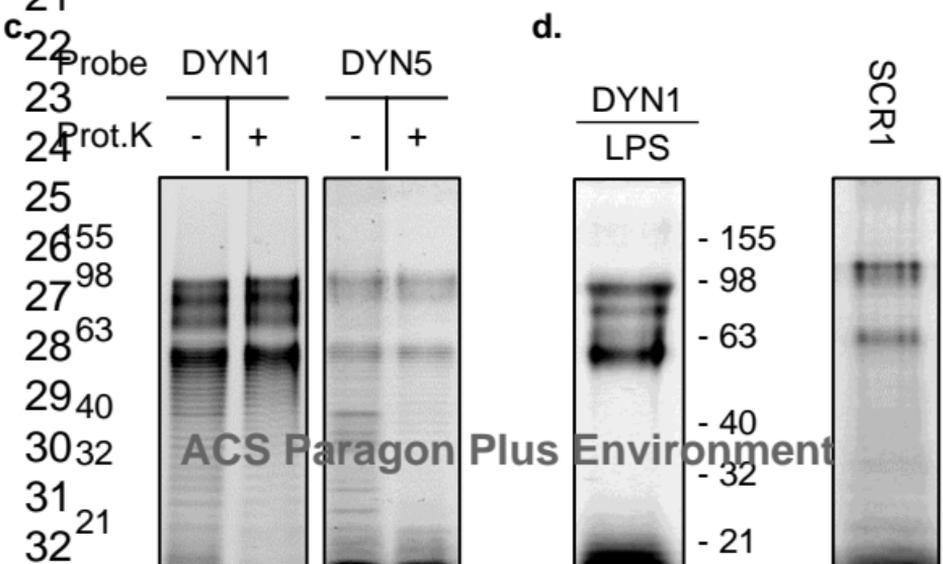
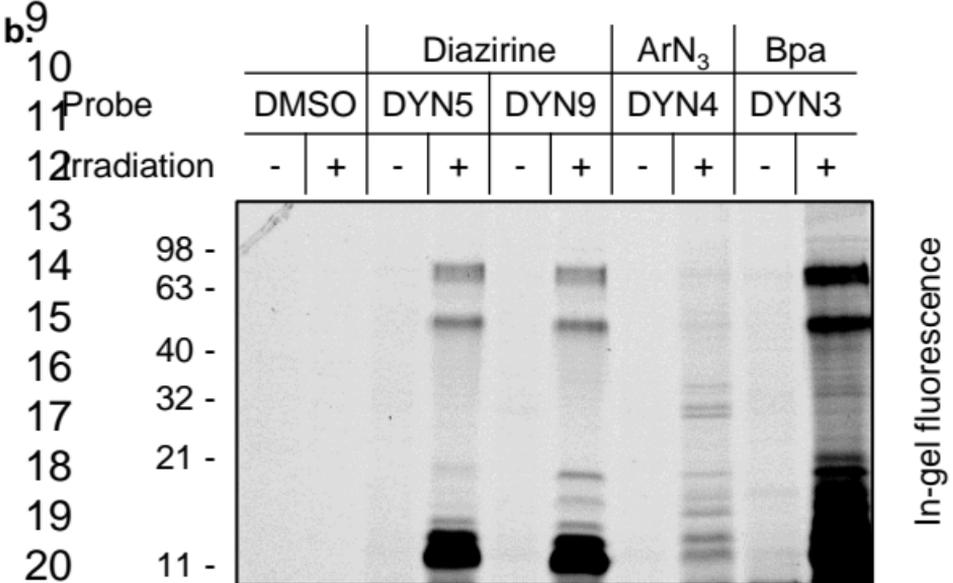
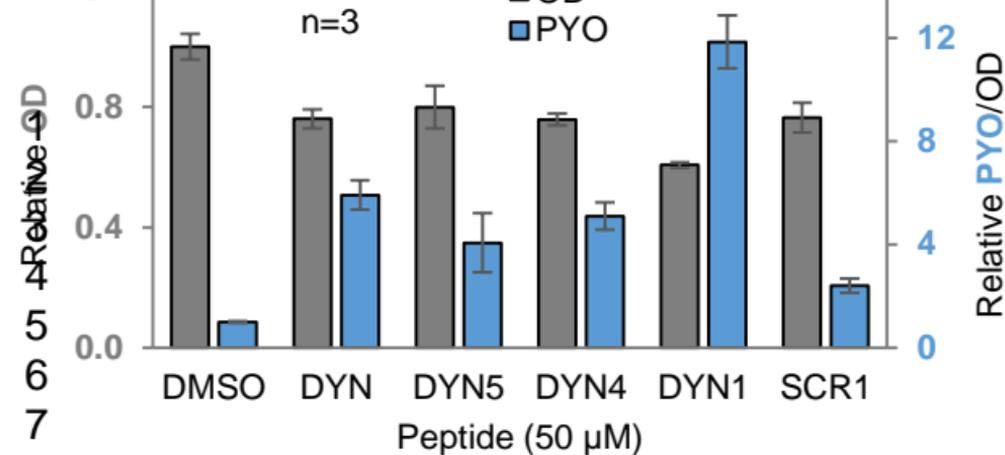
DYN	Y	G	G	F	L	R	R	I	R	P	K	L	K	-NH ₂
SCR	L	F	K	Y	R	R	I	G	P	R	G	K	L	-NH ₂
CC	L	S	I	A	T	R	H	Q	R	V	R	V	K	-NH ₂

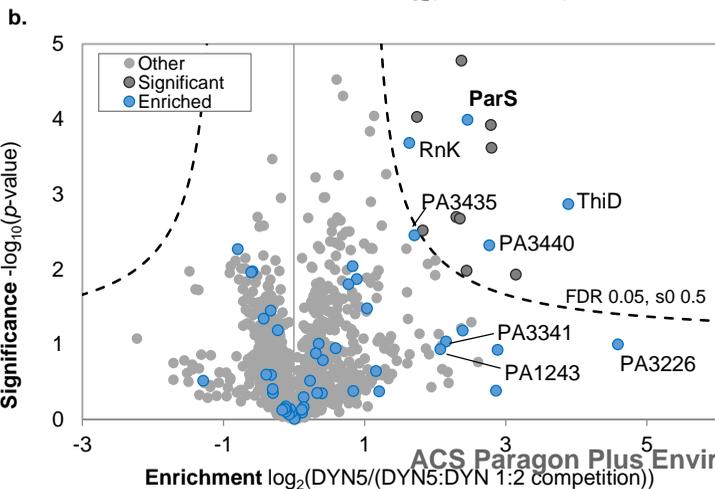
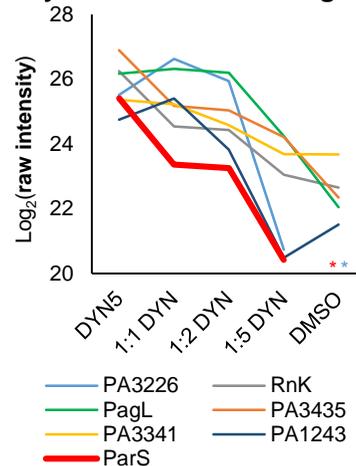
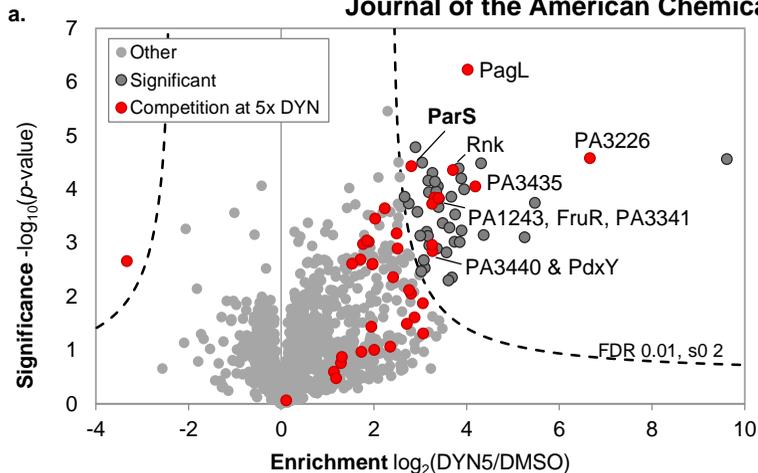




b.

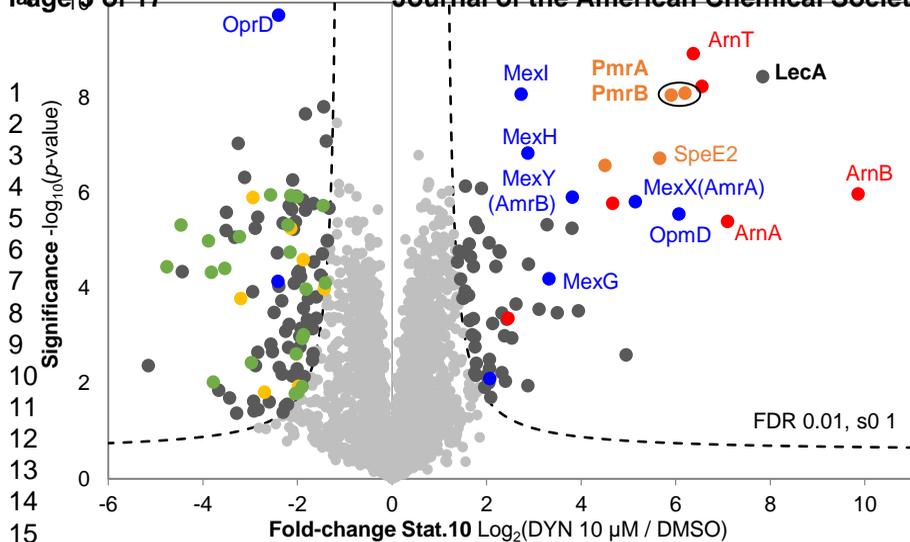
Residue	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
DYN	Tyr	Gly	Gly	Phe	Leu	Arg	Arg	Ile	Arg	Pro	Lys	Leu	Lys		
DYN1	Tyr	Gly	Gly	Bpa	Leu	Arg	Arg	Ile	Arg	Pro	Lys	Leu	Lys	Pra	
DYN3	Tyr	Gly	Gly	Phe	Leu	Arg	Arg	Ile	Arg	Pro	Lys	Leu	Lys	Bpa	Pra
SCR1	Pra	Bpa	Lys	Tyr	Arg	Arg	Ile	Gly	Pro	Arg	Gly	Lys	Leu		
DYN4	Tyr	Gly	Gly	ArN ₃	Leu	Arg	Arg	Ile	Arg	Pro	Lys	Pra	Lys		
DYN5	Tyr	Gly	Gly	Phe	Leu	Arg	Arg	Ile	Arg	P(Di)	Lys	Pra	Lys		
DYN9	Tyr	Gly	Gly	Phe	Leu	Arg	Arg	Ile	K(Di)	Pro	Lys	Pra	Lys		



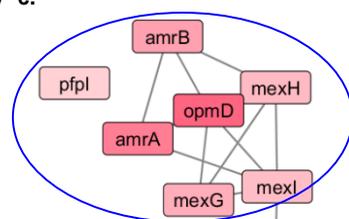
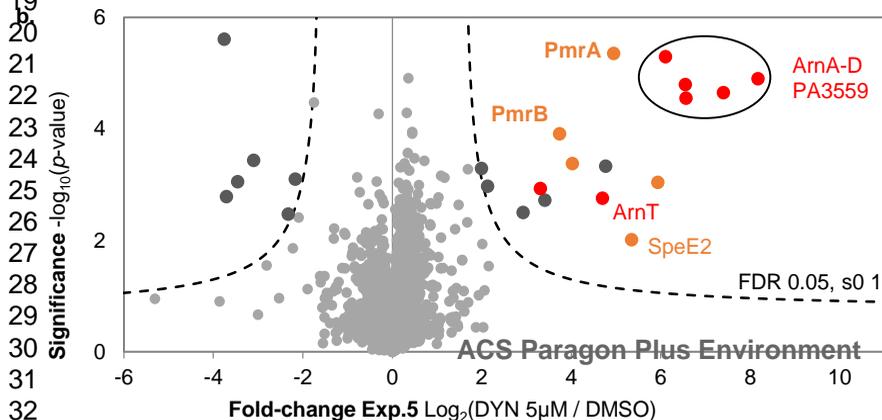


d.

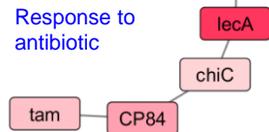
Uniprot	Gene	Protein
Q9HYQ4	PA3341	transcriptional regulator
Q9HZ14	PA3226	hydrolase
Q9HTS6	PA5274	Nucleoside diphosphate kinase regulator (Rnk)
Q9HYH1	PA3435	Uncharacterized (oxidoreductase)
Q9HVD1	PA4661	Lipid A deacylase PagL
Q9I495	PA1243	sensor/response regulator hybrid
Q9I2U4	PA1798	Two-component sensor, ParS



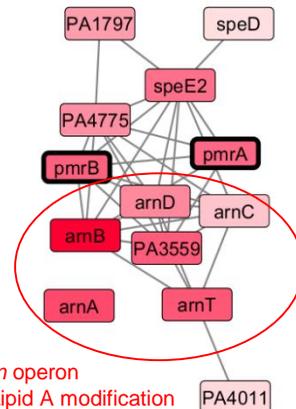
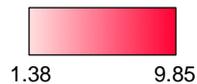
● siderophore ● response antibiotic/efflux ● *arm* operon
● sulfur cpd metab. ● PA4774-7 (PmrA, B & others)



Response to antibiotic

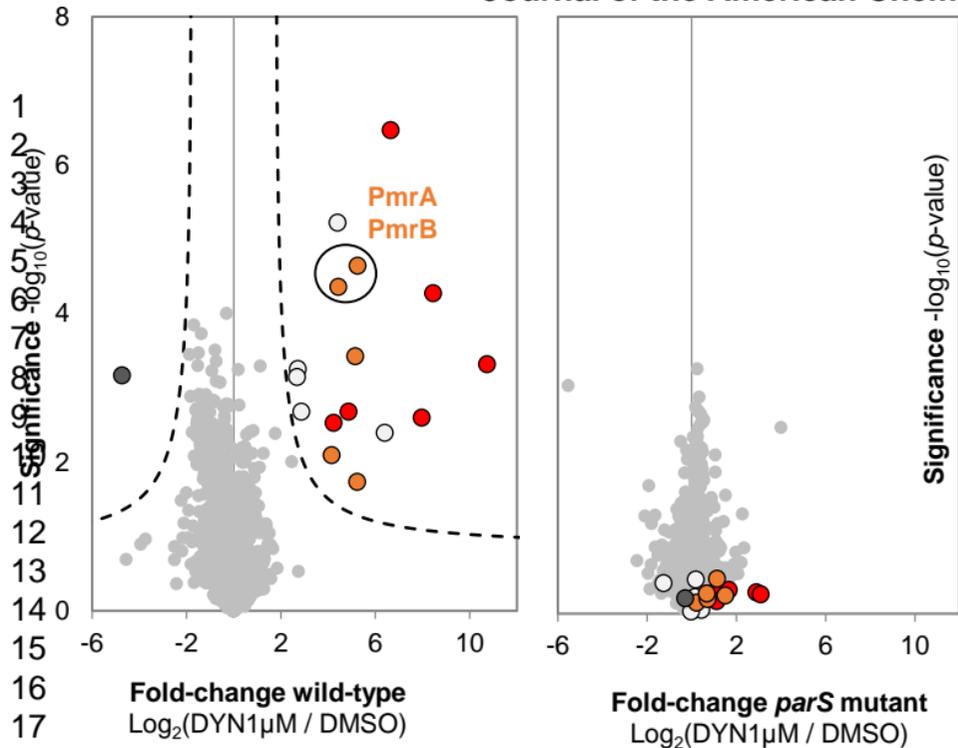


Log₂(DYN/DMSO)

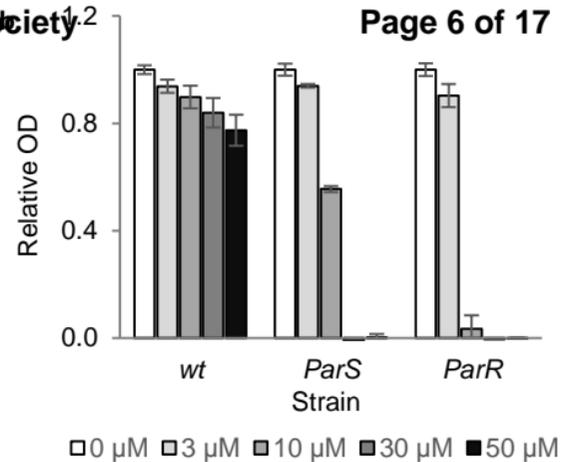


● *arm* operon
● Lipid A modification

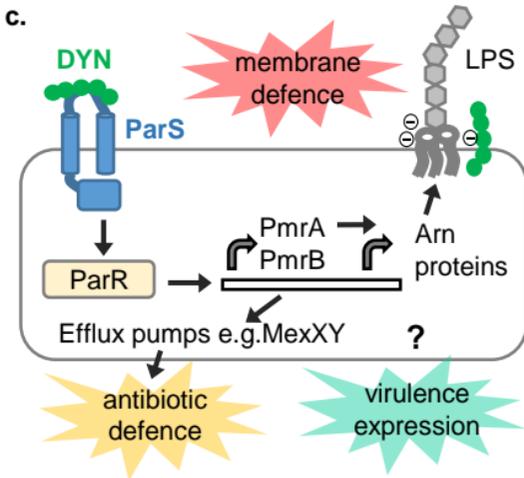
a.



- Proteins in *arn* operon
- other hit
- PA4774-7 (PmrA, B & others)
- hit but protein differs significantly between wt and *parS* mutant



c.



Chemical probes unravel an antimicrobial defense response triggered by binding of the human opioid dynorphin to a bacterial sensor kinase.

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ABSTRACT: Host-microbe communication via small molecule signals is important for both symbiotic and pathogenic relationships, but is often poorly understood at the molecular level. Under conditions of host stress, levels of the human opioid peptide dynorphin are elevated, triggering virulence in the opportunistic pathogenic bacterium *Pseudomonas aeruginosa* via an unknown pathway. Here we apply a multilayered chemical biology strategy to unravel the mode of action of this putative interkingdom signal. We designed and applied dynorphin-inspired photoaffinity probes to reveal the protein targets of the peptide in live bacteria via chemical proteomics. ParS, a largely uncharacterized membrane sensor of a two-component system, was identified as the most promising hit. Subsequent full proteome studies revealed that dynorphin(1-13) induces an antimicrobial peptide-like response in *Pseudomonas*, with specific upregulation of membrane defence mechanisms. No such response was observed in a *parS* mutant, which was more susceptible to dynorphin-induced toxicity. Thus, *P. aeruginosa* exploits the ParS sensing machinery to defend itself against the host in response to dynorphin as a signal. This study highlights interkingdom communication as a potential essential strategy not only for induction of *P. aeruginosa* virulence but also for maintaining viability in the hostile environment of the host.

Introduction

The dynorphins are important endogenous human peptide hormones that act primarily through binding to the κ -opioid receptor (OPRK) and have roles in pain response, stress and addiction, amongst others.¹ Opioids and their receptors are important components of the peripheral nervous system, but they also accumulate during host stress at sites of inflammation.² Coupled with evidence that bacteria activate virulence in response to host stress,³ these observations suggest that opioids may be recognized by bacteria as signaling molecules. A small molecule OPRK agonist and the peptide hormone dynorphin-A (Dyn-A) were shown to activate quorum sensing and result in enhanced virulence of the opportunistic pathogen *Pseudomonas aeruginosa* (PA).⁴ PA is one of the most commonly isolated microbes in patients with bloodstream and lung infections in acute care settings in Europe, and the leading cause of death in patients with cystic fibrosis.⁵ The pathogen is also very challenging to treat due to its high intrinsic resistance to antibiotics and its ability to modulate its behavior in widely different environments and in response to a host. This ability may stem in part from the organism's large number of two-component sensors, its large genome, and its complex quorum sensing networks.⁶ In a rodent stress model, Dyn-A was shown to be released in the intestine where it bound to and was taken up by bacteria.⁴ A subsequent study showed that PA regulates its response to a small molecule opioid depending on the abundance of nutrients

in the environment, integrating multiple inputs in the decision to switch to the costly virulence phenotype.⁷ Bacteria have receptors that recognize other host molecules (e.g. interferon- γ ⁸ and adrenergic hormones⁹). However, the putative dynorphin receptor and the majority of the response circuitries mediating bacterial response to opioids are completely unknown (Figure 1a). Targeting virulence or manipulating host-microbe or microbe-microbe communications have been put forward as possible non-traditional approaches to tackle the rise of multidrug resistant bacteria. However, the pathways and environmental conditions, such as host stress and interkingdom signaling, that lead to microbial virulence are poorly explored.

Here, we use a chemical biology approach to unravel the mechanism of action of the human opioid peptide Dyn-A in PA. Phenotypic studies first confirm that the virulence-inducing effect of a truncated dynorphin analogue (DYN) is peptide sequence-dependent. We then employ a chemical proteomic approach using DYN-inspired photoaffinity probes to identify the membrane sensor ParS as a putative binding partner of DYN in live bacteria. In-depth global proteomic analyses further define the specific defense response evoked in PA by DYN. Finally, we show that this defense response is abrogated in a mutant lacking ParS, functionally linking peptide binding to ParS with the phenotypic response.

Results

Dynorphin and derivatives induce *Pseudomonas* virulence

Since *PA* virulence is known to be highly environment-dependent, we first sought to define the conditions under which dynorphin induced a response. Previous work has linked the peptide Dyn-A (dynorphin(1-17)-OH) and the small molecule opioid U50,488 to induction of virulence in *PA* strain PAO1.^{4,7} We confirmed that in our hands these compounds also induced the production of the blue-green toxin pyocyanin under similar conditions of nutrient limitation, as did a truncated Dyn-A analogue with a C-terminal amide (DYN, dynorphin(1-13)-NH₂; Figure S1). The N-terminus of dynorphin is critical for signaling via human opioid receptors and this truncated peptide is equipotent in the human system.¹⁰ However, although they induced a similar response to each other, the two peptides behaved quite differently compared to the small molecule: there was significant growth reduction at higher concentrations of peptide and a corresponding drop-off of pyocyanin production, whereas U50,488 was less toxic to bacteria and only induced pyocyanin at higher concentrations (Figure S1). It is therefore not clear that dynorphin-based peptides and U50,488 have the same phenotype and thus mode of action. We decided to focus on the endogenous peptide signals, and, given the similar responses of the two peptides, chose the shorter dynorphin analogue, DYN, for synthetic tractability. We used a chemically defined MOPS-buffered succinate medium to optimize the iron and phosphate contents, since these components are important for the virulence responses of *PA* (Figure S1).⁷ Our optimized conditions generated more reproducible data and we observed that DYN (Figure 1b) induced mild toxicity and production of pyocyanin in a dose-dependent manner (Figure 1c).

To establish whether the sequence of DYN is important for the observed phenotype, we tested a sequence-scrambled peptide (SCR) and a charge-control peptide (CC), which has a different composition but is still highly basic (Figure 1b). The charge control showed no significant effect on either growth or virulence, and the scrambled peptide a greatly reduced effect (Figure 1d), suggesting a highly specific response to DYN.

Photoaffinity profiling of dynorphin targets

Intrigued by this phenotype, we next sought to identify protein interacting partners of DYN in live bacteria using photoprobes. Photoaffinity labelling is a technique used to explore small molecule-protein binding.^{11,12} A chemical probe mimicking the molecule of interest is equipped with a photoreactive group and a label or tag for detection. The probe is incubated with lysates or live cells, and, upon UV irradiation, a highly reactive intermediate is generated from the photoreactive group which crosslinks the probe to its biomolecular binding partners (Figure 2a). Common photoreactive groups include benzophenone (Bpa), aryl azides (ArN₃) and diazirines (Di) (Figure 2b), which possess different photo-properties and steric footprints.¹³ Given their distinct and complementary

properties, it is difficult to predict which photogroup will perform best in any one system; we therefore investigated all three. Rather than modify the peptide with a large label for detection, we incorporated a terminal alkyne into the structure of probes; this pre-tag allows subsequent attachment of a fluorophore or biotin affinity label via copper-catalysed azide-alkyne cycloaddition (CuAAC) for enrichment and visualization (Figure 2a). Probes were synthesized using Fmoc-SPPS chemistry. The bulkiest photogroup, Bpa, was incorporated at two different positions: in place of phenylalanine (probe DYN₁) and at the C-terminus of the sequence (DYN₃; Figure 2b).

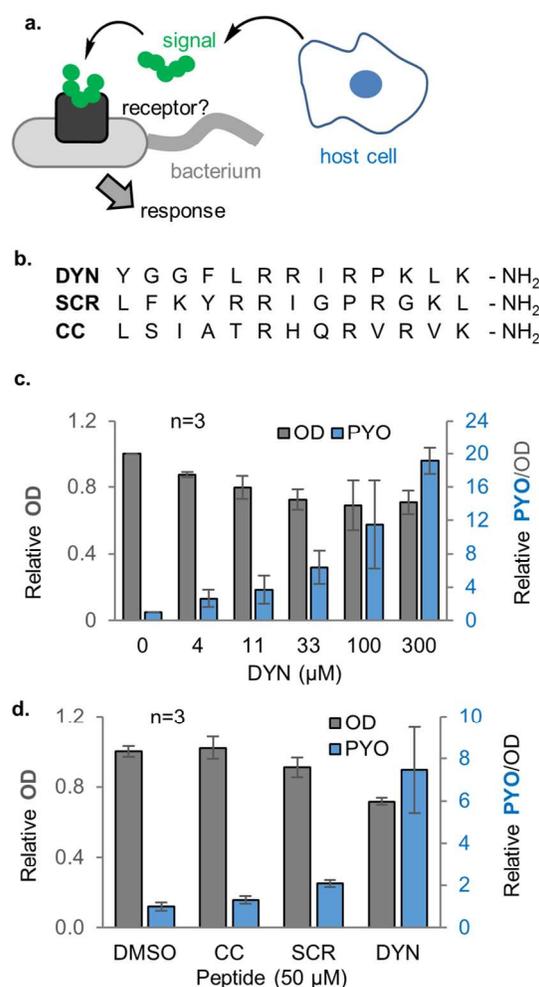


Figure 1. DYN induces virulence in *Pseudomonas aeruginosa* in a sequence-specific manner. **a.** Dynorphin is a human peptide hormone released by host cells during stress. It triggers a virulence response in the bacterium *PA* via an unknown pathway and putative receptor. **b.** Sequences of DYN and control peptides. **c.** The effect of DYN on growth, as measured by optical density (OD, grey bars), and on the production of the virulence factor pyocyanin (PYO, blue bars) in PAO1. Error bars show standard deviation from 3 independent biological experiments. OD and PYO were normalized to DMSO control in each case. **d.** Analyzing the sequence specificity of the DYN response using the peptides shown in b.

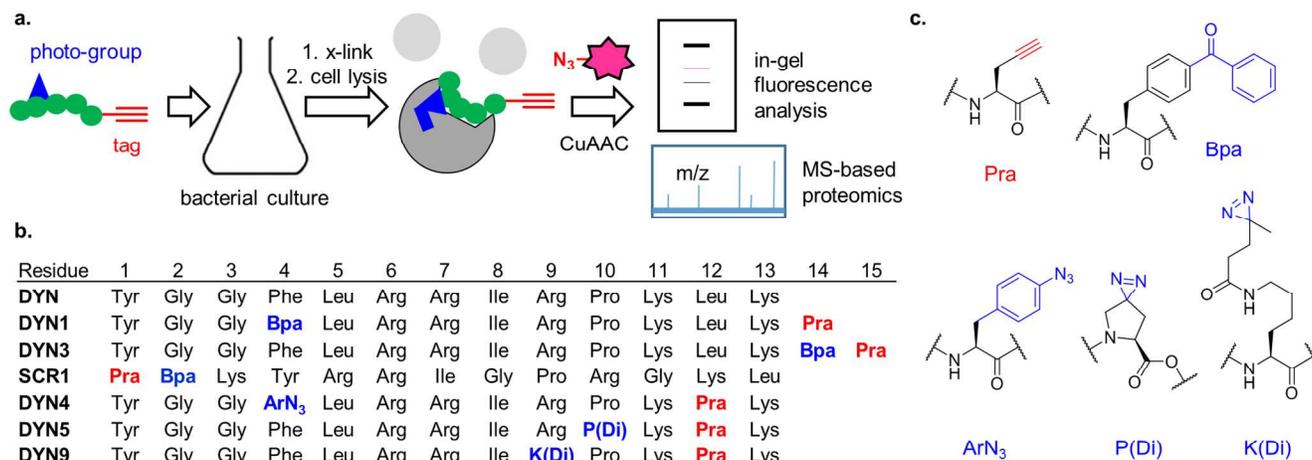


Figure 2. Design of photoreactive probes mimicking DYN. **a.** Workflow for identifying putative DYN protein interactors via chemical proteomics. Probes are incubated with bacterial cultures and the cultures are irradiated with UV light, thereby photocrosslinking the probe to its protein binding partners. Probes contain a clickable alkyne for ligation to azide-functionalised fluorophores (for visualization by in-gel fluorescence) or biotin (for affinity enrichment and gel-free LC-MS/MS analysis of tryptic peptides). **b.** Sequences of DYN and probes bearing different photocrosslinkers (all C-terminal amides). Bpa = benzophenone; ArN₃ = aryl azide; P(Di) = photoproline (diazirine-bearing proline); K(Di) = diazirine-modified lysine; Pra: propargyl-glycine. **c.** Structures of the unnatural amino acid residues.

Similarly, an aryl azide moiety was incorporated in place of phenylalanine in DYN4, and diazirine-containing ‘photoproline’²⁴ in place of proline in DYN5. A synthetically facile orthogonal protection strategy also yielded probe DYN9, where a short diazirine building block was coupled to a lysine side chain. Probes DYN5, DYN4, DYN3 and DYN1 all triggered a clear virulence response in *PA*, whilst the scrambled Bpa probe SCR1 showed very little effect, as anticipated (Figure 3a, S2). Unexpectedly, DYN9 had little effect on pyocyanin production (Figure S2), possibly indicating high sequence sensitivity of the virulence response.

To perform labelling experiments in live cells, *PA* was grown to early stationary phase, resuspended in PBS, incubated with probes at 4 μ M (the onset of virulence induction) and samples were irradiated. Cells were washed, lysed and lysates subject to CuAAC to attach a rhodamine fluorophore. Separation of proteins by SDS-PAGE and fluorescence scanning revealed some striking differences between probes bearing different photoreactive groups (Figure 3b). All probes exhibited irradiation-dependent labelling, but whilst aryl azide probe DYN4 labelled distinct bands, both diazirine and Bpa probes additionally gave rise to diffuse bands and a ‘laddering’ pattern, reminiscent of heterogeneous glycolipids. Lipopolysaccharide (LPS) forms a large part of the surface coat of *PA* and other gram-negative bacteria and consists of lipid A, a core oligosaccharide and a variable O-antigen polysaccharide side chain.¹⁵ Treatment of samples after CuAAC with a protease, proteinase K, indeed showed that the diffuse labeling and laddered bands were not protein (Figure 3c). Furthermore, isolated *PA* LPS labelled with probes generated a similar pattern (Figure 3d; Figure S2). The DYN probes are positively charged at physiological pH and charge-charge interactions may mediate binding to the negatively charged phosphates of LPS. Supportive of this

hypothesis, a scrambled Bpa-containing probe (SCR1, Figure 2b), designed based on the sequence of the scrambled control peptide SCR, gave a similar LPS-dominated labelling pattern (Figure 3d).

We initially focused on the Bpa probe DYN1, since probes with this photogroup gave the strongest labelling and high pyocyanin induction in *PA*. Proteins were subject to CuAAC with biotin-azide, enriched on avidin beads and digested. Peptides from control (DMSO vehicle treated) or DYN1 samples were isotopically dimethyl labelled¹⁶ for quantification, mixed and analyzed by gel-free LC-MS/MS. Consistent with the high level of labelling observed on-gel, large numbers of proteins were apparently enriched in DYN1 over control samples, suggestive of high non-specific binding or photoreactivity (Figure S3).¹⁷⁻¹⁹

We next performed chemical proteomic analyses with DYN4 and using a similar workflow but label-free MS intensity-based quantification (MaxLFQ),²⁰ which enables comparison across more samples. We included a competition experiment where bacteria were first incubated with 5-fold excess DYN and then with DYN4, with the aim of differentiating between genuine DYN binders over high abundance or false positive hits. DYN4 was more specific than DYN1 but still gave a large number of potential hits (Figure S3, Table S1). Outer membrane proteins (OMPs) figured prominently and biochemical separation of inner and outer membranes supported this result (Figure S4). We also observed a surprising enrichment of ribosomal proteins (Table S1). To check for effects of DYN on protein synthesis, we treated early log-phase bacteria with DYN at 10 μ M for 2 hours and then incubated aliquots for 30 min with the methionine analogue azidohomoalanine (AHA), which is incorporated into newly synthesized proteins.^{21,22} Although total protein yield was lower for the

DYN-treated bacteria due to reduction in growth, the rate of protein synthesis as measured by pulsed incorporation of AHA was the same (Figure S4). Furthermore, a simple aryl azide 'minimal' probe¹⁹ labelled proteins in the soluble fraction in a similar pattern to DYN₄ (Figure S4). Together these data suggest that aryl azide probes exhibit a high degree of non-specific binding in this system. Lack of LPS labelling by DYN₄ (Figure S4) also suggested that this probe is biased in its reactivity. This may be related to the propensity of aryl azides to undergo ring expansion upon irradiation.¹³

Finally, to investigate the protein targets of diazirine probe DYN₅, bacteria were incubated with DYN at varying concentrations followed by DYN₅ (ratios of 1:1, 1:2 and 1:5 of DYN₅:DYN) to generate competition data. Enrichment and label-free proteomics were carried out as before. Analysis of the data revealed more selective labelling compared to DYN₁ and DYN₄ (Figure 4a), as expected given the lower intensity of in-gel labelling. A subset of enriched proteins was significantly outcompeted at 5-fold excess DYN (Figure S5) and three proteins were also outcompeted at 2-fold excess DYN (Figure 4b; Table S2). Of these, the protein ParS was alone in being completely absent from DMSO controls. ParS was also detected in DYN₄ labelling, although only quantified in two out of three replicates. We analyzed the competition response profiles (LFQ intensity, raw intensity and sequence coverage) of all possible hits (Figure S5; Figure 4c); ParS showed the most pronounced dose-dependent response.

ParS is a putative membrane sensor kinase and is predicted to be co-expressed with response regulator ParR in a two-component system.²³ The ParRS system has been linked to the response of *PA* to cationic antimicrobial peptides (AMPs).²⁴ AMPs include endogenous host peptides involved in the immune defense against infection and synthetic compounds in clinical use. Although in many cases their mode of action is unclear, AMPs often bind to the bacterial membrane and rapidly kill cells through formation of pores or membrane disruption.²⁵ Select peptides appear to act via ParRS to induce adaptive resistance to other AMPs – a response where the bacterial cell activates specific mechanisms that render it temporarily more resistant to attack.²⁴ Although DYN only slightly reduced growth (Figure 1c), it is cationic. Thus we selected ParS out of the list of possible hits (Figure 4d) as the most promising protein for further analysis.

Total proteome analysis of DYN-treated *Pseudomonas* reveals an antimicrobial peptide-like defense response

If DYN binds ParS to induce an AMP-like response in *PA*, changes in the expression of specific proteins and pathways should be detectable. We therefore undertook a global proteome analysis of bacteria treated with the peptide. Bacteria were grown for 16 hours in the presence of 10 μ M DYN (a concentration resulting in a small drop in growth and a measurable increase in pyocyanin; Figure S6).

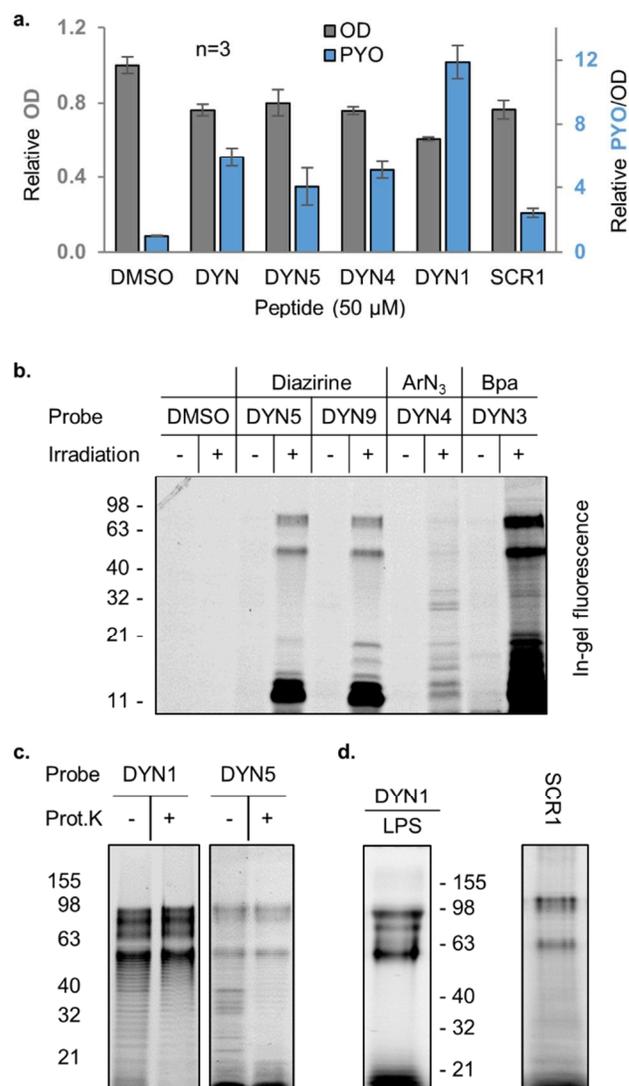


Figure 3. Photoaffinity labelling in live bacteria. **a.** Determination of growth (OD, grey) and PYO (blue) induction by probes. Error bars show standard deviation from 3 biological replicates. **b.** Gel-based analysis after photolabeling with probes (DYN₅, 9, 4 and 3; see Figure 2) in live *PA*. Probes were incubated at 5 μ M. Irradiation with UV was performed as indicated; after lysis and separation into soluble (PBS) and insoluble fractions, proteins were subject to CuAAC with rhodamine-azide (RhN₃), precipitated with acetone, separated by gel and imaged for fluorescence. Insoluble fraction shown here. **c.** Analysis of probe labeling of bacterial lipopolysaccharide (LPS). Bacteria were labeled with DYN₁ or DYN₅ and lysed in 0.2% TX100 in PBS. Following CuAAC with RhN₃ and precipitation, samples were resuspended, split into two portions and treated with proteinase K (Prot.K) or buffer (control) to digest proteinaceous material. Samples were separated on SDS PAGE and gels fluorescently scanned. **d.** Left: probe DYN₁ labelling of purified LPS. Right: Scrambled Bpa probe SCR1 labelling of bacteria (as for c). See full gels and Coomassie staining in Figure S2.

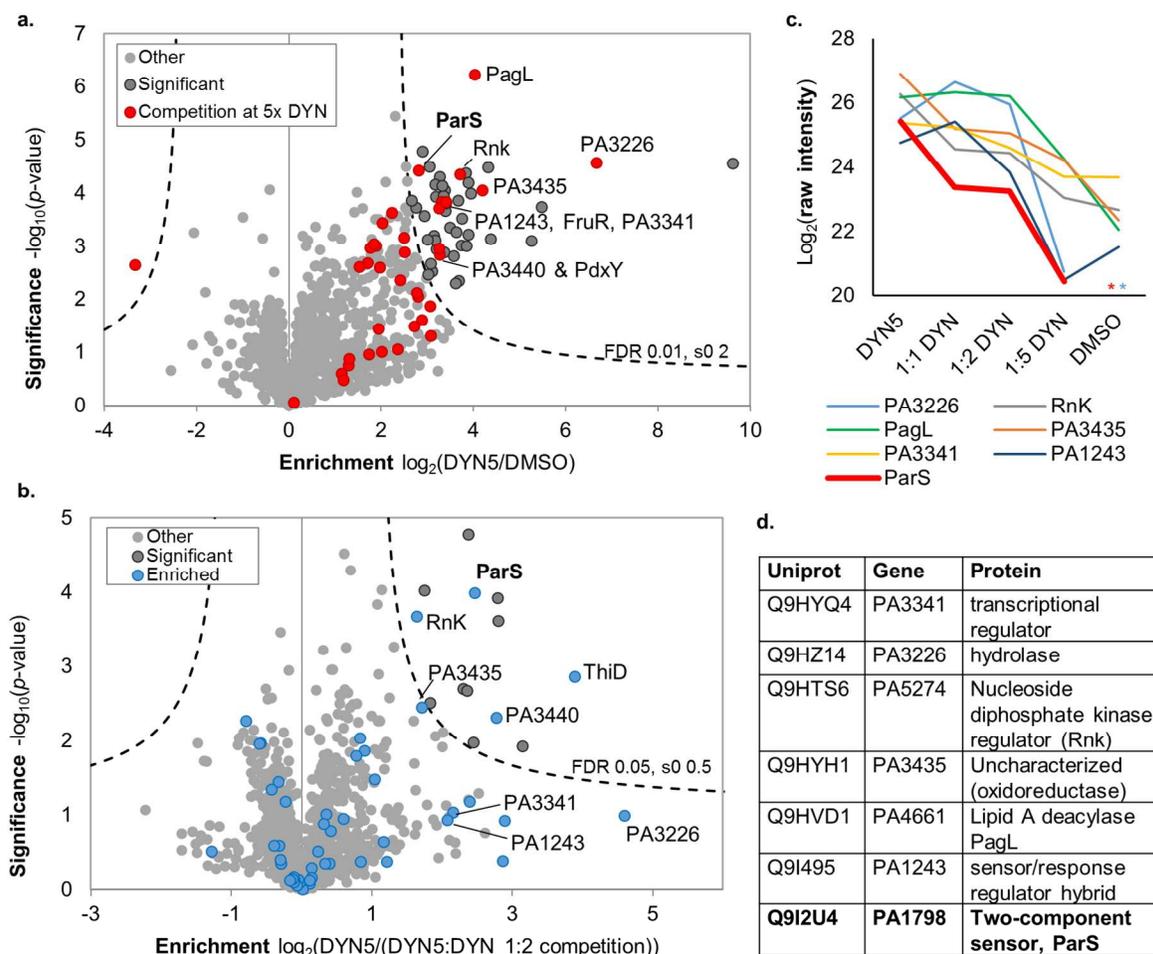


Figure 4. Chemical proteomics with diazirine probe DYN5 reveals ParS as a potential target. Bacteria were incubated with $3 \mu\text{M}$ DYN5, or DYN5 plus increasing concentrations of DYN (1, 2 or 5-fold), or DMSO control, irradiated, lysed, proteins ligated by CuAAC to biotin- N_3 , enriched on avidin beads, digested by trypsin and peptides analyzed by LC-MS/MS. Three independent biological replicate labelling experiments were performed on different days. Data were analyzed by MaxLFQ, filtered to retain only proteins identified by multiple peptides and quantified in $2/3$ biological replicates, and missing values were imputed to mimic noise. Two-sample permutation-corrected t -tests with FDR (false discovery rate) control were performed to compare samples. **a.** Volcano plot depicting enrichment and significance of enrichment in chemical proteomics experiments with $3 \mu\text{M}$ DYN5. In red: proteins shown to be outcompeted by 5x excess DYN (see Figure S5). $n=3$ biological replicates. **b.** Volcano plot showing results of competition between DYN5 and 2x excess DYN. In blue: proteins enriched over DMSO controls (see a). **c.** Profile plots of potential hits (raw MS intensity). * = value of zero in DMSO control for these proteins. Proteins selected showed a dose-response (see Figure S5), significant enrichment over DMSO controls and significant out-competition by DYN. FruR, PA33440 and PdxY did not show a consistent dose-response. **d.** The most promising hits based on competition and enrichment, and their putative roles. See Table S2.

Cells were subsequently lysed, lysates normalized based on total protein amount and samples prepared for analysis using the FASP protocol (filter-aided sample preparation), which enables lysis of samples in buffers containing high detergent concentrations to maximize solubilization of membrane proteins.²⁶ Peptides were analyzed by LC-MS/MS and quantified by MaxLFQ.²⁰ Over 3000 proteins were quantified in at least three biological replicates, with high reproducibility (Pearson correlations of 0.96-0.99, Figure S6). We observed a specific response, with only 5.5% of proteins significantly affected by DYN treatment (t -test; Figure 5a and Table S3). Gene ontology analysis suggested that siderophore biosynthesis and transport, sulfur compound metabolism, localization and transport

processes were overrepresented amongst downregulated proteins, indicating moderate changes in iron homeostasis and uptake (Figure S7). Proteins upregulated by DYN treatment included those involved in lipid A biosynthesis and belong to the *arn* operon (ArnA, B, C, D and T; Figure 5 and Figure S8), which is upregulated in response to diverse AMPs. ArnT adds 4-aminoarabinose to lipid A phosphate groups, reducing the negative charge of the membrane and promoting resistance to AMPs.¹⁵ The *arn* operon is under the control of various two-component systems in PA, including ParRS, PmrAB and CprRS.^{24,27,28} *pmrA* & *pmrB* are also upregulated by AMPs,²⁷ and expression levels of these two proteins increased strongly in response to DYN in our analysis.

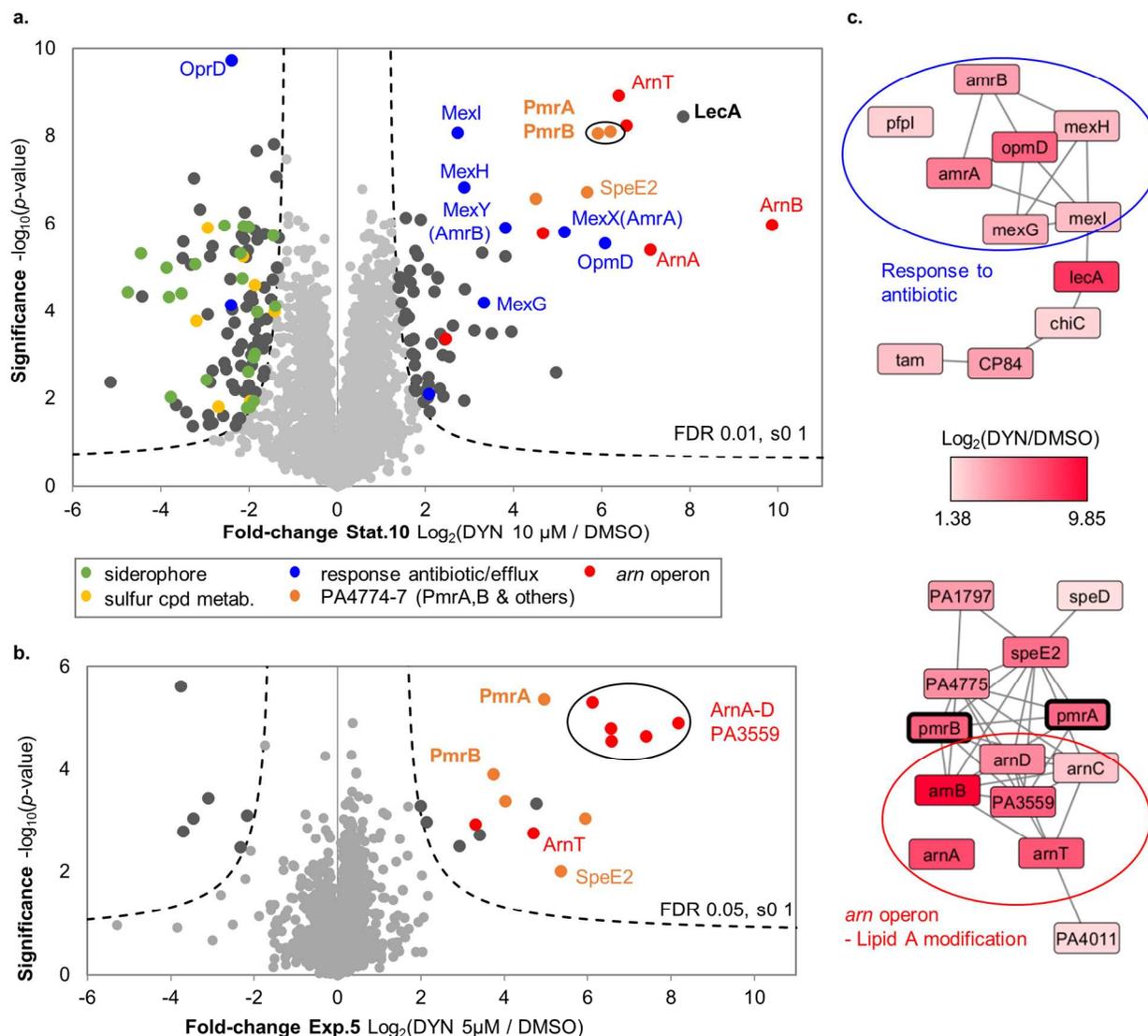


Figure 5. Global proteomics reveals specific changes in response to DYN. **a.** Volcano plot visualization of global proteomics experiment "Stat.10". *PA* was grown in the presence of DYN at 10 μM for 16 h until stationary phase. Bacteria were harvested, lysed and samples prepared by FASP for LC-MS/MS. Quantification was carried out with MaxLFQ. $n=5$ (biological replicates). After filtering to discard proteins not identified in at least four replicates, missing values were imputed and a two-sample t -test (permutation-based, FDR 0.01, s_0 1) carried out to compare DMSO and DYN treated samples. Proteins of particular interest are labelled and color-coded by putative function/pathway. See also Table S3. **b.** Global proteomics - exponential phase, dataset "Exp.5". *PA* was grown to OD 0.2-0.3 then incubated with DYN at 5 μM for 2 h during exponential growth. Samples were analyzed as above. $n=3$ (replicates grown from independent starter cultures). Only proteins identified in at least duplicate were used in analysis. Missing values were imputed (downshift 1.8, width 0.3) and a two-sample t -test (permutation-based, FDR 0.05, s_0 1) carried out to compare DMSO and DYN treated samples. See also Supplementary Table S4. **c.** Cytoscape analysis of upregulated proteins (from a.; see also Figure S8). Edges between nodes represent predicted functional or physical interactions. Analysis of downregulated proteins is shown in Figure S7.

Another highly upregulated protein with a known role in enhanced AMP resistance was SpeE2 (PA4774),²⁹ which, together with another upregulated product (PA4775), is predicted to be in the same operon as *pmrAB*. Another set of proteins that responded to DYN belong to the resistance-nodulation-division (RND) family and have known or putative roles in drug efflux. These tripartite complexes consist of an RND transporter, a periplasmic membrane fusion protein and an outer membrane factor, and act to pump antibiotics across the cell membrane.^{30,31}

All proteins from the *mexGHI-opmD* operon (PA4205-8) were significantly upregulated, as were MexX and MexY (PA2018-19; Table. S3). In contrast, the porin OprD, annotated as a basic amino acid, basic peptide and imipenem outer membrane transporter, was downregulated. Interestingly, the ParRS system has been previously linked to these changes.³²

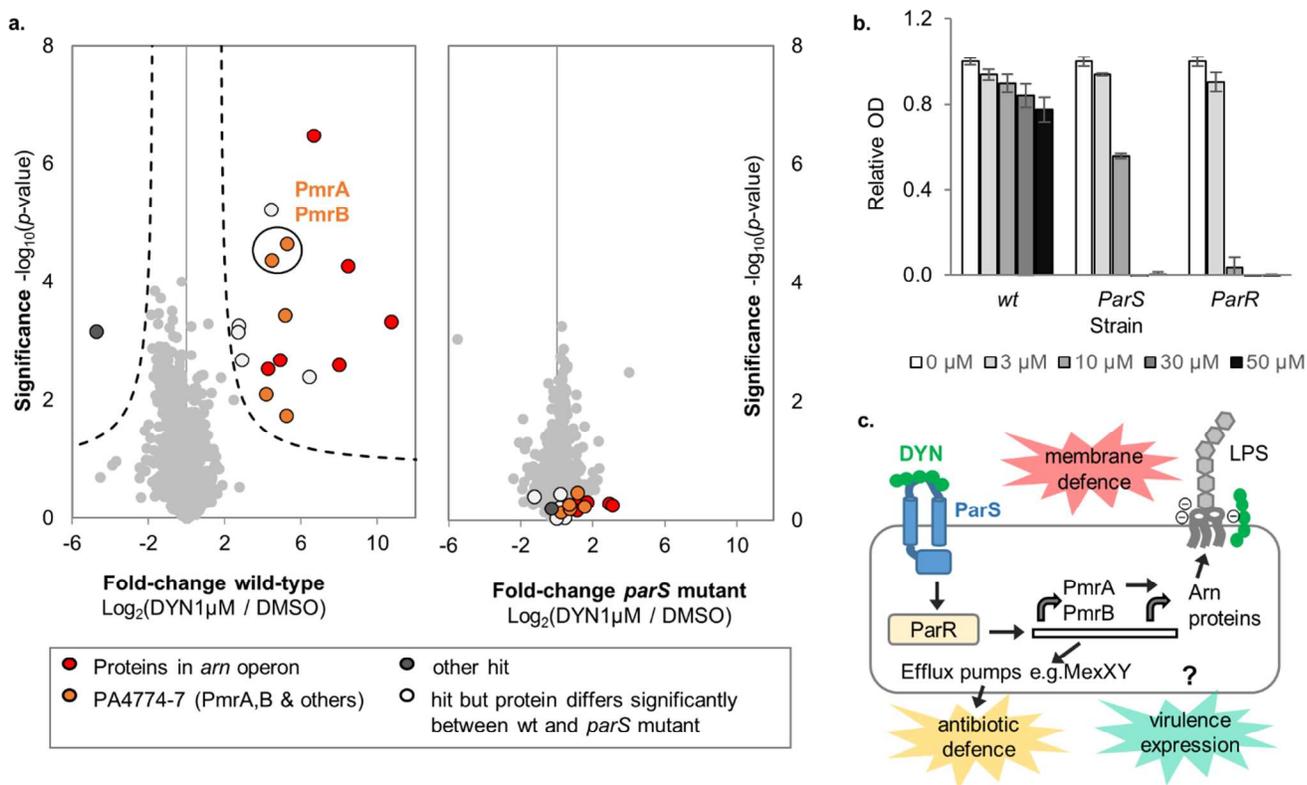


Figure 6. ParS mediates the AMP-response induced by DYN treatment. **a.** Volcano plot visualization of Stat.1 dataset (wt or *parS* mutant bacteria grown for 16 h in the presence of 1 μM DYN or DMSO control). *n*=3 replicates (grown from separate starter cultures on the same day). Bacteria were harvested, lysed and samples prepared by FASP for LC-MS/MS. Quantification was carried out with MaxLFQ. After filtering to discard proteins not identified in at least two replicates, missing values were imputed (downshift 1.8, width 0.3) and a two-sample *t*-test (permutation-based, FDR 0.05, *so* 1) carried out to compare DMSO and DYN treated samples for each strain. No significant hits were obtained in the *parS* mutant. See also Table S5. The wt and mutant proteomes are compared in Figure S10. **b.** *parS* and *parR* transposon mutants are more susceptible to treatment with DYN. Error bars show standard deviation from technical duplicates. Response to pyocyanin for these mutants and others is shown in Figure S9. **c.** Proposed model for DYN signaling via ParS to promote membrane defence mechanisms and induce virulence. DYN interacts with ParS sensor kinase, presumably triggering phosphorylation of the response regulator ParR and transcription of proteins in the *pmrAB* and *arn* operons. Proteins from these operons are involved in the antimicrobial peptide-like response, specifically modifying the structure of LPS lipid A in the case of ArnT. Efflux pumps are also upregulated. Thus ParS signaling triggers bacterial defence against antimicrobial peptides and antibiotics. The link to virulence expression (e.g. pyocyanin induction and up-regulation of biofilm-related protein LecA) is yet to be elucidated.

Another virulence-related protein, PA-I galactophilic lectin (LecA) was strongly upregulated in our data. LecA is a cytotoxic lectin and adhesion molecule implicated in biofilm formation,³³ and induced in response to another host signal, interferon- γ .⁸ The data described above were collected incubation of DYN throughout growth until stationary phase. To explore the effects of transient DYN exposure, bacteria were grown to early exponential phase and then incubated with a slightly lower concentration of 5 μM DYN (to limit the mild inhibition of growth that DYN induces) and the global proteome analyzed (Table S4). Only 0.75% of proteins showed a significant response under these conditions: again, proteins in the *arn* and *pmrAB* operons were the most prominent upregulated hits (Figure 5b). Thus, *arn* and *pmrAB* upregulation is a rapid, direct response to DYN under different growth stages.

In all analyses ParR and ParS proteins themselves were unaffected by DYN treatment; however, this is not inconsistent with their role as mediators since a previous study by Fernández *et al.* reported that the AMP indolicidin had no effect on *parRS* expression, although this peptide triggered responses via *parRS*. We compared our dataset to the microarray transcriptome data of Fernández *et al.*²⁴ Proteins in the *arn* and *pmr* operons, significantly upregulated by DYN treatment in our analysis, were indeed induced by indolicidin treatment in wild-type relative to a *parR* mutant (Table S6).

ParS mediates the DYN-induced phenotype

We hypothesized that of the possible DYN targets (Figure 4d), ParS was most likely responsible for the AMP-defence phenotype. To test this hypothesis more directly, a PAO1 *parS* transposon mutant was obtained (PA Two-Allele Library, University of Washington), validated by PCR (Figure S9) and assayed for response to DYN. Both this

1 and a *parR* mutant were dramatically more susceptible to
2 DYN-induced toxicity than the original wild-type (wt)
3 strain and we did not observe any pyocyanin production
4 in response to the peptide at non-toxic concentrations
5 (Figure S9). Using a low DYN concentration of 1 μ M to
6 minimize effects on growth, we repeated the global pro-
7 teome analysis of the *parS* mutant or wt *PA* treated with
8 DYN for 16 hours (Figure S6; Table S5). Even at this low
9 concentration, there was strong upregulation of Arn pro-
10 teins and PmrA & B in wt bacteria, whilst cells lacking
11 ParS showed no significant response at all (Figure 6b).
12 Although there are differences between the *parS* mutant
13 and wt strain proteomes, most proteins altered by DYN
14 treatment in the wt were expressed at comparable levels
15 (Figure S10). Furthermore, although *parR* and *parS* mu-
16 tants were more susceptible to DYN treatment (Figure
17 6b), other strains from the library, such as a strain mutant
18 in the unrelated response regulator *phoP*, showed a simi-
19 lar growth and phenotypic response to the wt (Figure S9).
20 Together these data strongly link ParS to the AMP-
21 defense response of *Pseudomonas* to DYN.

22 Discussion

23 Here we use chemical probes to identify a membrane sen-
24 sor kinase, ParS, as a binding partner of the human pep-
25 tide dynorphin and the mediator of a highly specific bac-
26 terial defense response.

27 Dynorphin(1-17) and the shorter (1-13) analogue (DYN)
28 both induced production of the virulence factor pyocya-
29 nin. Interestingly this phenotype was highly medium-
30 dependent, indicating that *PA* integrates multiple envi-
31 ronmental signals in its response to these compounds.
32 Pyocyanin induction was also peptide sequence-
33 dependent; however, binding of DYN-based photoaffinity
34 probes to the LPS was subsequently shown to be se-
35 quence-independent, suggesting that LPS interaction is
36 not responsible for induction of virulence but may be im-
37 portant for initial charge-mediated coordination with the
38 cell membrane. Photoaffinity probes with different photo-
39 reactive groups exhibited very distinct profiles, and the
40 lack of specificity exhibited by both benzophenones and
41 aryl azides, as noted recently by us and others,^{17,19} led us
42 to focus on diazirine-containing probes. We identified the
43 membrane protein ParS, the sensing partner of a two-
44 component response system, as a promising hit.

45 Subsequent in-depth global proteome analyses of bacteria
46 treated with DYN at different growth stages revealed a
47 highly specific, *parS*-dependent, response: a dramatic
48 increase in the levels of proteins that boost membrane
49 defense mechanisms against cationic peptides. Upregu-
50 lated proteins included those in the *arn* operon that mod-
51 ify lipid A, reducing the negative charge of the membrane
52 and thereby increasing AMP resistance. Such modifica-
53 tions also alter outer membrane permeability and host
54 immune recognition of LPS, and are common in clinical
55 isolates of *PA* from cystic fibrosis patients.³⁴ These data
56 are consistent with previous reports of *parRS*-dependent
57 *arn* upregulation and adaptive AMP resistance.²⁴ Previous
58 work showed that ParRS and a second two-component

system CprRS mediate the response to diverse AMPs in
PA in a peptide-dependent manner.²⁸ CprRS was not de-
tected in our analyses, suggesting that it is not involved in
DYN-dependent signaling. Interestingly, DYN is only
weakly toxic but induces strong upregulation of defense
pathways, consistent with previous data indicating that
the signaling and growth inhibition functions of AMPs are
not directly coupled.^{24,27,28} More work is needed to un-
derstand the structure-activity relationships of AMP signal-
ing and the exact binding site of the peptides, but it is
tempting to speculate that DYN binds at the predicted
negatively charged periplasmic loops of ParS, in a similar
manner to peptide binding to sensor PhoQ in
Salmonella.³⁵ The mechanism of mild bacterial growth
inhibition by DYN also remains to be explored, but may
be due to stress induced by membrane binding.

Treatment of *PA* with Dyn-A has been previously linked
to the PQS (*Pseudomonas* Quinolone Signal) quorum
sensing (QS) system.⁴ AMPs, such as colistin, also induce
expression of QS genes,³⁶ and ParRS has been linked to
modest changes in quorum sensing pathways in *PA*.³⁷
However, we observed no upregulation of any QS pro-
teins in our proteomic analyses. This could be due to the
timing of analysis, or differences in the culture medium,
which is a key determinate in the phenotypic response.
Our proteomic data also do not readily explain the in-
crease in pyocyanin production observed in response to
DYN; of all the proteins involved in the synthesis of this
toxin, only PhzB2 was upregulated. Again, this could re-
flect timing of analysis. How ParRS signaling may be
linked to the circuitry for enhanced pyocyanin production
is unclear, and it is possible that other sensors and path-
ways are involved in this aspect of the response. The other
chemical proteomics hits identified here (Figure 4d; e.g.
putative sensor/response regulator PA1243) are interest-
ing starting points for further study.

We also observed DYN-induced upregulation of drug ef-
flux pumps. A previous study showed that, unlike wt bac-
teria, a *parRS* mutant did not upregulate MexXY and
downregulate the porin OprD in response to AMPs, and
that spontaneous mutants in *parR* & *S* had heightened
intrinsic resistance to diverse antibiotics.³² This may be
clinically relevant since the AMP colistin and other anti-
biotics are given sequentially to cystic fibrosis patients.^{32,38}
The present study thus adds to the increasing body of
evidence linking virulence and antibiotic resistance in
Pseudomonas, and suggests that host factors can trigger a
multi-resistant phenotype in this pathogen.

59 Conclusion

60 In summary, we reveal that a human opioid peptide in-
duces a specific defense mechanism in *P. aeruginosa* via
direct binding of the sensor ParS. We provide strong evi-
dence that the antimicrobial peptide (AMP) response is
mediated by ParS. Concentrations of up to ~ 0.4 μ M Dyn-
A have been reported in the *PA*-infected, injured gut⁴ – in
the same range as DYN concentrations that induced a
AMP response – indicating that this effect could be bio-
logically relevant *in vivo*. Our data thus supports the in-
triguing possibility that *Pseudomonas* can specifically

recognize human stress hormones and initiate mechanisms to defend itself against the hostile environment of the host. This work highlights ParS as a promising drug target for blocking virulence induction and for sensitizing bacteria to antimicrobial therapies.

ASSOCIATED CONTENT

Supporting Information. Detailed synthetic and experimental procedures, analytical (NMR, LCMS) characterization of compounds and peptides. Supporting data: Figures S1-S10, Tables S1-S5 (separate Excel files) and S6. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Proteomics datasets. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE³⁹ partner repository with the dataset identifier PXD006172.

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