

## 1 Lipopolysaccharide integrity primes bacterial sensitivity to a cell wall-degrading intermicrobial toxin

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3 Kristine L Trotta<sup>1</sup>, Beth M Hayes<sup>1</sup>, Johannes P Schneider<sup>2</sup>, Jing Wang<sup>2</sup>, Horia Todor<sup>3</sup>, Patrick Rockefeller  
4 Grimes<sup>1</sup>, Ziyi Zhao<sup>1</sup>, William L Hatleberg<sup>4</sup>, Melanie R Silvis<sup>3</sup>, Rachel Kim<sup>1</sup>, Byoung Mo Koo<sup>3</sup>, Marek Basler<sup>2</sup>,  
5 Seemay Chou<sup>1\*</sup>

6  
7 <sup>1</sup> Department of Biochemistry & Biophysics, University of California – San Francisco, San Francisco, CA, USA

8 <sup>2</sup> Focal Area Infection Biology, Biozentrum, University of Basel, Klingelbergstrasse 50/70, CH - 4056

9 Basel, Switzerland

10 <sup>3</sup> Department of Cell and Tissue Biology, University of California – San Francisco, San Francisco, CA, USA

11 <sup>4</sup> Independent Researcher, Pittsburgh, Pennsylvania, USA

12  
13 \*Corresponding author: Seemay Chou (seemaychou@gmail.com)

### 14 15 **ABSTRACT**

16  
17 Gram-negative bacteria can antagonize neighboring microbes using a type VI secretion system (T6SS) to deliver  
18 toxins that target different essential cellular features. Despite the conserved nature of these targets, T6SS  
19 potency can vary across recipient species. To understand the molecular basis of intrinsic T6SS susceptibility, we  
20 screened for essential *Escherichia coli* genes that affect its survival when antagonized by a cell wall-degrading  
21 T6SS toxin from *Pseudomonas aeruginosa*, Tae1. We revealed genes associated with both the cell wall and a  
22 separate layer of the cell envelope, surface lipopolysaccharide, that modulate Tae1 toxicity *in vivo*. Disruption of  
23 lipopolysaccharide synthesis provided *Escherichia coli* (*Eco*) with novel resistance to Tae1, despite significant cell  
24 wall degradation. These data suggest that Tae1 toxicity is determined not only by direct substrate damage, but  
25 also by indirect cell envelope homeostasis activities. We also found that Tae1-resistant *Eco* exhibited reduced cell  
26 wall synthesis and overall slowed growth, suggesting that reactive cell envelope maintenance pathways could  
27 promote, not prevent, self-lysis. Together, our study highlights the consequences of co-regulating essential  
28 pathways on recipient fitness during interbacterial competition, and how antibacterial toxins leverage cellular  
29 vulnerabilities that are both direct and indirect to their specific targets *in vivo*.

## 30 INTRODUCTION

31  
32 Many bacteria live in mixed-species microbial communities where they compete with each other for limited space  
33 and resources<sup>1</sup>. Intermicrobial competition is mediated by a diverse array of molecular strategies that can exclude  
34 or directly interfere with other microbes, both near and far<sup>2</sup>. Nearly 25% of Gram-negative bacteria encode a type  
35 VI secretion system (T6SS)<sup>3</sup>, which antagonizes neighboring cells by injection of toxic protein effectors into a  
36 recipient cell<sup>4-6</sup>. The opportunistic human pathogen *Pseudomonas aeruginosa* (*Pae*) harbors an interbacterial  
37 T6SS (H1-T6SS)<sup>7</sup> that can kill the model bacterium *Escherichia coli* (*Eco*)<sup>8-10</sup>. Studies of H1-T6SS-mediated  
38 competition between these genetically tractable species have provided fundamental insights into the molecular  
39 basis of T6SS function and regulation.

40  
41 Key to *Pae* H1-T6SS toxicity are its seven known effectors, each with a unique biochemical activity<sup>6,11-15</sup>. The T6S  
42 amidase effector 1 (Tae1) from *Pae* plays a dominant role in H1-T6SS-dependent killing of *Eco* by degrading  
43 peptidoglycan (PG), a structural component of the cell wall that is critical for managing cell shape and turgor<sup>16,17</sup>.  
44 Early efforts to understand Tae1 toxicity focused on its *in vitro* biochemical activity against PG, which offered key  
45 insights about how H1-T6SS targets select bacterial species. Tae1 specifically digests  $\gamma$ -D-glutamyl-meso-2,6-  
46 diaminopimelic acid (D-Glu-*m*DAP) peptide bonds, which are commonly found in PG from Gram-negative bacteria  
47 <sup>8,18</sup>. Tae1 toxicity is further restricted to non-kin cells through a *Pae* cognate immunity protein, T6S amidase  
48 immunity protein 1 (Tai1), which binds and inhibits Tae1 in kin cells<sup>11,19,20</sup>.

49  
50 However, biochemical specificity is not sufficient to explain the toxicity and organismal selectivity of T6SS  
51 effectors *in vivo*. Bacteria antagonized by T6SSs ('recipients') can actively regulate effector toxicity through  
52 adaptive stress responses. *Eco* upregulates its envelope stress responses Rcs and BaeSR after exposure to the  
53 *Vibrio cholerae* (V52) T6SS effectors TseH (a PG hydrolase)<sup>21</sup> and TseL (a lipase)<sup>22</sup>, suggesting that *Eco* could  
54 counter cell envelope damage by re-enforcing its surface<sup>23</sup>. Similarly, *Bacillus subtilis* triggers protective  
55 sporulation in response to a *Pseudomonas chlororaphis* (PCL1606) T6SS effector, Tse1 (a muramidase)<sup>24</sup>.  
56 Additional recipient-cell coordinators of T6SS effector toxicity include reactive oxygen species<sup>25</sup> and glucose-  
57 dependent gene expression<sup>26</sup>. These studies demonstrate that T6SS effector toxicity *in vivo* may also depend on  
58 downstream adaptive features of recipient cells.

59  
60 The cell wall is a complex and dynamic substrate that is actively regulated to protect the cell<sup>27-32</sup>, yet *Eco* is highly  
61 susceptible to lysis by Tae1 *in vivo*. We therefore hypothesized that Tae1 activity promotes H1-T6SS-mediated  
62 lysis in *Eco* through a unique strategy to overcome neutralization by the recipient cell. In this study, we  
63 investigated the *Eco* cellular features that drive its intrinsic sensitivity to H1-T6SS and the Tae1 toxin during  
64 interbacterial competition with *Pae*. Using conditional gene knockdowns, we screened the entire complement of  
65 essential *Eco* genes for the most physiologically-relevant Tae1 susceptibility determinants. While cell wall-related  
66 genes indeed impact *Eco* susceptibility to Tae1, we also discovered a strong relationship between survival and  
67 another component of the cell envelope, lipopolysaccharide (LPS). Perturbation of LPS synthesis genes *msbA*  
68 and *lpxK* rendered *Eco* conditionally resistant to lysis by Tae1 from *Pae*. Our work revealed that LPS-related  
69 resistance was mediated through cell-biological processes that were independent of the biochemical Tae1-PG  
70 interaction. Our findings suggest that beyond biochemical specificity and adaptive stress responses lies a role for  
71 essential homeostatic processes in defining T6SS effector toxicity *in vivo*.

## 72 RESULTS

### 73 Adaptation of native T6SS competitions to study *Eco* susceptibility to Tae1

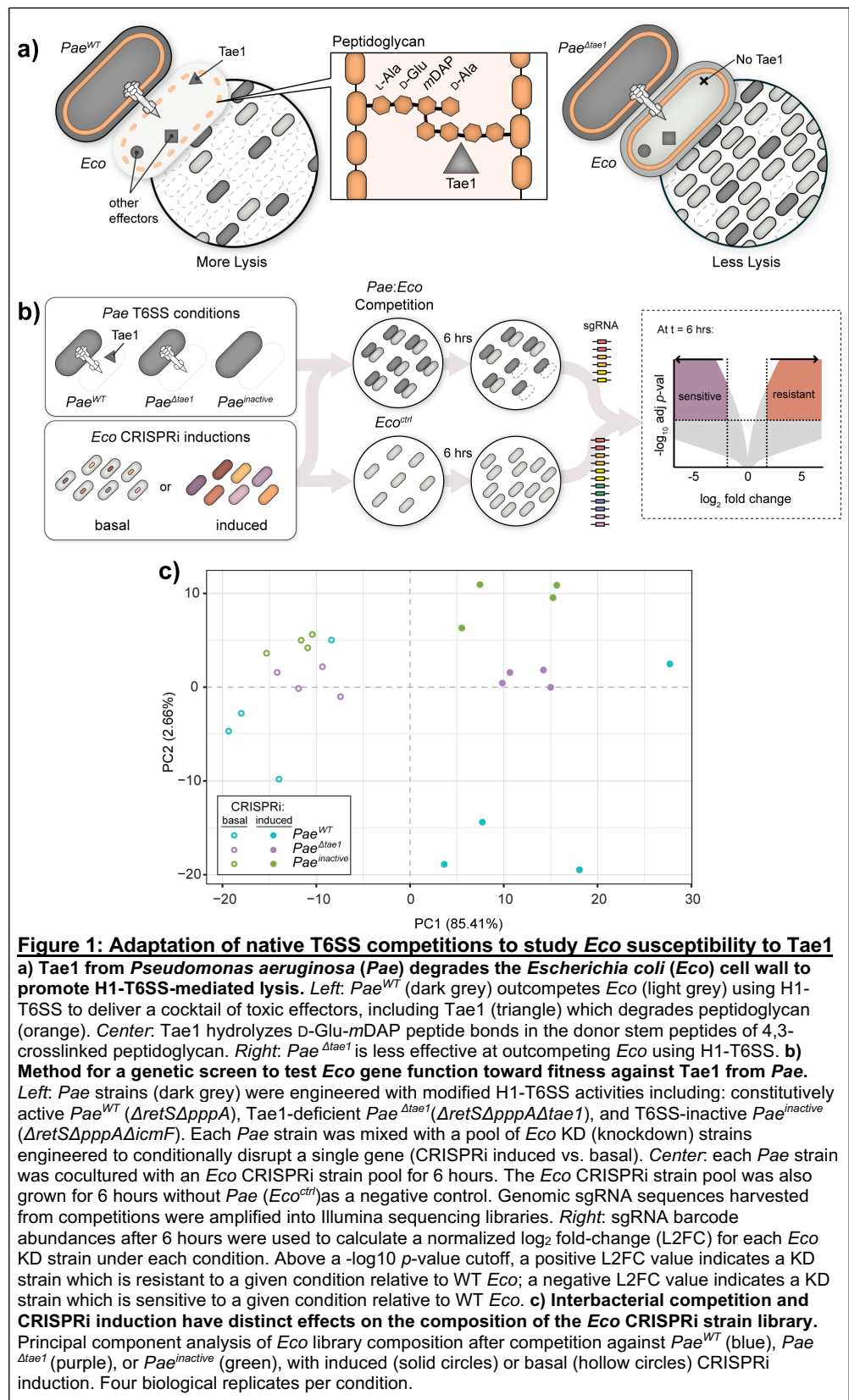
74  
75 We developed an *in vivo* screen for genetic interactions between the cell wall-degrading H1-T6SS effector Tae1  
76 from *Pae* and the model target bacterium *Eco*. Our screen had two fundamental design requirements: (1) the  
77 ability to distinguish between general (T6SS-dependent) and specific (Tae1-dependent) genetic interactions, and  
78 (2) the capacity to test a broad array of target cell features. We adapted an established interbacterial competition  
79 co-culture assay between H1-T6SS-active *Pae* and *Eco*, the outcome of which is sensitive to the specific  
80 contribution of Tae1<sup>8</sup>. In this assay *Eco* exhibits a greater fitness advantage when competed against *Pae* missing  
81 *tae1* (*Pae* <sup>$\Delta$ t<sub>t<sup>ae1</sup></sub></sup>) relative to an equivalent control strain (*Pae*<sup>WT</sup>) (**Figure 1a**). We hypothesized that the *Pae*:*Eco* co-  
82 culture assay could be leveraged to quantitatively compare recipient cell fitness against both Tae1 (toxin-specific  
83 fitness) and the H1-T6SS (Tae1-independent fitness) in interbacterial competition.

84  
85  
86  
87 To screen broadly for *Eco* determinants, we adopted an established *Eco* CRISPR interference (CRISPRi)  
88 platform that generates hypomorphic mutants through intermediate gene expression knockdowns (KDs)<sup>33</sup>. In

89 contrast to knock-out or transposon mutagenesis approaches, CRISPRi is amenable to essential genes and thus  
 90 provided an opportunity to make unique insights about genes that are typically challenging to screen for. This  
 91 includes many essential (or conditionally essential) genes related to peptidoglycan (PG) metabolism, whose KDs  
 92 we predicted would impact *Tae1* toxicity. In this CRISPRi system, inducible sgRNA expression is coupled with  
 93 constitutive dCas9 expression to conditionally repress transcription at specific loci with and without induction

94 (“induced” and “basal”  
 95 CRISPRi, respectively)  
 96 **(Supplemental Figure 1a)**.  
 97 In total, our CRISPRi  
 98 collection was composed of  
 99 596 *Eco* strains with KDs  
 100 representing most cellular  
 101 functions as defined by the  
 102 NCBI clusters of  
 103 orthologous genes (COG)  
 104 system **(Supp. Fig. 1b)**.  
 105 Our collection also included  
 106 50 negative control strains  
 107 with non-targeting sgRNAs,  
 108 including *rfp-KD*, to ensure  
 109 CRISPRi alone did not  
 110 impact inherent *Eco*  
 111 susceptibility to *Pae* **(Supp.**  
 112 **Fig. 2a)**.

114 For the interbacterial  
 115 competition screen, we co-  
 116 cultured *Pae* with the  
 117 pooled *Eco* CRISPRi  
 118 collection to test  
 119 competitive fitness across  
 120 all KD strains in parallel  
 121 **(Fig. 1b)**. To compare  
 122 *Tae1*-dependent and -  
 123 independent fitness  
 124 determinants, we  
 125 conducted screens against  
 126 H1-T6SS-active *Pae*  
 127 strains that either secrete  
 128 *Tae1* (*Pae*<sup>WT</sup>;  $\Delta retS\Delta pppA$ )  
 129 or are *Tae1*-deficient  
 130 (*Pae* <sup>$\Delta tae1$</sup> ;  
 131  $\Delta retS\Delta pppA\Delta tae1$ ). As  
 132 negative controls, we also  
 133 competed the *Eco*  
 134 collection against a  
 135 genetically H1-T6SS-  
 136 inactivated *Pae* strain  
 137 (*Pae*<sup>inactive</sup>;  
 138  $\Delta retS\Delta pppA\Delta icmF$ ) and  
 139 included a condition in  
 140 which the collection was  
 141 grown without *Pae* present  
 142 (*Eco*<sup>ctrl</sup>). Experiments were  
 143 performed under both  
 144 induced and basal  
 145 CRISPRi conditions to  
 146 distinguish between  
 147 general *Eco* fitness



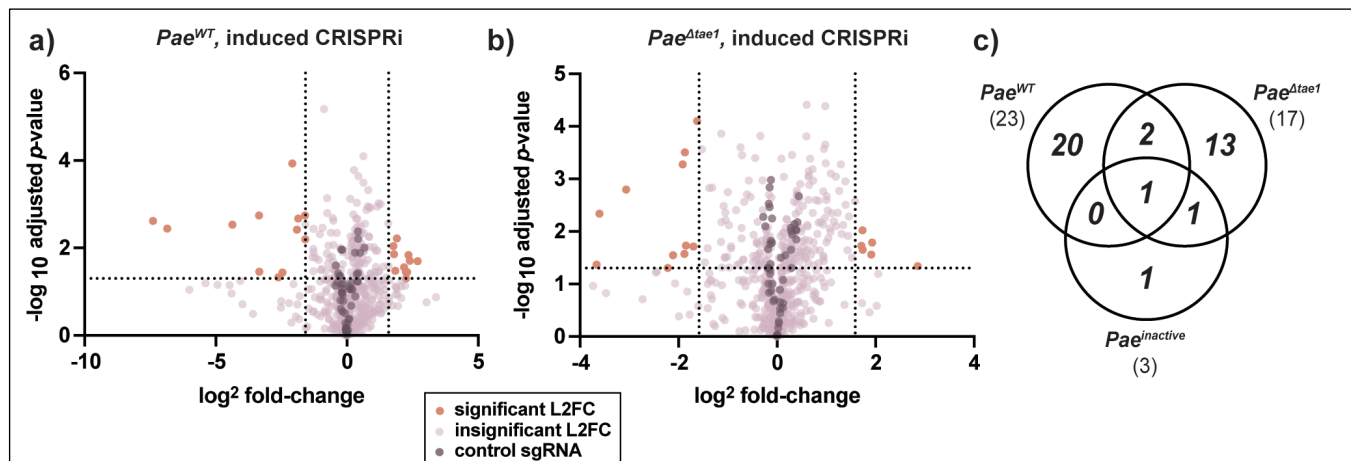
148 changes and those due to transcriptional knockdown. We used high-throughput sequencing to quantify KD strain  
 149 abundance at the beginning and end of each six-hour competition. To understand the contribution of each KD to  
 150 *Eco* survival against *Pae* in the presence or absence of H1-T6SS or *Tae1*, we calculated  $\log_2$  fold-change (L2FC)  
 151 values for each KD strain after competition and normalized against abundance after growth without competition  
 152 (*Eco<sup>ctrl</sup>*)<sup>34,35</sup>. Across four biological replicates per condition, L2FC values were reproducible (**Supp. Fig. 3a**;  
 153 median Pearson's *r* between all replicates = 0.91). L2FC was used as a proxy for competitive fitness of KD strains  
 154 across different competition conditions.

155  
 156 To determine if our screen was sensitive to the effects of *Tae1*, H1-T6SS, and CRISPRi, we conducted a principal  
 157 component analysis of L2FC values for each strain under every competition condition (**Fig. 1c**). We observed  
 158 clear separation of datasets by CRISPRi induction (induced versus basal) across the first principal component  
 159 (PC1; 85.41%), indicating that KD induction was a major contributor to the performance of the KD library in the  
 160 pooled screen. We also observed clustering of datasets according to *Pae* competitor (PC2; 2.66%). These results  
 161 indicate that each *Pae* competitor yielded a distinct effect on the fitness of the CRISPRi library and demonstrates  
 162 that our screen was sensitive to the presence (*Pae<sup>WT</sup>*) or absence (*Pae<sup>Δtae1</sup>*) of *Tae1* delivery from H1-T6SS.  
 163 From these data we conclude that our screen successfully captured the unique impacts of CRISPRi, *Tae1*, and  
 164 H1-T6SS on pooled *Eco* CRISPRi libraries during interbacterial competition.

### 165 166 **CRISPRi reveals toxin-specific and non-specific determinants of *Eco* fitness against H1-T6SS**

167  
 168 To reveal specific *Eco* genes that shape intrinsic susceptibility to H1-T6SS-mediated antagonism, we identified  
 169 KD strains which were significantly depleted or enriched at least three-fold (L2FC < -1.585 for depletion or  
 170 L2FC > 1.585 for enrichment, and  $-\log_{10}$  p-adj < 0.05) after competition against *Pae<sup>WT</sup>*, *Pae<sup>Δtae1</sup>*, or *Pae<sup>inactive</sup>*. Our  
 171 goal was to prioritize KDs which had a unique effect on fitness against *Pae<sup>WT</sup>* relative to conditions lacking *Tae1*.  
 172 With CRISPRi induced, we found a select cohort of KDs with significant loss of fitness (*n*=12) or gain of fitness  
 173 (*n*=11) against *Pae<sup>WT</sup>* (**Fig. 2a**). We were surprised that some KDs caused resistance to *Tae1* despite the  
 174 combined challenge of essential gene depletion and H1-T6SS antagonism.

175  
 176 Competition against *Pae<sup>WT</sup>* with basal CRISPRi diminished the pool of significant candidate KDs (**Supp. 4a**),  
 177 reinforcing our observation that KD strains' fitness changes against *Pae* are dependent on CRISPRi induction.  
 178 Against *Pae<sup>Δtae1</sup>* (CRISPRi induced), we observed seventeen KDs with significant fitness changes (**Fig. 2b**) which  
 179 were also CRISPRi-dependent (**Supp. 4b**). These KDs were mostly distinct from those that affected *Eco* fitness  
 180 against *Pae<sup>WT</sup>* (**Fig. 2c**). These results indicate that the presence or absence of *Tae1* had a unique effect on the  
 181 T6SS competition and thus had a distinct impact on KD fitness. Finally, we found few candidate KDs that affected  
 182 fitness against *Pae<sup>inactive</sup>* regardless of CRISPRi induction condition (**Supp. 4a-b**), suggesting that most significant  
 183 phenotypes were H1-T6SS-dependent, if not *Tae1*-dependent. In fact, L2FC values in *Pae<sup>inactive</sup>* and *Eco<sup>ctrl</sup>*



**Figure 2: CRISPRi reveals toxin-specific and non-specific determinants of *Eco* fitness against H1-T6SS**

**a-b** CRISPRi knockdowns promote *Eco* survival against *Pae<sup>WT</sup>* (a) and *Pae<sup>Δtae1</sup>* (b). Volcano plots showing  $\log_2$ -fold change (L2FC) values for each KD strain after interbacterial competition (induced CRISPRi). Data shown: mean from four biological replicates. Statistical test: Wald test. Vertical dotted lines indicate arbitrary cutoffs for L2FC at  $x = -1.58$  and  $x = 1.58$  (absolute FC  $x = -3$  or  $x = 3$ ). Horizontal dotted line indicates statistical significance cutoff for  $\log_{10}$  adjusted p-value ( $\leq 0.05$ ). Orange points represent KDs with L2FC  $\geq 1.58$  or  $\leq -1.58$  and  $\log_{10}$ -adj. p-value  $\leq 0.05$ . Dark purple points represent non-targeting negative control KDs (*n*=50). Lavender points represent KDs that do not meet cutoffs for L2FC or statistical test. **c** T6SS competitions identify CRISPRi strains with distinct fitness changes against T6SS and *Tae1*. Venn diagram of total KDs significantly enriched OR depleted after competition against *Pae<sup>WT</sup>* (*n*=23), *Pae<sup>Δtae1</sup>* (*n*=17), and *Pae<sup>inactive</sup>* (*n*=5).

184 datasets had high correlation  
 185 (**Supp. 4 c-d**, median Pearson  
 186 correlation  $r = 0.98$ ), indicating that  
 187 *Pae* is a neutral co-culture partner  
 188 with its H1-T6SS inactivated.  
 189 With our interest in Tae1-specific  
 190 determinants, we focused our  
 191 attention on the 20 KDs which had  
 192 a unique effect on *Eco* fitness  
 193 against Tae1 (*Pae*<sup>WT</sup>+CRISPRi  
 194 induced; **Table 1**). Most KDs in this  
 195 group targeted genes related to the  
 196 cell envelope (COG category M:  
 197 cell wall/membrane/envelope  
 198 biogenesis,  $n = 13/20$ ). Composed  
 199 of concentric layers of inner  
 200 membrane (IM), cell wall PG, outer  
 201 membrane (OM), and  
 202 lipopolysaccharide (LPS)<sup>36</sup>(**Fig.**  
 203 **3b**), the cell envelope is a critical  
 204 structure for protecting *Eco* against  
 205 environmental stress. Tae1-  
 206 sensitized strains were dominated  
 207 by gene targets related to the  
 208 synthesis of PG (*murA*, *ftsI*, *murC*,  
 209 *murI*, *mcrB*, *murJ*). Given that Tae1  
 210 targets the cell wall, these results  
 211 support our initial hypothesis that  
 212 PG structural integrity or  
 213 composition are direct  
 214 determinants of Tae1 susceptibility.  
 215 *In addition, we were surprised to*  
 216 *find most KDs that rendered Eco*  
 217 *resistant to Pae*<sup>WT</sup> *were related to*  
 218 *LPS and lipid membrane synthesis.*  
 219 *Tae1 is not known to directly*  
 220 *interact with membranes as part of*  
 221 *its molecular mechanism.*

222 However, metabolic crosstalk does occur between the PG, LPS, and lipid biosynthesis pathways<sup>31,37</sup>. Thus, our  
 223 data raised the possibility that regulation of other cell envelope structures could also be implicated in mediating  
 224 cell wall attack.

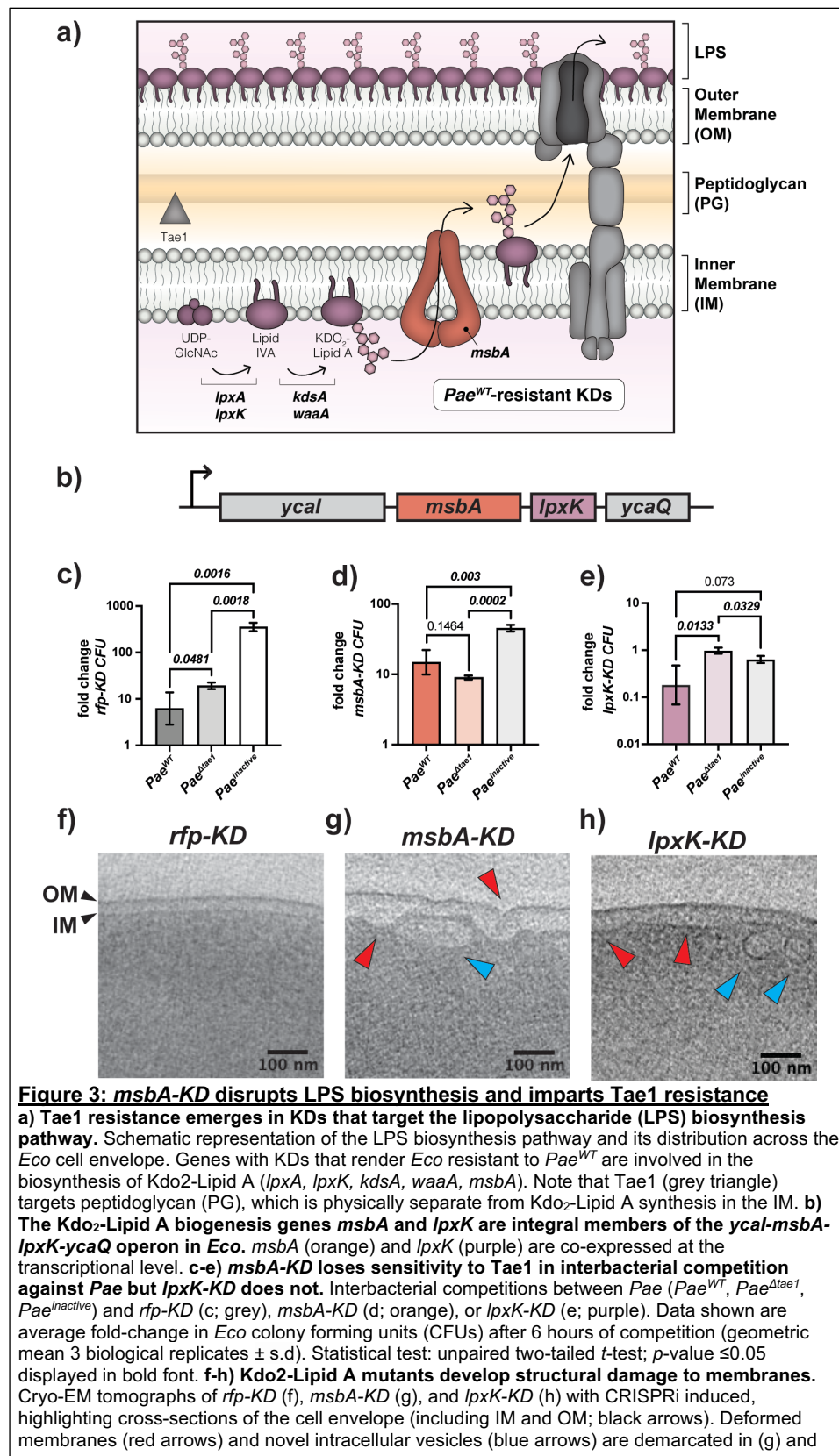
### 225 ***msbA*-KD disrupts LPS biosynthesis and imparts Tae1 resistance**

226  
 227  
 228 To investigate the hypothesis that non-PG components of the cell envelope may also shape Tae1 toxicity, we  
 229 focused downstream studies on Tae1-resistant KDs related to the synthesis of LPS, an essential lipidated surface  
 230 sugar that offers protection and structure to the OM<sup>38</sup>. Candidate KDs targeted highly-conserved, essential genes  
 231 in Kdo<sub>2</sub>-Lipid A synthesis (*lpxA*, *lpxK*, *kdsB*, *waaA*, *msbA*) (**Fig. 3a**). Kdo<sub>2</sub>-Lipid A synthesis is the most-upstream  
 232 arm of LPS biosynthesis with rate-limiting control over the entire pathway<sup>39,40</sup>. In our screen, the strongest  
 233 resistance phenotypes we observed were in KDs targeting *lpxK* (*lpxK*<sub>-1as</sub> and *lpxK*<sub>32as</sub>) (**Table 1**). *LpxK* is a  
 234 kinase that phosphorylates the Lipid-A intermediate tetraacyldisaccharide 1-phosphate to form Lipid IV<sub>A</sub><sup>41,42</sup>. In  
 235 *Eco*, *lpxK* is in an operon with *msbA* (**Fig. 3b**), which encodes the IM Kdo<sub>2</sub>-Lipid A flippase *MsbA*<sup>43,44</sup>. A KD of  
 236 *msbA* (*msbA*<sub>40as</sub>) also conferred resistance to *Pae*<sup>WT</sup> in our screen(**Table 1**).

237  
 238 We first experimentally validated pooled screen results by individually testing *lpxK*-KD and *msbA*-KD fitness in  
 239 binary competitions against *Pae*. We regenerated and validated KD strains for *lpxK* (*lpxK*<sub>-1as</sub>; "*lpxK*<sub>-KD</sub>") and  
 240 *msbA* ("*msbA*-KD") for use in these experiments (**Supp. 6**). Consistent with our screen, *msbA*-KD gained Tae1-  
 241 specific resistance in H1-T6SS-mediated competitions (**Fig. 3d**), exhibiting loss of sensitivity to *Pae*<sup>WT</sup> relative to

KD target	pathway/process	Avg. L2FC ( <i>Pae</i> <sup>WT</sup> )	fitness against <i>Pae</i> <sup>WT</sup>
<i>murA</i>	PG synthesis	-7.40	sensitive
<i>ftsI</i>	Cell division	-6.85	sensitive
<i>accD</i>	Lipid metabolism	-4.37	sensitive
<i>lptC</i>	LPS synthesis	-3.35	sensitive
<i>murC</i>	PG synthesis	-2.61	sensitive
<i>bamA</i>	OM protein assembly	-2.46	sensitive
<i>murI</i>	PG synthesis	-1.86	sensitive
<i>mrcB</i>	PG synthesis	-1.60	sensitive
<i>murJ</i>	PG synthesis	-1.59	sensitive
<i>pssA</i>	Lipid metabolism	1.77	resistant
<i>hemE</i>	Heme metabolism	1.79	resistant
<i>msbA</i>	LPS synthesis	1.84	resistant
<i>waaA</i>	LPS synthesis	1.91	resistant
<i>lpxA</i>	LPS synthesis	2.18	resistant
<i>ffs</i>	Membrane trafficking/ secretion	2.25	resistant
<i>acpP</i>	Lipid metabolism	2.25	resistant
<i>ffh</i>	Membrane trafficking/ secretion	2.30	resistant
<i>kdsB</i>	LPS synthesis	2.35	resistant
<i>lpxK</i> ( <i>lpxK</i> <sub>-1as</sub> )	LPS synthesis	2.39	resistant
<i>lpxK</i> ( <i>lpxK</i> <sub>32as</sub> )	LPS synthesis	2.69	resistant

**Table 1: Cell envelope gene KDs develop strong fitness changes against Tae1 in competition.** KDs that target PG synthesis can increase *Pae*<sup>WT</sup> sensitivity, while targeting other cell envelope processes can result in sensitivity or resistance. Data shown: normalized L2FC values for all 20 KD strains with unique and significant fitness changes against *Pae*<sup>WT</sup> (which secretes Tae1); average of four biological replicates.



*Pae*<sup>Δ*tae1*</sup>. In contrast, we could not validate *Tae1* resistance for *lpxK-KD* (Fig. 3e). Like *rfp-KD* (Fig. 3c), *lpxK-KD* maintains sensitivity to *Pae*<sup>WT</sup> relative to *Pae*<sup>Δ*tae1*</sup>. The gene expression of *msbA* and *lpxK* are co-dependent, so we were surprised that *msbA-KD* and *lpxK-KD* did not equally reproduce *Tae1* resistance. However, CRISPRi-dependent phenotypes could be controlled by factors such as transcriptional polar effects or off-target CRISPRi effects. To address their phenotypic disparities, we quantified transcriptional KD efficacy and specificity for *lpxK-KD* and *msbA-KD* with qRT-PCR. For *msbA-KD* with CRISPRi induced, we found repression of *msbA* (29-fold), *lpxK* (15-fold), and *ycaQ* (3.6-fold) expression (Supp. Fig. 6a). Thus, owing to downstream polar effects, our *msbA-KD* strain is a KD of both LPS candidate genes, *msbA* and *lpxK*. Conversely, *lpxK-KD* only repressed *lpxK* (71-fold) and *ycaQ* (11-fold) (Supp. Fig. 6b), but not *msbA*. Therefore, *msbA-KD* and *lpxK-KD* yield distinct transcriptional consequences despite targeting the same operon using CRISPRi.

Next, we investigated phenotypic consequences of inducing CRISPRi in *msbA-KD* and *lpxK-KD* by comparing their cellular morphologies with cryo-electron tomography. Disruption of *msbA* and *lpxK* typically leads to structural deformation in the *Eco* cell envelope from aberrant accumulation of Kdo<sub>2</sub>-Lipid A intermediates in the IM<sup>42,44,45</sup>. Unlike *rfp-KD* negative control cells (Fig. 3e), *msbA-KD* cells

296

297 developed irregular buckling in the IM and OM (Fig. 3f, red arrows). We also observed vesicular or tubular  
 298 membrane structures within the cytoplasm (Fig. 3f, blue arrows). Such structural abnormalities are consistent with  
 299 physical crowding of Kdo<sub>2</sub>-Lipid A intermediates in the IM that are relieved by vesicular internalization. On the  
 300 other hand, while *lpxK-KD* had a distended IM and vesicles (Fig. 3g, red and blue arrows), the OM appeared

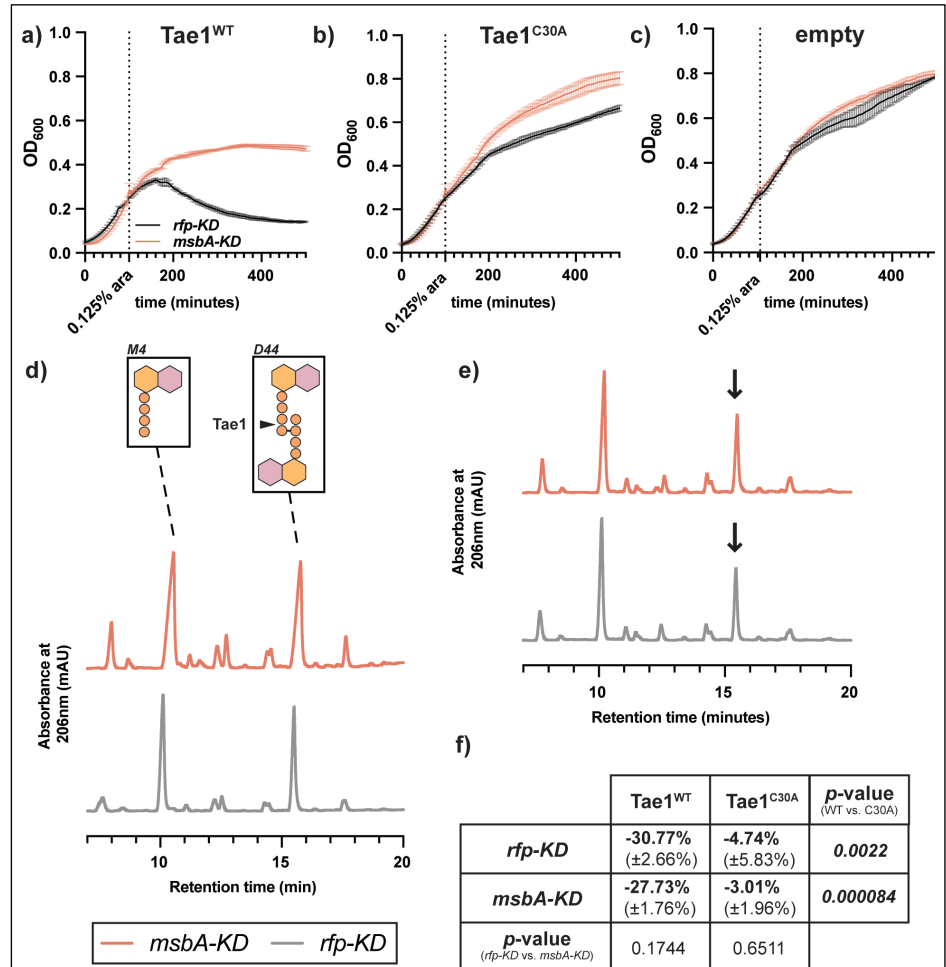
301 smooth and regular. This phenotypic divergence points to two distinct KD effects: defects in the IM (both *msbA*-  
 302 *KD* and *lpxK*-*KD*) and defects in the OM (*msbA*-*KD* only). Together with our transcriptional analyses, these results  
 303 demonstrate that *msbA*-*KD* and *lpxK*-*KD* have unique consequences for LPS integrity and *Tae1* susceptibility  
 304 despite targeting the same operon. We focused the remainder of our study on the validated *msbA*-*KD* strain  
 305 which damages the IM and OM.

### 306 Resistance to *Tae1* in *msbA*-*KD* is independent of cell wall hydrolysis

307 Identifying *msbA* and *lpxK* as potential *Tae1* resistance determinants provided us a chance to study mechanisms  
 308 by which LPS impacts susceptibility to cell wall damage. Such mechanisms could span several scales including:  
 309 direct *Tae1*-PG interactions (Fig. 4), cellular responses to *Tae1* hydrolysis (Fig. 5), broad physiological conditions  
 310 that affect mechanical lysis (Fig. 6), or some combination of these. To investigate, we used an orthogonal *in vivo*  
 311 assay to directly test the effect of *Tae1* activity in *msbA*-*KD* cells in the absence of *Pae* and other co-delivered  
 312 H1-T6SS toxins. We measured

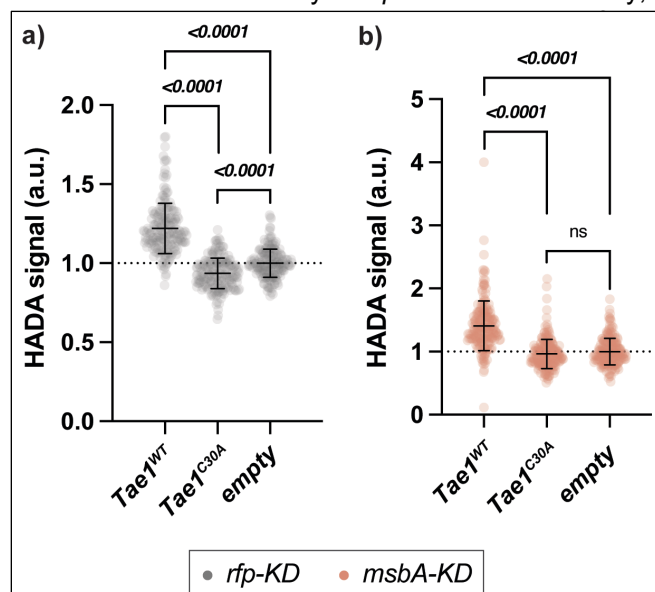
313 lysis for *rfp*-*KD* and *msbA*-*KD*  
 314 upon induction of exogenous  
 315 wild-type *Tae1* (*Tae1*<sup>WT</sup>)  
 316 expression in the cell wall-  
 317 containing periplasm<sup>8,46</sup> and  
 318 found that *msbA*-*KD* had  
 319 increased survival against  
 320 *Tae1*<sup>WT</sup> relative to *rfp*-*KD* (Fig.  
 321 4a). *Eco* resistance was  
 322 dependent on *Tae1* activity, as  
 323 evidenced by loss of the *msbA*-*KD*  
 324 resistance phenotype with  
 325 catalytically-attenuated  
 326 *Tae1*<sup>C30A</sup> (Fig. 4b) and no-  
 327 enzyme (empty) (Fig. 4c)  
 328 controls. There were no major  
 329 differences in *Tae1* expression  
 330 levels across conditions (Supp.  
 331 Fig. 7a-b), which ruled out the  
 332 possibility that fitness was tied  
 333 to toxin dose. Complementation  
 334 of *msbA* by overexpression  
 335 partially rescued *Tae1*<sup>WT</sup>  
 336 susceptibility in *msbA*-*KD*  
 337 (Supp. Fig. 9a-c,g), while *lpxK*  
 338 overexpression did not (Supp.  
 339 Fig. 9d-f,h). Given the  
 340 multigenic knockdown in *msbA*-  
 341 *lpxK*-*ycaQ* in *msbA*-*KD*, these  
 342 data suggest that *msbA* is a  
 343 partial determinant of *Tae1*  
 344 susceptibility in the strain.

345  
 346  
 347  
 348 Next, we tested whether *msbA*-  
 349 *KD* directly impacts *Tae1*-PG  
 350 physical interactions by  
 351 triggering changes to the  
 352 chemical composition of *Eco*  
 353 PG, which can occur  
 354 downstream of OM stress<sup>31</sup>. PG  
 355 remodeling could alter intrinsic  
 356 *Tae1* susceptibility by changing  
 357 the relative abundance of  
 358 targetable peptides in the cell  
 359 wall. We isolated and



**Figure 4: Resistance to *Tae1* in *msbA*-*KD* is independent of cell wall hydrolysis**  
**a-c) *msbA*-*KD* populations have a *Tae1*-dependent growth advantage.** OD<sub>600</sub> growth curves of *msbA*-*KD* (orange) and *rfp*-*KD* (black) with CRISPRi induced, overexpressing (a) *pBAD24::pelB-tae1*<sup>WT</sup> (*Tae1*<sup>WT</sup>), (b) *pBAD24::pelB-tae1*<sup>C30A</sup> (*Tae1*<sup>C30A</sup>), or (c) *pBAD24* (empty). Data shown: average of 3 biological replicates ± s.d. Dotted vertical line indicates plasmid induction timepoint (at OD<sub>600</sub>=0.25). **d) The mucopeptide composition of *msbA*-*KD* is identical to control *rfp*-*KD*.** HPLC chromatograms of mucopeptides purified from *msbA*-*KD* (orange) and *rfp*-*KD* (grey) expressing *pBAD24* (empty). Inset: major mucopeptide species in *Eco* include tetrapeptide monomers (M4; r.t. ~10 minutes) and 4,3-crosslinked tetra-tetra dimers (D44; r.t. ~15.5 minutes). *Tae1* digests D44 peptides (black arrow). Data shown: representative from 3 biological replicates. **e) *Tae1*<sup>WT</sup> digests PG from both *msbA*-*KD* and *rfp*-*KD* PG *in vivo*.** HPLC chromatograms of mucopeptides purified from *msbA*-*KD* (orange) and *rfp*-*KD* (grey) expressing *pBAD24::pelB-tae1*<sup>WT</sup> (*Tae1*<sup>WT</sup>). Black arrow indicates D44 peptide partially digested by *Tae1*. Data shown: representative from 3 biological replicates. **f) *Tae1* is equally efficient at digesting PG in *msbA*-*KD* and *rfp*-*KD*.** Percent loss of D44 peptide after 60 minutes of periplasmic *Tae1*<sup>WT</sup> or *Tae1*<sup>C30A</sup> expression. Data shown: average of 3 biological replicates (± s.d.). Statistical test: two-tailed unpaired *t*-test; *p*-value ≤0.05 displayed in bold font.

360 characterized the composition of PG purified from *rfp-KD* and *msbA-KD* by HPLC muropeptide analysis. Both  
 361 strains had highly similar and stereotypical *Eco* muropeptide profiles (**Fig. 4d**). PG peptides containing the scissile  
 362 bond and structural context for Tae1 recognition (4,3-crosslinked dimers; D44)<sup>8</sup> were found at an approximate 1:1  
 363 ratio with another dominant species of muropeptide (tetrapeptide monomers; M4)<sup>47</sup>. Our results suggest that the  
 364 PG composition of *msbA-KD* is not modified downstream of LPS damage, indicating that Tae1 resistance cannot  
 365 be explained by biochemical changes to the Tae1:PG interaction.  
 366  
 367 We tested an alternative hypothesis that resistance may derive from decreased efficiency in Tae1 hydrolysis. We  
 368 reasoned that structural deformations in the *msbA-KD* cell envelope (**Fig. 3f**) could occlude or delay the  
 369 accessibility of PG to Tae1, thus slowing the kinetics of cell wall degradation and cell lysis. To test this, we  
 370 monitored the relative degradation of D44 peptides after Tae1 induction in *rfp-KD* and *msbA-KD* populations.  
 371 Empty-vector and Tae1<sup>C30A</sup> conditions were included as negative controls (**Fig4d,f; Supp. Fig. 9a**). At 60 minutes  
 372 of induction (just prior to lysis in *rfp-KD* populations), we found that D44 peptides were similarly hydrolyzed  
 373 between strains, with a 32.58% loss in *rfp-KD* and 27.73% of in *msbA-KD* (**Fig. 4e-f**). Thus, Tae1 hydrolyzes  
 374 *msbA-KD* PG as efficiently as *rfp-KD* PG. Collectively, these data show that both cell wall recognition and



**Figure 5: PG synthesis is suppressed in *msbA-KD* but sensitive to Tae1 activity**

**a-b) PG synthesis activity is sensitive to Tae1 overexpression.** Single-cell fluorescence intensity measurements for *rfp-KD* (a; grey) or *msbA-KD* (b; orange) after incorporating the fluorescent D-amino acid HADA into PG after 60 minutes of overexpressing *pBAD24::peIB-tae1<sup>WT</sup>* (Tae1<sup>WT</sup>), *pBAD24::peIB-tae1<sup>C30A</sup>* (Tae1<sup>C30A</sup>), or *pBAD24* (empty), with CRISPRi induced. Data shown: 600 cells (200 cells x 3 biological replicates), with average  $\pm$  s.d. Statistical test: unpaired two-tailed *t*-test; *p*-value  $\leq 0.05$  displayed in bold font.

		% change (intra-strain)	% change ( <i>rfp-KD</i> norm.)
<i>rfp-KD</i>	Tae1 <sup>WT</sup>	22% ( $\pm 3.6\%$ )	
	Tae1 <sup>C30A</sup>	-6.5% ( $\pm 2.6\%$ )	
	empty	0% ( $\pm 1.6\%$ )	
<i>msbA-KD</i>	Tae1 <sup>WT</sup>	26.5% ( $\pm 2.5\%$ )	12% ( $\pm 2.5\%$ )
	Tae1 <sup>C30A</sup>	2.82% ( $\pm 3.2\%$ )	-9% ( $\pm 3.2\%$ )
	empty	0% ( $\pm 2.0\%$ )	-11.5% ( $\pm 2.0\%$ )

**Table 2: PG synthesis activity is sensitive to CRISPRi and Tae1 overexpression.** Descriptive statistics for normalized percent change in HADA fluorescence in *rfp-KD* and *msbA-KD* as related to **Fig. 5** and **Supp. Fig. 10**. Data shown: average of 600 single-cell measurements  $\pm$  s.d.

hydrolysis by Tae1 are unchanged in *msbA-KD*, ruling out the possibility that direct changes to PG are responsible for differential cellular lysis outcomes.

### PG synthesis is suppressed in *msbA-KD* but sensitive to Tae1 activity

Given that we did not find any effects on direct Tae1–cell wall interactions in *msbA-KD*, we next explored indirect resistance mechanisms. The PG sacculus is dynamically synthesized, edited, and recycled *in vivo* to maintain mechanical support to the cell during growth and stress<sup>27,48</sup>. We hypothesized that Tae1 hydrolysis could also impact PG synthesis activity in *Eco* by generating a need to replace damaged PG with new substrate. The ability to repair PG could thus be a valuable determinant of Tae1 susceptibility. To determine if PG synthesis is sensitive to Tae1 exposure, we measured the incorporation of the fluorescent D-amino acid HADA into *rfp-KD* cell walls both with and without exogenous Tae1 expression. When normalized against control cells (*empty*), PG synthesis in *rfp-KD* cells increased by 22% in response to Tae1<sup>WT</sup> and decreased by 6.5% in response to Tae1<sup>C30A</sup> (**Fig. 5a; Table 2**). These data show that PG synthesis is stimulated by Tae1 exposure, and this response is dependent on toxin activity.

PG synthesis is also coordinated to other essential processes in *Eco*, and sensitive to their genetic or chemical perturbations<sup>31,49</sup>. We investigated if *msbA-KD* impacts the dynamic PG synthesis response to Tae1. Tae1<sup>WT</sup> exposure yielded a 26.5% increase in PG activity in *msbA-KD*, and no significant change in activity with Tae1<sup>C30A</sup> (**Fig. 5b; Table 2**). These results indicate that PG synthesis is still actively regulated in *msbA-KD* in accordance with relative Tae1 activity. However, when normalized against baseline *rfp-KD* activity, all PG synthesis measurements for *msbA-KD* were significantly diminished (**Supp. Fig. 10; Table 2**). This observation indicates that PG synthesis activity is globally suppressed as a consequence of CRISPRi in *msbA*. Thus, we conclude that PG dynamism in *Eco* is



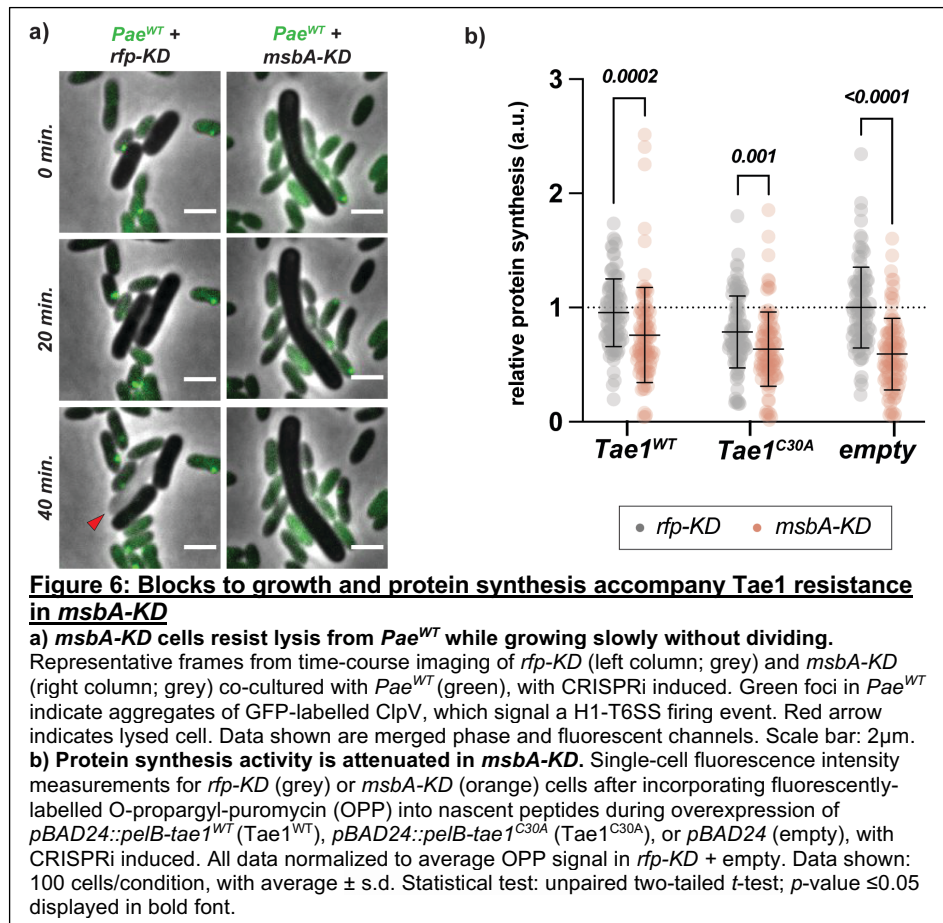
417 sensitive to Tae1 hydrolysis of  
418 PG, and that *msbA-KD* alters  
419 the global capacity for PG  
420 synthesis activity without  
421 altering its sensitivity to Tae1.  
422 Furthermore, these data  
423 demonstrate a reactive crosstalk  
424 between LPS and PG synthesis  
425 activities *in vivo*.

### 427 **Blocks to growth and protein** 428 **synthesis accompany Tae1** 429 **resistance in *msbA-KD***

430  
431 Based on its responsiveness to  
432 Tae1 exposure, we might  
433 hypothesize that *Eco* stimulates  
434 PG synthesis to attempt  
435 protection against lysis by Tae1.  
436 However, suppressed PG  
437 synthesis activity alongside  
438 tolerance to wildtype-levels of  
439 PG damage in *msbA-KD*  
440 suggested that *msbA-KD* may  
441 survive lysis by Tae1 using an  
442 additional strategy to support or  
443 even supersede PG integrity.  
444 *Eco* can resist lysis upon acute  
445 PG stress by entering a  
446 transient dormant state called  
447 persistence in which  
448 homeostatic growth processes like DNA replication, protein synthesis, and cell division are arrested to prioritize stress responses to critical damage<sup>50–52</sup>. Additionally, a recent study showed that a CRISPRi KD in *lpxA*, the first enzyme in Lipid A biosynthesis, triggered hallmark signs of a dormancy stress response called the stringent response<sup>53</sup>. We hypothesized that decreased PG synthesis activity in *msbA-KD* may be symptomatic of persistence, which could protect against Tae1 activity by passive tolerance.

454 To observe the effects of Tae1 and CRISPRi on cellular growth and lysis behaviors over time, we performed  
455 timelapse microscopy of *rfp-KD* and *msbA-KD* cells in competition with *Pae*. Across all *Pae* competitions, *msbA-KD*  
456 *KD* cells grew slowly without dividing or lysing (Fig. 6a; Supp. Fig. 11a-b). By contrast, *rfp-KD* cells grew and  
457 divided rapidly, but lysed when in competition against *Pae* strains with active H1-T6SSs (*Pae*<sup>WT</sup>, *Pae*<sup>Δ*tae1*</sup>) (Fig.  
458 6a; Supp. Fig. 11a-b). These data demonstrate that stunted cell growth and division are additional consequences  
459 of CRISPRi in *msbA-KD*. Another hallmark of persistence is slowed protein production in the cell. To assess  
460 whether global protein synthesis is affected in *msbA-KD*, we used pulse-chase fluorescent labelling of nascent  
461 protein synthesis in *msbA-KD* and *rfp-KD*. Overall protein synthesis levels were significantly lower in *msbA-KD*  
462 relative to *rfp-KD* under all conditions tested (Fig. 6a). From these data we conclude that *msbA-KD* cells show  
463 signs of persistence, suggesting that broad changes in cellular physiology may underscore the ability of *msbA-KD*  
464 cells to resist the toxic activity of Tae1.

466 We propose a model in which Tae1 susceptibility *in vivo* is determined at multiple levels of specificity in *Eco*: not  
467 only at the level of local PG damage but also by crosstalk between essential cell envelope pathways and the  
468 general growth state of the cell. As mediated through damage to LPS in *msbA-KD*, we posit that such crosstalk  
469 between essential cell functions can be helpful for slowing reactivity and thus increasing tolerance to acute PG  
470 stress. By the same token, the enmeshment of essential pathways may render fast-growing *Eco* vulnerable to  
471 Tae1 by creating a sudden chain-reaction of imbalances in critical functions which the cell must also resolve  
472 alongside the initial PG damage.



## 473 DISCUSSION

474

475 The species composition of mixed-microbial communities can be driven by competitive strategies that bacteria  
476 use to antagonize their neighbors. However, our understanding of microbial weapons is primarily derived from *in*  
477 *vitro* studies of their molecular mechanisms. In this study, we wanted to understand how Tae1, a PG-degrading  
478 H1-T6SS effector toxin, specifically aided *Pae* in antagonizing *Eco* *in vivo*. By combining T6SS-mediated  
479 competition with CRISPRi against essential *Eco* genes, our high-throughput genetic screen was poised to  
480 uncover new molecular details about the interaction between Tae1 and essential functions in recipient cells. We  
481 found that dynamic regulation of both direct (PG) and indirect (LPS, growth) aspects of cell wall hydrolysis had  
482 critical roles in defining the intrinsic sensitivity to Tae1-mediated lysis. Thus, Tae1 toxicity *in vivo* is driven not only  
483 by its ability to destroy PG but also by broader physiological and regulatory context.

484

485 Through the lens of LPS perturbation (*msbA-KD*), we discovered that cellular persistence is associated with  
486 resistance to Tae1-dependent lysis. The protective nature of dormancy has been demonstrated for survival  
487 against other cell wall-degrading enzymes, lytic bacteriophages, and antibiotics<sup>54-58</sup>. However, previous work on  
488 interbacterial competition has shown that fast growth protects recipient cells from T6SS by establishing stable  
489 microcolonies more quickly than T6SS can kill the recipient cell type<sup>59,60</sup>. This study suggests that slow recipient  
490 growth could also offer a fitness advantage against lytic T6SS effectors. Similarly to how dead (unlysed) cells can  
491 physically block T6SS-wielding competitors from progressing in space<sup>61</sup>, transiently dormant cells could also  
492 absorb T6SS attacks to protect their kin in community settings.

493

494 A surprising feature of lysis resistance in *msbA-KD* was its tolerance to PG damage by Tae1 alongside additional  
495 damage to its IM and OM. Structural destabilization of the cell envelope commonly renders *Eco* hypersensitive to  
496 lysis<sup>62,63</sup>. However, our observations suggest that integrity of individual envelope components is not always  
497 sufficient to explain cell lysis. Indeed, PG and the OM can work together to bear cellular turgor pressure changes  
498 by sharing the mechanical load across both surfaces<sup>64</sup>. The damaged OM observed in *msbA-KD* could therefore  
499 maintain its turgor-bearing properties to protect cells against lysis when Tae1 hydrolyzes PG. Additionally, the  
500 mechanical integrity of the cell envelope in *msbA-KD* may be fortified by covalently-bound Braun's lipoprotein or  
501 changes to membrane composition which could increase cell envelope stiffness<sup>65,66</sup>. Thus, our study indicates  
502 that there is still much to learn about the complex underpinnings of mechanical integrity within the bacterial cell  
503 envelope.

504

505 Another key insight from our study is that PG synthesis is stimulated in response to Tae1, indicative of an active  
506 *Eco* counterresponse. However, wild-type levels of PG synthesis were coincident with, not counter to, lytic death.  
507 Diminished PG synthesis activity in *msbA-KD* could therefore enable resistance by suppressing a toxic  
508 dysregulation of homeostatic activities. We propose that Tae1 activity leads to *Eco* cell death, in part, by triggering  
509 a futile cycle of Tae1 hydrolysis and PG synthesis that does not resolve in cell wall homeostasis. An exciting  
510 prospect for future studies could involve determining the molecular mechanisms that control PG synthesis  
511 stimulation after Tae1 hydrolysis, including whether Tae1 may synergize or hijack endogenous cell wall enzymes  
512 to amplify its damage to PG<sup>67</sup>.

513

514 In conclusion, our work highlights how recipient susceptibility in interbacterial competition may be more complex  
515 than direct toxin-substrate interactions alone. Toxins with essential targets not only impact specific molecules but  
516 also a dynamic network of interconnected pathways. T6SSs often encode multiple toxins that antagonize different  
517 essential features<sup>68</sup>, including components of the cell envelope and other metabolic pathways. We posit that  
518 T6SSs deploy a cocktail of toxins that can act in coordination to disrupt the network beyond repair, or even  
519 weaponize protective homeostatic mechanisms themselves. This study points to the importance of studying the  
520 role of essential genes in the context of T6S-mediated bacterial antagonism.

## 521 METHODS

522

### 523 Bacterial growth and selection

524 *Escherichia coli* strains were cultured in LB or LB-no salt (LBNS) at 37°C with orbital shaking. *Pseudomonas*  
525 *aeruginosa* strains were cultured in LB+ 0.01% Triton at 37°C with orbital shaking. Interbacterial competitions  
526 between *Eco* and *Pae*, and all *Eco* assays requiring solid growth, were conducted on LB+agar or LBNS+agar  
527 plates at 30°C. Where necessary, bacterial strains and plasmids were selected for growth using the following  
528 antibiotics: carbenicillin (Carb; 50 µg/ml) (Grainger), chloramphenicol (Chl; 25 µg/ml) (MP Biomedicals),  
529 gentamicin (Gent; 50 µg/ml)(Alfa Aesar), irgasan (Irg; 25 µg/ml) (Sigma-Aldrich), trimethoprim (Trm;15 µg/ml)  
530 (Sigma-Aldrich), or kanamycin (Kan; 50 µg/ml.) (VWR).

531

### 532 Eco CRISPRi library construction and use

533 The *Eco* CRISPRi collection was received in pooled format as a gift from the laboratory of Carol Gross (UCSF).  
534 CRISPRi strains were derived from K12 strain BW25113<sup>69</sup> and are each engineered with a chromosomal insertion  
535 of *dcas9* (constitutive expression) and a custom sgRNA sequence for inducible dCas9-mediated knockdown of a  
536 single gene-of-interest<sup>33</sup>. Transcriptional knockdown is induced with addition of 100µM IPTG (“induced”) into  
537 growth media, though growth without inductant also results in a mild knockdown phenotype (“basal”)<sup>33</sup>. Except  
538 where indicated, CRISPRi knockdown is induced in this study. CRISPRi strains *msbA-KD* and *lpxK-KD* were  
539 reconstructed from the parent strain for individual use in this study. Reconstructed strains were validated by  
540 Sanger sequencing (of the sgRNA and dCas9 chromosomal inserts), qRT-PCR (for knockdown efficiency), and  
541 Western blot (for dCas9 expression). See **Table S1** for strain descriptions and **Table S2** for primer sequences  
542 used for construction and validation.

543

### 544 Pae strain construction

545 *Pae* <sup>$\Delta$ tae1</sup> ( $\Delta$ retS $\Delta$ pppA $\Delta$ tae1; *clpV-GFP*) and *Pae*<sup>inactive</sup> ( $\Delta$ retS $\Delta$ pppA $\Delta$ icmF; *clpV-GFP*) strains were constructed  
546 from biparental mating of parent strain *Pae*<sup>WT</sup> (B515: PAO1  $\Delta$ retS $\Delta$ pppA; *clpV-GFP*)<sup>70</sup> with *Eco* SM10  $\lambda$ pir<sup>71</sup>  
547 bearing suicide vector pEXG2 cloned with homology to the gene(s) of interest and a spacer sequence for  
548 replacement. pEXG2 plasmids were cloned using splice-overlap extension<sup>11</sup>. After mating, transformants were  
549 isolated by negative selection on LB-agar + 5% sucrose and confirmed as scarless knockout mutants by colony  
550 PCR of the locus of interest. See **Table S1** for strain descriptions and **Table S2** for primer sequences used for  
551 construction and validation.

552

### 553 Pooled interbacterial competition screen

554 Competition assays were performed with overnight *Pae* cultures (*Pae*<sup>WT</sup>, *Pae* <sup>$\Delta$ tae1</sup>, *Pae*<sup>inactive</sup>) and pooled *Eco*  
555 CRISPRi libraries. Flash-frozen glycerol stocks of *Eco* pools were resuspended in LB, backdiluted to OD<sub>600</sub>=0.25,  
556 and recovered for 90 minutes at 37°C with shaking. All cultures were washed twice with fresh LB, then OD<sub>600</sub>-  
557 adjusted to 2.0 (for *Pae*) or 1.0 (for *Eco*) in either LB (basal CRISPRi) or LB+100µM IPTG (induced CRISPRi) . An  
558 aliquot of each CRISPRi pool was reserved by pelleting and flash-freezing for sequencing-based analysis of strain  
559 abundances in the starting population. Media-matched *Pae* and *Eco* were mixed at a 1:1 volumetric ratio, except  
560 for *Eco*<sup>ctrl</sup> populations (for which *Eco* pools were not mixed with *Pae*). Six, 10µl aliquots of coculture were applied  
561 to nitrocellulose membranes (0.2µm, GVS) atop LB-agar (basal CRISPRi) or LB-agar +100µM IPTG (induced  
562 CRISPRi) plates to match liquid media conditions. Covering the agar surface with nitrocellulose allows for nutrient  
563 transfer from the media to the bacteria, while aiding in bacterial recovery from the surface after competition.  
564 Cocultures were dried down to the membrane under flame-sterilization, then incubated at 30°C for 6h. Cocultures  
565 were removed from the plate by scalpel-excision of surrounding nitrocellulose and resuspended into 1ml fresh  
566 PBS by bead-beating for 45s on a tabletop vortex. The six aliquots per experiment were pooled, centrifuged (2min  
567 at 9000xG, RT), and PBS was decanted. Pellets were flash frozen in liquid nitrogen and stored at -80°C.

568

### 569 Sequencing library preparation

570 Genomic DNA was extracted from frozen bacterial pellets by phenol: chloroform extraction and RNase  
571 treatment<sup>74</sup>, followed by quantification on a Nanodrop 2000 spectrophotometer (Thermo Scientific). PCR  
572 amplification was used to isolate *Eco* sgRNA sequences from mixed genomic DNA and to attach Illumina Truseq  
573 index adapters for high-throughput sequencing. Sequencing libraries were purified by gel electrophoresis on 8%  
574 TBE gels (Invitrogen Novex), stained with SYBR Gold (Invitrogen) to visualize library bands, and scalpel-excised  
575 (200-300bp region) under blue light imaging (Azure Biosystems c600). Excised libraries were gel-extracted and  
576 precipitated<sup>75</sup>, then resuspended in nuclease-free distilled water (Invitrogen UltraPure). Library concentration was  
577 quantified on a Qubit 2.0 fluorimeter (Invitrogen) using the dsDNA high-sensitivity assay, and assayed for purity  
578 on a 2100 Bioanalyzer (Agilent) using the high-sensitivity DNA assay. Single-end sequencing was performed on  
579 an Illumina NextSeq 500 using a custom sequencing primer and a read length of 75bp. Multiplexed samples were

580 spiked with 5% PhiX Control v3 DNA (Illumina) to account for low diversity among sgRNA sequences. See **Table**  
581 **S2** for custom primers used for library preparation and sequencing.

582

### 583 **Sequencing data analysis**

584 Raw FASTQ files were aligned to the library oligos and counted using ScreenProcessing  
585 (<https://github.com/mhorlbeck/ScreenProcessing>). Counts were normalized to a total of 20,000,000 reads,  
586 pseudocounts of 1 were added, and log<sub>2</sub> fold change (L2FC) from t<sub>0</sub> was calculated for each strain with at least  
587 100 counts at t<sub>0</sub>. L2FC was further corrected by subtracting the median L2FC of the non-targeting control  
588 sgRNAs from that sample<sup>76</sup>. The L2FC of each sgRNA were averaged across four biological replicates to  
589 calculate the L2FC for that condition. Finally, to account for differences in the number of generations experienced  
590 (growth) in each of the experimental conditions, L2FC values for the *Pae*<sup>WT</sup>, *Pae*<sup>Δtae1</sup>, *Pae*<sup>inactive</sup> experiments were  
591 corrected by the coefficient of a robust (MM-type) intercept free linear regression between the experimental L2FC  
592 values and the CRISPRi induction-matched (induced/basal) *Eco*<sup>ctrl</sup> experiment. See Table S3 for correction  
593 coefficients and corrected L2FC values. Differences between conditions were then calculated for each sgRNA as:

594

$$595 \text{Diff} = (\text{L2FC} [\textit{condition}]) - (\text{L2FC} \textit{Eco}^{\textit{ctrl}})$$

596

597 Final Diff values are listed in **Table S4** and were used for all further analyses.

598

### 599 **COG analysis**

600 Gene ontology information was compiled from the NIH Database of Clusters of Orthologous Genes (COGs)  
601 (<https://www.ncbi.nlm.nih.gov/research/cog>) and reported previously<sup>33</sup>.

602

### 603 **Data availability and software**

604 Illumina sequencing data from this study is accessible at the NCBI Sequence Read Archive under accession  
605 PRJNA917770. Principal component analysis was performed using R<sup>77</sup> and visualized using ggplot2<sup>78</sup>. All other  
606 data visualizations were prepared using GraphPad Prism 9.4.1 (GraphPad Software, San Diego, California USA,  
607 [www.graphpad.com](http://www.graphpad.com)).

608

### 609 **Pairwise Interbacterial T6SS competition assay**

610 Competition assays were performed with overnight liquid cultures of *Pae* and *Eco* CRISPRi strains. *Eco* cultures  
611 were backdiluted 1:4 in LB-no salt (LBNS; cite) + 100μM IPTG and grown for 1h at 37°C with shaking to pre-  
612 induce CRISPRi before competition. Strains were washed and mixed in a 1:1 volumetric ratio of *Pae* (OD<sub>600</sub>=2)  
613 and *Eco* (OD<sub>600</sub>=1) in LBNS+100μM IPTG. Three, 10μl aliquots of each liquid co-culture applied to nitrocellulose  
614 membranes (0.2μm, GVS) atop LB-agar+100μM IPTG and dried down by flame-sterilization to encourage  
615 interbacterial competition. Cocultures were incubated at 30°C for 6h. For initial *Eco* colony-forming unit  
616 measurements (CFU<sub>t=0h</sub>), 20μl of each liquid co-culture input was serially diluted (10-fold dilutions x 8) in a 96-well  
617 plate (Corning) and plated onto LB-agar + Gent (*Eco*-selective). After the competition, coculture spots were  
618 harvested from the plate by scalpel-excision of the surrounding nitrocellulose, and pooled by resuspension into  
619 1ml fresh PBS by bead-beating for 45s on a tabletop vortex. Resuspensions were serially diluted (10x8) and  
620 plated onto LB+Gent. All serial dilution plates were incubated overnight at 37°C. Dilution plates with  
621 approximately 20-200 colonies-per-plate were counted for *Eco* CFU abundance (CFU<sub>t=0h</sub>, CFU<sub>t=6h</sub>). Fold-change  
622 in *Eco* CFUs was determined by back-calculating CFUs per ml from dilution plates, and then calculating  
623 CFU<sub>t=6h</sub>/CFU<sub>t=0h</sub>. Experiment was performed for three biological replicates. Statistical test: two-tailed unpaired t-  
624 test.

625

### 626 **qRT-PCR**

627 Overnight cultures of *Eco* were washed and OD<sub>600</sub>-corrected to 1.0 in LB or LBNS +/-100μl IPTG. Three, 10μl  
628 aliquots of each culture were applied to nitrocellulose membranes (0.2μm, GVS) atop LB-agar+100μM IPTG or  
629 LBNS-agar+100μM IPTG and dried down by flame-sterilization. After growing 6 hours at 30°C, the spots were  
630 scalpel-excised, pooled, and resuspended into PBS by bead beating, then pelleted for RNA extraction. RNA was  
631 extracted using TRIzol Reagent (Invitrogen) with Max Bacterial Enhancement Reagent (Invitrogen), followed by  
632 treatment with Turbo DNA-free kit (Invitrogen) to remove contaminating DNA. After quantification by Nanodrop  
633 (Thermo Scientific), total RNA was reverse transcribed into cDNA using qScript cDNA Supermix (QuantaBio). A  
634 1:5 dilution of cDNA and custom primers were input into qPCR reactions with PowerUP SYBR Green Master Mix  
635 (Applied Biosystems).qRT-PCR was performed using a QuantStudio 3 Real Time PCR system (ThermoFisher  
636 Scientific) using cycling parameters as defined by the master mix instructions. Fold-change in transcript levels  
637 was calculated using ΔΔC<sub>t</sub> analysis, using *rpoD* as a control gene. Three biological and three technical replicates

638 were used per experiment. Statistical test: two-tailed unpaired t-test. Custom primers for qPCR of *Eco* genes can  
639 be found in Table 3.

640

### 641 **Cryo-ET imaging**

642 Overnight cultures of *E. coli* strains were diluted in LB 1:100 and grown at 37°C. At OD<sub>600</sub>=0.2, 150 μM IPTG was  
643 added to the liquid culture to induce CRISPRi knockdown. Bacteria were grown for another 90 min and then flash-  
644 frozen in liquid nitrogen. Cell cultures were mixed with 10 nm protein A gold at 20:1 ratio (Utrecht), then aliquots  
645 of 3 μL mixtures were applied to glow-discharged R2/2, 200 mesh copper Quantifoil grids (Quantifoil Micro Tools).  
646 The sample was blotted for 3 s at 20°C and at 80% humidity. The grids were plunge-frozen in liquid ethane using  
647 Leica EM GP system (Leica Microsystems) and stored in liquid nitrogen. Cryo-ET was performed on a Talos  
648 electron microscope equipped with a Ceta CCD camera (ThermoFisher). Images were taken at magnification  
649 22,000x corresponding to a pixel size of 6.7 Å. Tilt series were collected using SerialEM<sup>79</sup> with a continuous tilt  
650 scheme (-48° to 48°, every 3° increment). The defocus was set to -6 to -8 μm and the cumulative exposure per tilt  
651 series was 150 e<sup>-</sup>/Å<sup>2</sup>. Tomograms were reconstructed with the IMOD software package<sup>80</sup>.

652

### 653 **Overexpression plasmid construction and use**

654 Plasmids for periplasmic Tae1 overexpression in *Eco* were constructed using splice-overlap extension cloning of  
655 *tae1*<sup>WT</sup> and *tae1*<sup>C30A</sup> coding sequences derived from *P. aeruginosa* (PAO1) into *pBAD24*<sup>46,72</sup>. A *pelB* leader  
656 sequence was fused to *tae1* for localization to the periplasm. Expression from *pBAD24* plasmids transformed into  
657 *Eco* was induced by addition of 0.125% arabinose (w/v) (Spectrum Chemical) into liquid LBNS media at early log  
658 phase (OD<sub>600</sub> ~0.25). Overexpression constructs for *msbA* and *lpxK* were constructed by cloning each full-length  
659 gene from *Eco* into the NdeI/HindIII restriction sites of *pSCRhaB2*<sup>73</sup>. Overexpression from *pSCRhaB2* plasmids  
660 transformed into *Eco* was induced by addition of 0.1% rhamnose (w/v) (Thermo Scientific) into liquid media. See  
661 **Table S2** for primer sequences used for cloning and PCR validation.

662

### 663 **Tae1 overexpression lysis assay**

664 Chemically competent *Eco* were transformed with Tae1 overexpression constructs (*pBAD24::tae1*<sup>WT</sup>,  
665 *pBAD24::tae1*<sup>C30A</sup>, *pBAD24*) by standard 42°C heat-shock and a 45-minute recovery in LB at 37°C with shaking.  
666 A transformant population was selected overnight in liquid LB+Carb; the more-traditional method of selecting on  
667 solid media was skipped to discourage the formation of Tae1-resistant compensatory mutations. Overnight  
668 transformant cultures were backdiluted to OD<sub>600</sub>=0.1 in LBNS+Carb +/- 100μM IPTG, then incubated in a Synergy  
669 H1 plate reader (BioTek) at 37°C with shaking (2 technical x 3 biological replicates). OD<sub>600</sub> reads were taken  
670 every five minutes to generate a growth curve. At OD<sub>600</sub>=0.25 (early log-phase), Tae1 expression was induced  
671 from *pBAD24* with the addition of 0.125% arabinose to each well, and grown for 500 minutes at 37°C with  
672 shaking. Bacterial growth curves were normalized to blank growth curves (LBNS+Carb, no bacteria), and average  
673 growth curves from all biological and technical replicates were plotted in Prism (GraphPad).

674

675 For *msbA* and *lpxK* complementation assays, *pSCRhaB2* plasmids were transformed alongside *pBAD24* plasmids,  
676 and overnight selection was performed in liquid LB+Carb+Trm. The next day, cultures were washed and  
677 backdiluted at OD<sub>600</sub>=0.1 into LBNS+Carb+Trm+0.1% rhamnose. The experiment then proceeded in the plate  
678 reader as described above.

679

### 680 **Western blotting**

681 *dCas9 detection*: Total protein was extracted from the organic layer of bacterial pellets treated with TRIzol  
682 Reagent (prepared as described in **qRT-PCR**), according to manufacturer's protocol. Protein samples were  
683 diluted to 1mg/ml in PBS + 1x Laemmli denaturing buffer, boiled for 10 minutes then centrifuged at 20,000xg at  
684 RT for 2 minutes. Fifteen μl of supernatant was loaded onto an anyKD MiniPROTEAN gel (BioRad), alongside  
685 ProteinPlus Ladder (BioRad). Gels were run according to manufacturer's protocol in 1x SDS-PAGE running buffer  
686 to separate proteins. Protein was transferred to nitrocellulose (0.2μm; GVS) via semi-dry transfer with a TransBlot  
687 Turbo transfer system (BioRad) and matching transfer buffer (BioRad) under the following conditions: 45 min @  
688 15V, 2.5 Amp. Transfer was validated by Ponceau stain. Blots were blocked for one hour at RT with shaking in  
689 3% milk+TBST. Primary antibody was applied: 1:1000 mouse anti-Cas9 (Abcam ab191468) in TBST, overnight,  
690 at 4C with shaking. Blots were washed four times in TBST. Secondary antibody was applied: 1:5000 anti-mouse  
691 HRP (Avansta R-05071-500) in TBST, for one hour at RT, with shaking. Blots were washed four times in TBST.  
692 Blots were treated with Clarity ECL Western blotting substrate (BioRad) for chemiluminescent detection on an  
693 Azure c400 imager. Visible light images were also taken to visualize protein ladder. Densitometry analysis was  
694 performed in Fiji<sup>81,82</sup>. Statistical test: two-tailed unpaired t-test. Three biological replicates.

695 *Tae1 detection*: Chemically competent *Eco* cells were transformed with Tae1 overexpression constructs  
696 (*pBAD24::tae1*<sup>WT</sup>, *pBAD24::tae1*<sup>C30A</sup>, *pBAD24*) by standard 42°C heat-shock and a 45-minute recovery in LB at

697 37°C with shaking. A transformant population was selected overnight in liquid LB+Carb. Cultures were  
698 backdiluted to OD<sub>600</sub>=0.1 in LBNS + Carb +100µM IPTG, then incubated in a Synergy H1 plate reader (BioTek) at  
699 37°C with shaking (2 technical x 3 biological replicates). OD<sub>600</sub> reads were taken every five minutes to track  
700 population growth. At OD<sub>600</sub>=0.25, Tae1 expression was induced with the addition of 0.125% arabinose to each  
701 well. Bacteria were grown for 60 minutes with Tae1 induction, before technical replicates were harvested and  
702 pooled. Samples were pelleted by centrifugation and media was decanted before cells were resuspended in PBS  
703 + 1x Laemmli denaturing buffer. Western blotting protocol then proceeded as above, excepting the use of a  
704 custom rabbit anti-Tae1 primary antibody (1:2500 in TBST) (ThermoFisher) and anti-rabbit HRP secondary  
705 antibody (1:5000 in TBST) (Advansta R-05072-500).

706

707

### Muropeptide analysis

708 Chemically competent *Eco* cells were transformed with Tae1 overexpression constructs (*pBAD24::tae1<sup>WT</sup>*,  
709 *pBAD24::tae1<sup>C30A</sup>*, *pBAD24*) by standard 42°C heat-shock and a 45-minute recovery in LB at 37°C with shaking.  
710 A transformant population was selected overnight in liquid LB+Carb. Cultures were backdiluted to OD<sub>600</sub>=0.1 in  
711 LBNS+Carb +100µM IPTG, and grown with shaking. At early log phase (OD<sub>600</sub>=0.25), 0.125% arabinose was  
712 added to induce *pBAD24* expression. Cells were grown for 60 minutes, then harvested by centrifugation. For PG  
713 purification, cells were boiled in 3% SDS to extract crude PG, then treated with Pronase E (100µg/ml in Tris-HCl  
714 (pH 7.2) + 0.06% NaCl) (VWR Chemicals) for 2 hours at 60C to remove proteins covalently bound to PG.  
715 Mutanolysin digestion (40µg/ml in Tris-HCl (pH 7.2) + 0.06% NaCl) was performed overnight at 37C to solubilize  
716 PG into muropeptides for HPLC analysis. Samples were reduced with sodium borohydride (Fisher Chemical) then  
717 pH-corrected to 3-4 using o-phosphoric acid(Fisher Chemical)<sup>83</sup>. Muropeptides were separated on a 1220 Infinity  
718 II HPLC (Agilent) with UV-visible detection (λ=206nm). Muropeptide separation was achieved over 54 minutes at  
719 0.5 ml/min using a Hypersil ODS C18 column (Thermo Scientific) and a gradient elution from 50mM sodium  
720 phosphate + 0.04% NaN<sub>3</sub> (Buffer A) to 75mM sodium phosphate +15% methanol (Buffer B). Chromatograms  
721 were integrated in ChemStation software (Agilent) to determine peak area, height, and elution time. Experimental  
722 chromatograms were normalized against a chromatogram from a blank run (ddH<sub>2</sub>O). Chromatograms were also  
723 internally normalized against the most abundant M4 (monomer muropeptide) peak; this allowed for direct relative  
724 comparisons of peak heights between samples.

725

726 To calculate the percent change in D44 (4,3-crosslinked dimer) peptides after Tae1 overexpression, the  
727 normalized area under the curve (AUC) for D44 was divided by the total chromatogram area to calculate the  
728 relative D44 peak area for each condition (AUC<sub>WT</sub>, AUC<sub>C30A</sub>, AUC<sub>EV</sub>). Then, within a given strain,  
729 (AUC<sub>WT</sub>/AUC<sub>EV</sub>)\*100 and (AUC<sub>C30A</sub>/AUC<sub>EV</sub>)\*100 were calculated to determine the percent of D44 peak area lost to  
730 Tae1<sup>WT</sup> or Tae1<sup>C30A</sup> treatment, relative to EV treatment. Three biological replicates were performed per condition.  
731 Statistical test: two-tailed unpaired t-test.

732

733

### HADA incorporation imaging

734 Chemically competent cells were transformed with *pBAD24* constructs: (*pBAD24::tae1<sup>WT</sup>*, *pBAD24::tae1<sup>C30A</sup>*, or  
735 *pBAD24*) and selected with Carb overnight in liquid LB. Transformant cultures were backdiluted to OD<sub>600</sub>=0.1 in  
736 1ml LBNS+Carb +100µM IPTG, and grown with shaking. At early log phase (OD<sub>600</sub>=0.25), 0.125% arabinose  
737 added to induce *pBAD24* expression. Cells were grown for 30 minutes, then 250µM HADA added to culture. Cells  
738 were grown an additional 30 minutes, then collected by centrifugation and washed 3x with cold PBS + sodium  
739 citrate (pH 3.0) to block hydrolysis of labelled septal PG<sup>84</sup>. Cells were fixed by treatment with 3% PFA for 15  
740 minutes on ice. Fixed cells were washed 3x in cold PBS, then resuspended in PBS +20% DMSO. Fluorescence  
741 imaging was performed on a Nikon Eclipse Ti2-E inverted microscope equipped with a 100x/1.40 oil-immersion  
742 phase objective and an EMCCD camera (Prime 95B). Fluorescence (DAPI channel) and phase-contrast images  
743 were captured using NIS-Elements AR Viewer 5.20. Images were analyzed for single-cell fluorescence intensity  
744 using MicrobeJ for Fiji<sup>82,85</sup>. 200 cells/sample measured, 3 biological replicates. Statistical test: unpaired t-test.

745

746

### Nascent protein synthesis imaging

747 Chemically competent cells were transformed with *pBAD24* constructs: (*pBAD24::tae1<sup>WT</sup>*, *pBAD24::tae1<sup>C30A</sup>*, or  
748 *pBAD24*) and selected with Carb overnight in liquid LB. Cultures were diluted by 1:100 and grown in LBNS+ Carb+  
749 100µM IPTG at 37 °C with shaking. At early log phase (~80 minutes) 0.125% arabinose was added to induce Tae1  
750 expression. After 35 minutes, 13µM O-propargyl-puromycin (OPP) was added to cultures to label new peptide  
751 synthesis before harvesting (Click-iT™ Plus OPP Alexa Fluor™ 488 Protein Synthesis Assay Kit, Invitrogen)<sup>86</sup>. After  
752 labelling, cells were pelleted and fixed in 3.7% formaldehyde in PBS. Cells were permeabilized with 0.3% Triton X-  
753 100 in PBS for 15 min, then labelled for imaging with Click-iT reaction cocktail for 20 min in the dark, washed then  
754 resuspended in PBS. Fluorescence imaging was performed on a Nikon Eclipse Ti2-E inverted microscope equipped  
755 with a 100x/1.40 oil-immersion objective and an EMCCD camera (Prime 95B). The 488-nm laser illumination

756 fluorescence and phase-contrast images were captured using NIS-Elements AR Viewer 5.20 and analyzed using  
757 MicrobeJ software for Fiji<sup>82,85</sup>.

758

### 759 **Time-lapse imaging of T6SS competitions**

760 Competition microscopy experiments were performed with overnight liquid cultures of *Pae* (LB) and *Eco* CRISPRi  
761 strains (LB+Gent+Cam). Cultures were diluted 1:50 in fresh medium and grown for 2h. *Pae* cells were diluted  
762 again 1:50 in fresh medium (LB) and grown at 37°C to OD 1.2 – 1.5 (~1 hour). Similarly, *E. coli* strains were  
763 diluted 1:100 in fresh medium (LB+150µM IPTG) supplemented with antibiotics (Gent / Cam) and grown at 37°C  
764 to OD 1.2 – 1.5 (~1 hour). Then, cultures were washed with LB, resuspended in LB + 150µM IPTG and mixed 2:1  
765 (*Pae:Eco*). 1 µl of the mixed cells was spotted on an agarose pad containing propidium iodide and imaged for 2h  
766 at 37°C. A Nikon Ti-E inverted motorized microscope with Perfect Focus System and Plan Apo 1003 Oil Ph3 DM  
767 (NA 1.4) objective lens was used to acquire images. If not indicated otherwise, time-lapse series of competitions  
768 were acquired at 10 s acquisition frame rate during 120 min. SPECTRA X light engine (Lumencore), ET-GFP  
769 (Chroma #49002) and ET-mCherry (Chroma #49008) filter sets were used to excite and filter fluorescence.  
770 VisiView software (Visitron Systems, Germany) was used to record images with a sCMOS camera pco.edge 4.2  
771 (PCO, Germany) (pixel size 65 nm). The power output of the SPECTRA X light engine was set to 20% for all  
772 excitation wavelengths. GFP, phase-contrast and RFP / propidium iodide (PI) images were acquired with 50-100  
773 ms exposure time. Temperature and humidity were set to 37°C, 95% respectively, using an Okolab T-unit  
774 objective heating collar as well as a climate chamber (Okolab). Fiji was used for imaging processing<sup>82</sup>. Acquired  
775 time-lapse series were drift-corrected using a custom StackReg based software<sup>87,88</sup>.

776

### 777 **SUPPLEMENTAL INFORMATION**

778 **Table S1.** Bacterial strains and plasmids used in this study

779 **Table S2.** Primer sequences

780 **Table S3.** Corrected L2FC values from screen

781 **Table S4.** Final Diff values from screen

782

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797 **AUTHOR CONTRIBUTIONS**

798 Kristine L Trotta (Conceptualization, Methodology, Research, Data analysis, Data visualization, Writing,  
799 Reviewing)

800 Beth M Hayes (Conceptualization, Research, Data analysis, Reviewing)

801 Johannes P Schneider (Methodology, Research, Data analysis, Reviewing)

802 Jing Wang (Methodology, Research, Data analysis, Reviewing)

803 Horia Todor (Data analysis, Data visualization, Reviewing)

804 Patrick Rockefeller Grimes (Research, Reviewing)

805 Ziyi Zhao (Methodology, Research, Data analysis, Reviewing)

806 William L Hatleberg (Data visualization, Reviewing)

807 Melanie R Silvis (Conceptualization, Methodology, Reviewing)

808 Rachel Kim (Methodology, Reviewing)

809 Byoung-Mo Koo (Methodology, Reviewing)

810 Marek Basler (Project administration, Reviewing)

811 Seemay Chou (Project administration, Funding, Conceptualization, Writing, Reviewing)

812

813 **COMPETING INTERESTS**

814 The authors declare no competing interests. Seemay Chou is the president and CEO of Arcadia Science.



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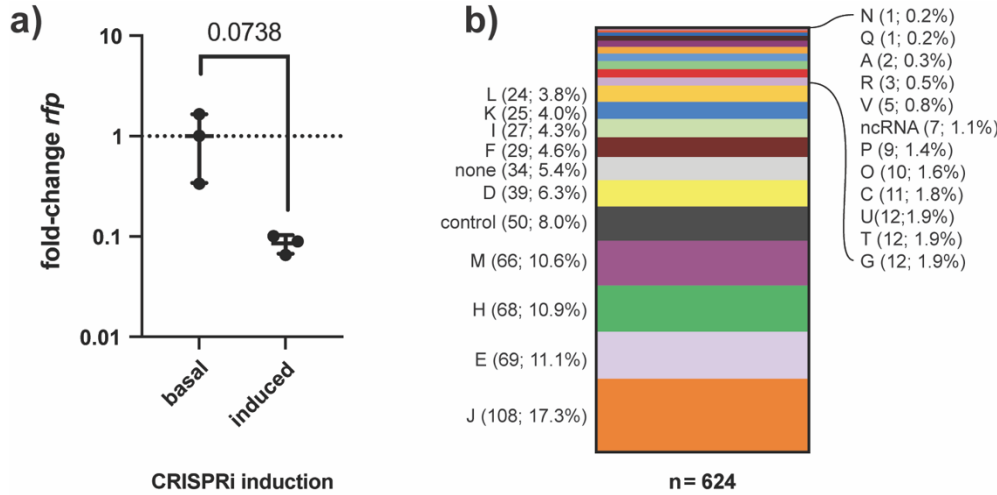
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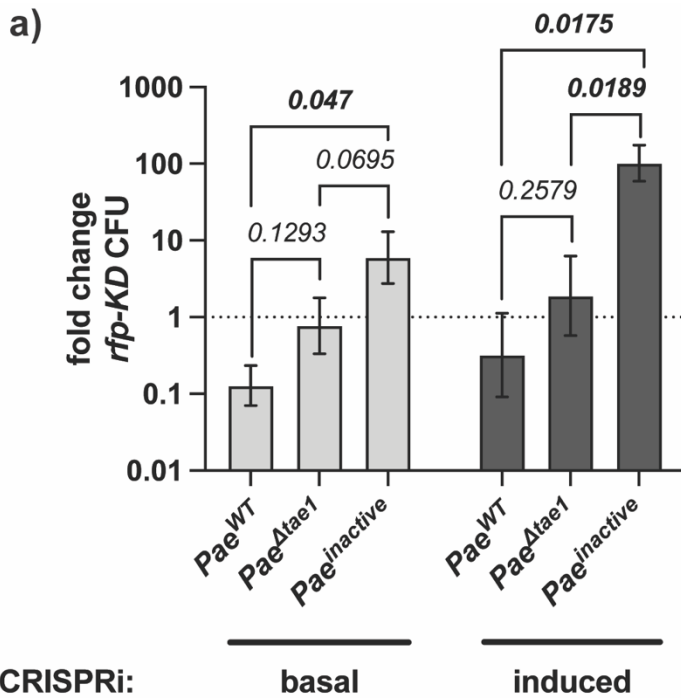
**SUPPLEMENTAL FIGURES**



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**Supplement 1: CRISPRi conditionally knocks down transcription across hundreds of *Eco* gene targets**

**a) CRISPRi induction produces mild transcriptional knockdown of endogenous *rfp* (11.7-fold decrease) in *Eco*.** qRT-PCR measurement of relative *rfp* RNA expression in *Eco* strain SC363 after 6 hours of growth on solid LB media with basal or induced CRISPRi. Data shown: 3 biological replicates with mean  $\pm$  s.d. Statistical test: unpaired two-tailed *t*-test. **b) CRISPRi targets *Eco* genes that collectively represent 21 clusters of orthogonal genes (COGs).** CRISPRi target genes ( $n=596$ ) were binned by their NCBI COG functional assignment. The relative representation of each COG in the strain collection is displayed as a percent of all COGs. Some genes are represented by multiple COGs, resulting in a greater number of COGs ( $n=624$ ) than target genes. Non-targeting negative controls (“control”,  $n=50$ ) genes without COG assignments (“none”,  $n=34$ ), and genes coding for non-coding RNAs (“ncRNA”,  $n=7$ ) were also binned.



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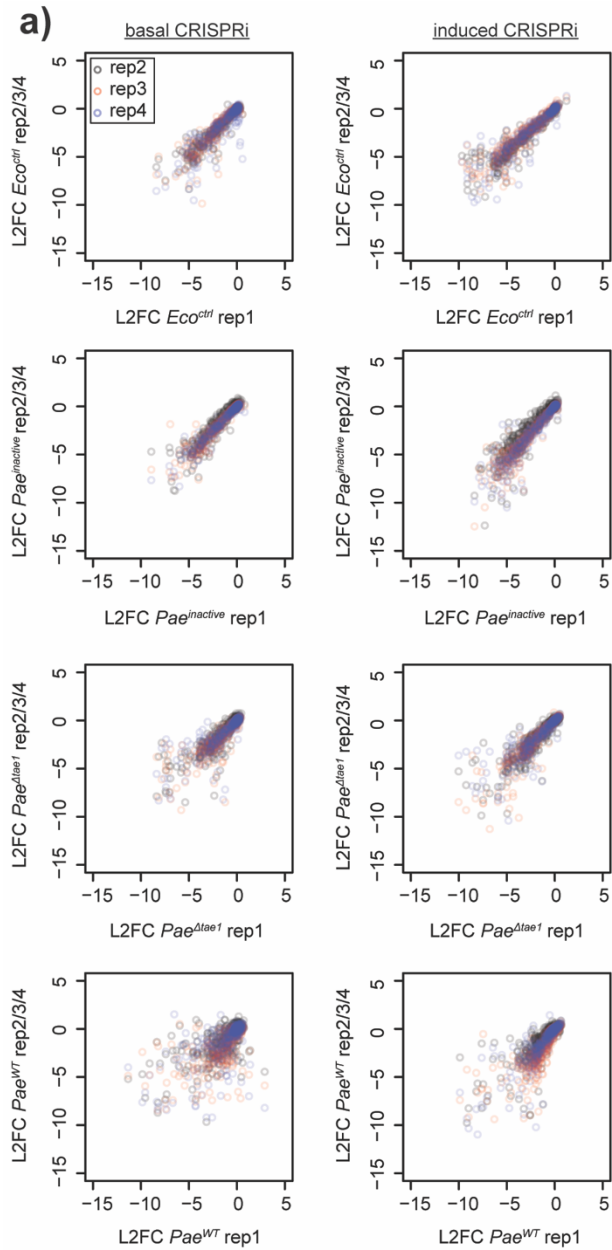
CRISPRi:

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**Supplement 2: Non-targeting CRISPRi induction has little effect on *Eco* fitness in T6SS competition**

a) CRISPRi induction does not disrupt T6SS- and *Tae1*-dependent targeting of *Eco* by *Pae*. Interbacterial competition between *Pae* (*Pae*<sup>WT</sup>, *Pae* <sup>$\Delta$ tae1</sup>, *Pae*<sup>inactive</sup>) and an *Eco* negative-control KD strain (*rfp*-KD), with induced or basal CRISPRi. Data shown: mean fold-change ( $\pm$  geometric s.d.) of *rfp*-KD colony forming units (CFUs) after six hours of competition against *Pae*. Statistical test: unpaired two-tailed *t*-test; *p*-value  $\leq 0.05$  displayed in bold font.

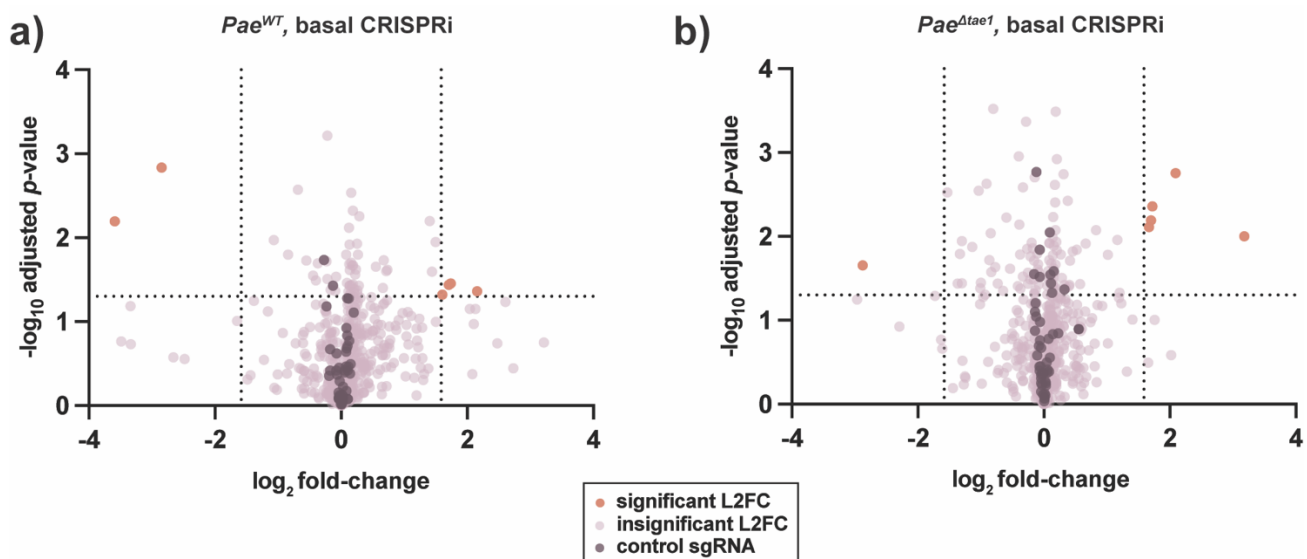


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**Supplement 3: CRISPRi library fitness in T6SS screen is reproducible across biological replicates**

a) CRISPRi library fitness in T6SS screen is reproducible across biological replicates. Replica plots showing the uncorrected L2FC values for each *Eco* CRISPRi strain after competition against *Pae*<sup>WT</sup>, *Pae*<sup>Δtae1</sup>, *Pae*<sup>inactive</sup>, for four biological replicates. For each plot, replicate 1 is compared to replicate 2 (grey), replicate 3 (red), or replicate 4 (blue). Median Pearson's *r* between all replicates = 0.91.



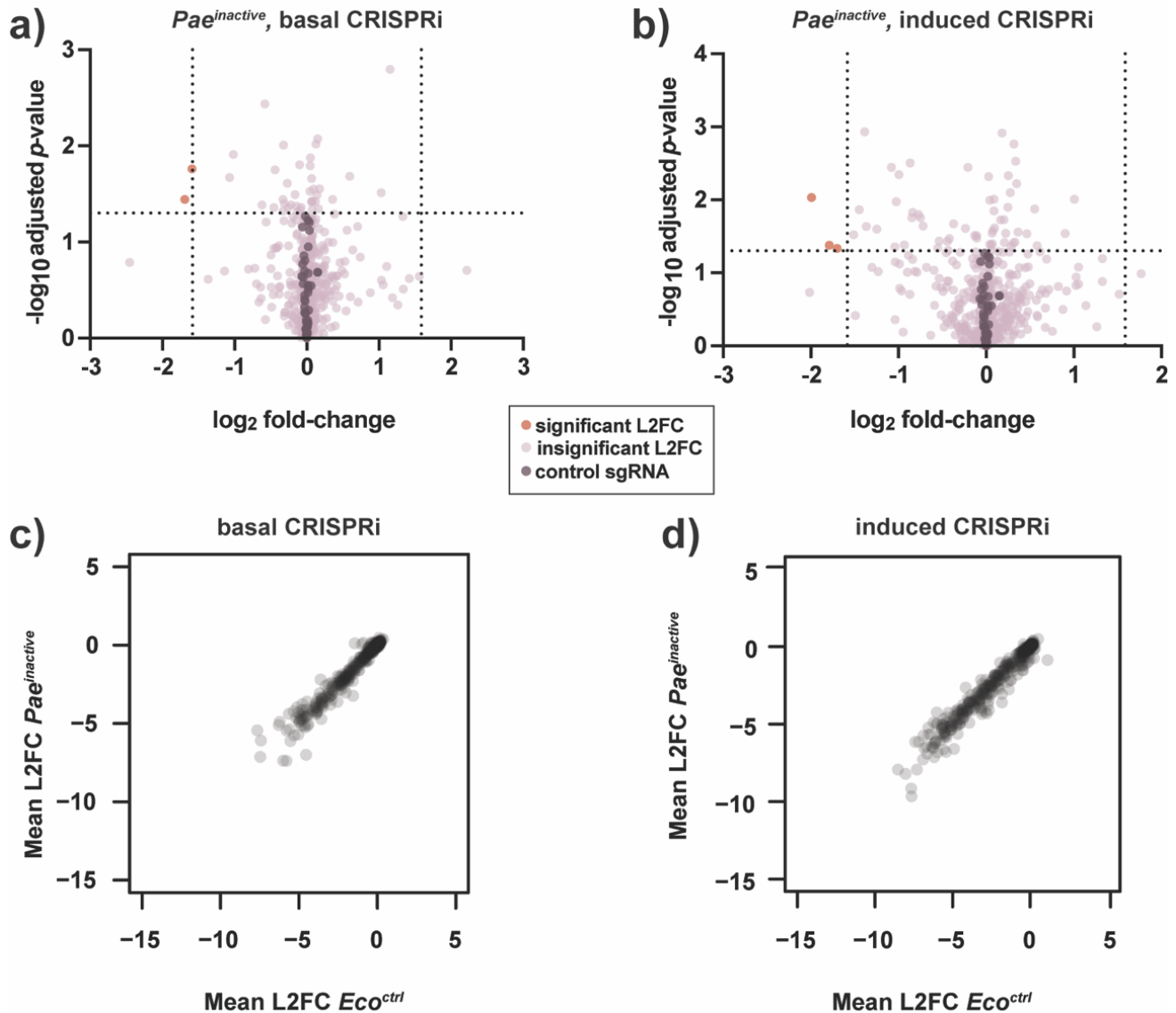


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**Supplement 4: Pooled T6SS competitions with basal CRISPRi attenuate significant fitness phenotypes**

a-b) Basal CRISPRi attenuates *Eco* fitness phenotypes against *Pae<sup>WT</sup>* (a) and *Pae<sup>Δetae1</sup>* (b). Volcano plots showing log<sub>2</sub>-fold change (L2FC) values for each KD strain after interbacterial competition (basal CRISPRi). Data shown: mean from four biological replicates. Statistical test: Wald test. Vertical dotted lines indicate arbitrary cutoffs for L2FC at x = -1.58 and x = 1.58 (absolute FC x = -3 or x = 3). Horizontal dotted line indicates statistical significance cutoff for log<sub>10</sub> adjusted p-value (≤ 0.05). Orange points represent KDs with L2FC ≥ 1.58 or ≤ -1.58 and log<sub>10</sub>-adj. p-value ≤ 0.05. Dark purple points represent non-targeting negative control KDs (n=50). Lavender points represent KDs that do not meet cutoffs for L2FC or statistical test.

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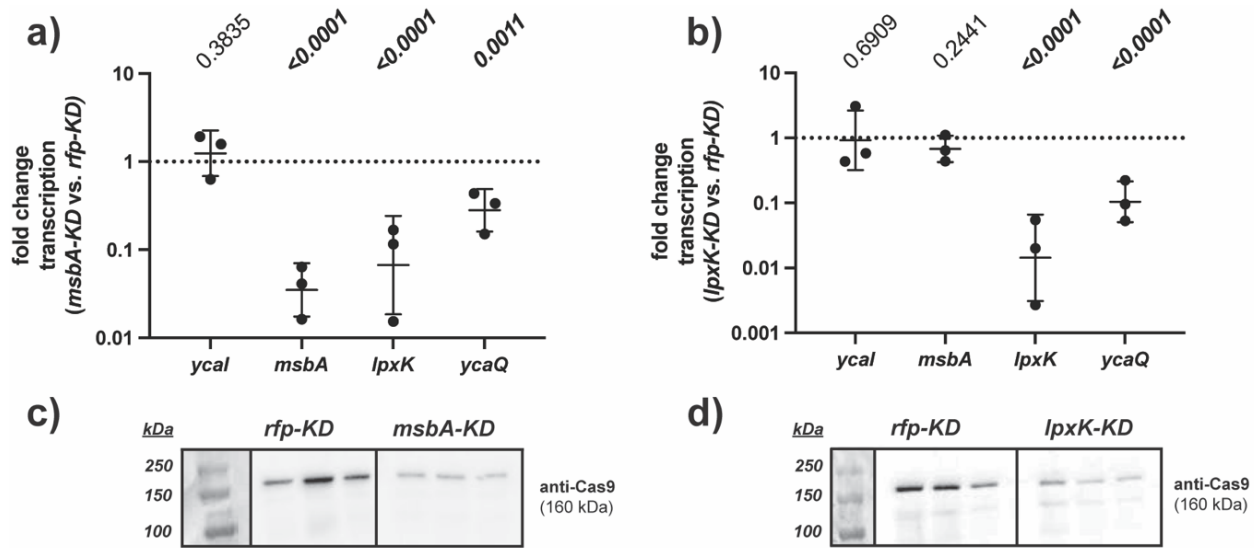
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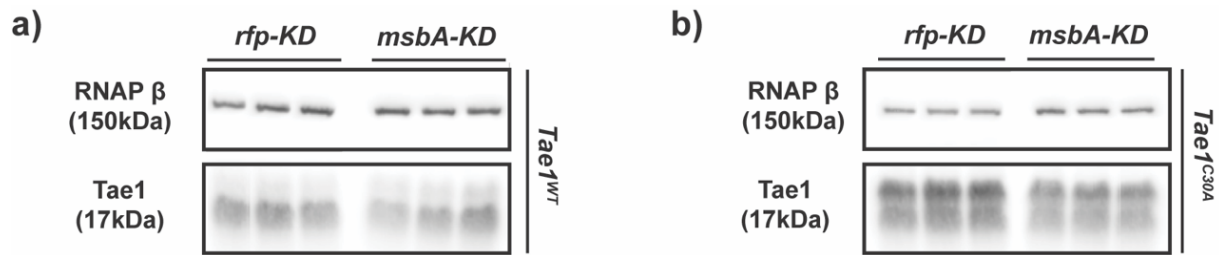
**Supplement 5: *Pae<sup>inactive</sup>* is a neutral co-culture partner for *Eco***

**a-b) Competition against *Pae<sup>inactive</sup>* reveals few *Eco* fitness determinants.** Volcano plots showing log<sub>2</sub>-fold change (L2FC) values for each KD strain after interbacterial competition with induced (a) or basal (b) CRISPRi. Data shown: mean from four biological replicates. Statistical test: Wald test. Vertical dotted lines indicate arbitrary cutoffs for L2FC at x=-1.58 and x=1.58 (absolute FC x=-3 or x=3). Horizontal dotted line indicates statistical significance cutoff for log<sub>10</sub> adjusted p-value (≤ 0.05). Orange points represent KDs with L2FC ≥ 1.58 or ≤ -1.58 and log<sub>10</sub>-adj. p-value ≤ 0.05. Dark purple points represent non-targeting negative control KDs (n=50). Lavender points represent KDs that do not meet cutoffs for L2FC or statistical test. **c-d) KD strain abundance is highly similar after competition with *Pae<sup>inactive</sup>* and after growth without competition (*Eco<sup>ctrl</sup>*).** Scatter plots comparing mean L2FC for each *Eco* KD strain after competition with *Pae<sup>inactive</sup>* or *Eco<sup>ctrl</sup>* treatment, with basal (c) or induced (d) CRISPRi. Median Pearson correlation r = 0.98.



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1114 **Supplement 6: *lpxK-KD* and *msbA-KD* modulate target gene expression and show polar effects**

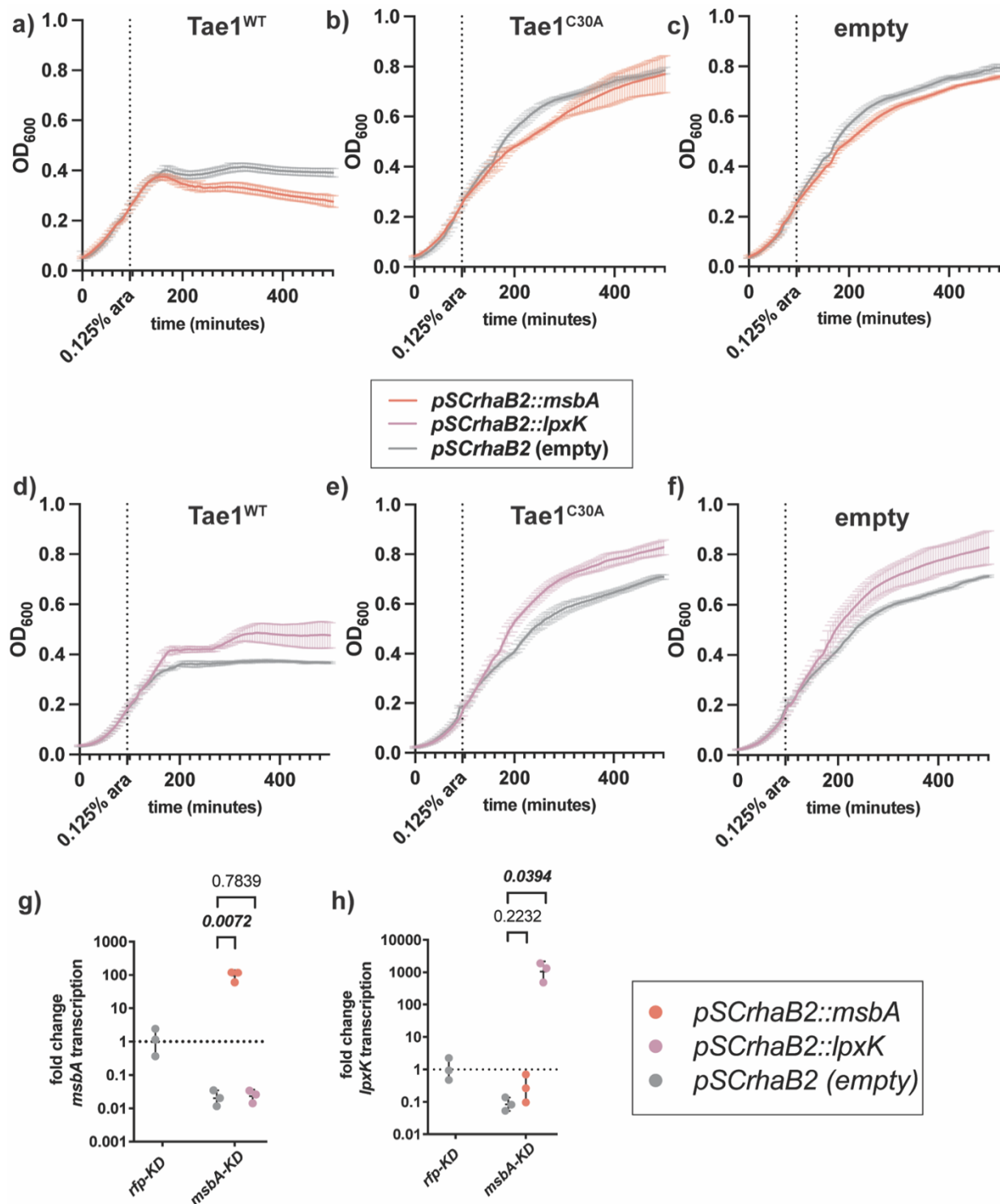
1115 **a-b) Transcriptional knockdowns in *msbA* and *lpxK* have off-target polar effects on transcription in their operon.** qRT-PCR analysis of  
1116 transcriptional fold-change in *ycal-msbA-lpxK-ycaQ* in *msbA-KD* (a) and in *lpxK-KD* (b) after growth for 6 hours with induced CRISPRi,  
1117 normalized to expression in *rfp-KD*. Data shown are geometric average of 3 biological replicates  $\pm$  s.d. Statistical test: unpaired two-tailed *t*-  
1118 test; *p*-value  $\leq 0.05$  displayed in bold font. **c-d) *msbA-KD* and *lpxK-KD* express a catalytically dead Cas9 (dCas9) enzyme for CRISPRi-**  
1119 **mediated transcriptional knockdown.** Western blot analysis of dCas9 protein expression (160 kDa) from *rfp-KD*, *msbA-KD* (c), and *lpxK-KD*  
1120 (d). Three independent biological replicates shown.  
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**Supplement 7: Tae1 protein expression is unaffected in *msbA-KD***

**a-b) Bulk Tae1 protein expression is similar between *msbA-KD* and *rfp-KD*.** Western blot analysis of periplasmic Tae1 protein (17kDa) from (a) *pBAD24::pelB-tae1<sup>WT</sup>* (*Tae1<sup>WT</sup>*) or (b) *pBAD24::pelB-tae1<sup>C30A</sup>* (*Tae1<sup>C30A</sup>*) in *rfp-KD* and *msbA-KD* (with induced CRISPRi). Protein expression of RNA polymerase (β subunit) (150kDa) is used as an internal loading control.

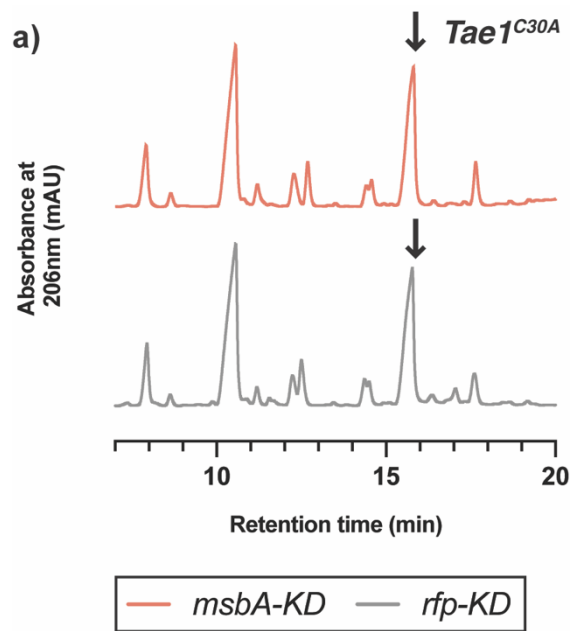
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**Supplement 8: Plasmid-borne overexpression of *msbA* partially rescues *Tae1* sensitivity in *msbA-KD***

a-c) Plasmid-borne *msbA* overexpression partially rescues *msbA-KD* resistance to lysis by *Tae1*. OD<sub>600</sub> growth curves of *msbA-KD* with induced CRISPRi, overexpressing *pSCRhaB2::msbA* (orange) or *pSCRhaB2* (empty) (grey) alongside (a) *pBAD24::pelB-tae1*<sup>WT</sup> (*Tae1*<sup>WT</sup>), (b) *pBAD24::pelB-tae1*<sup>C30A</sup> (*Tae1*<sup>C30A</sup>), or (c) *pBAD24* (empty). Data shown: average of 3 biological replicates ± s.d. Dotted vertical line indicates *pBAD24* induction timepoint (at OD<sub>600</sub>=0.25) (0.125% arabinose w/v). d-f) Plasmid-borne *lpxK* overexpression enhances *msbA-KD* resistance to lysis by *Tae1*. OD<sub>600</sub> growth curves of *msbA-KD* with CRISPRi induced, overexpressing *pSCRhaB2::lpxK* (purple) or *pSCRhaB2* (empty) (grey) alongside (d) *pBAD24::pelB-tae1*<sup>WT</sup> (*Tae1*<sup>WT</sup>), (e) *pBAD24::pelB-tae1*<sup>C30A</sup> (*Tae1*<sup>C30A</sup>), or (f) *pBAD24* (empty). Data shown: average of 3 biological replicates ± s.d. Dotted vertical line indicates *pBAD24* induction timepoint (at OD<sub>600</sub>=0.25) (0.125% arabinose w/v). g-h) *pSCRhaB2* vectors selectively rescue transcription of their target gene by overexpression. qRT-PCR analysis of transcriptional fold-change in (g) *msbA* or (h) *lpxK* expression with constitutive rhamnose induction of *pSCRhaB2::msbA* (orange), *pSCRhaB2::lpxK* (purple), or (c) *pSCRhaB2* (empty; grey) in *msbA-KD* with induced CRISPRi. Expression normalized against basal *msbA* expression in *rfp-KD* + *pSCRhaB2* (empty). Data shown: geometric average of 3 biological replicates ± s.d. Statistical test: unpaired two-tailed t-test; p-value ≤ 0.05 displayed in bold font.

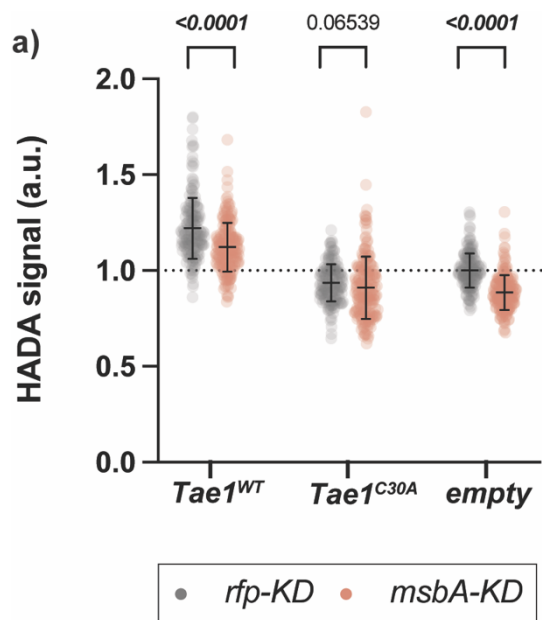
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**Supplement 9: *Tae1<sup>C30A</sup>* hydrolyzes D44 muropeptides in *rfp-KD* and *msbA-KD***

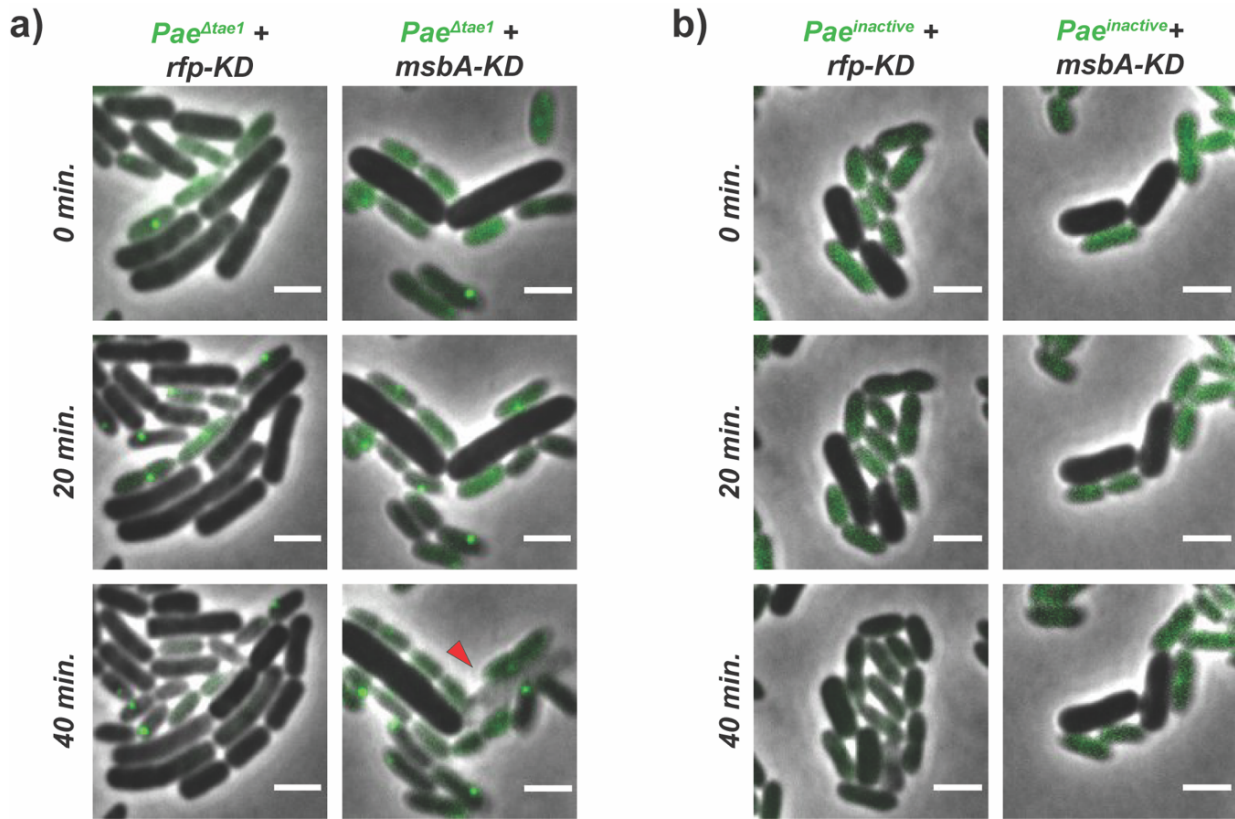
a) *Tae1<sup>C30A</sup>* overexpression yields minor digestion of D44 muropeptides. HPLC chromatograms of muropeptides purified from *msbA-KD* (orange) and *rfp-KD* (grey) expressing *pBAD24::peIB-tae1<sup>C30A</sup>* (*Tae1<sup>C30A</sup>*). Black arrow indicates D44 peptide partially digested by *Tae1<sup>C30A</sup>*. Data shown: representative from 3 biological replicates.



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**Supplement 10: PG synthesis activity in *msbA-KD* is suppressed across all conditions**

a) **PG synthesis activity in *msbA-KD* is attenuated under all tested conditions.** Single-cell fluorescence intensity measurements for *rfp-KD* (grey) or *msbA-KD* (orange) incorporating the fluorescent D-amino acid HADA into PG after 60 minutes of overexpressing *pBAD24::pelB-tae1<sup>WT</sup>* ( $Tae1^{WT}$ ), *pBAD24::pelB-tae1<sup>C30A</sup>* ( $Tae1^{C30A}$ ), or *pBAD24* (empty), with CRISPRi induced. All data normalized to average HADA signal in *rfp-KD* + empty. Data shown: 600 cells (200 cells x 3 biological replicates), with average  $\pm$  s.d. Statistical test: unpaired two-tailed *t*-test; *p*-value  $\leq 0.05$  displayed in bold font.



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**Supplement 11: *msbA-KD* growth defects are independent of *Pae* T6SS activity**

*a-b* *msbA-KD* cells maintain growth defects regardless of *Pae* competitor. Representative frames from time-course imaging of *rfp-KD* (left column; grey cells) and *msbA-KD* (right column; grey cells) co-cultured with *Pae* <sup>$\Delta$ tae1</sup> (*a*) or *Pae*<sup>inactive</sup> (*b*) (green cells), and with induced CRISPRi. Green foci in *Pae*<sup>WT</sup> indicate aggregates of GFP-labelled ClpV, which signal H1-T6SS firing events. Red arrow indicates lysed cell. Data shown are merged phase and fluorescent channels. Scale bar: 2 $\mu$ m.