Perturbation-Specific Transcriptional Mapping facilitates unbiased target elucidation of antibiotics.

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Abstract

The rising prevalence of antibiotic resistance threatens human health. While more sophisticated strategies for antibiotic discovery are beginning to combat the threat, target elucidation of new chemical entities remains challenging. In the post-genomic era, expression profiling can play an important role in mechanism-of-action (MOA) prediction based on the principle that molecules with similar MOAs elicit similar transcriptional responses. However, the broad application of transcriptomics-based MOA prediction remains limited by technical confounding that encumber experimental throughput. Here we report an unbiased strategy for MOA prediction, called Perturbation-Specific Transcriptional Mapping (PerSpecTM), in which large-throughput transcriptional sequencing enables the identification of perturbation-specific gene responses to either target inhibition or transcriptional repression. We applied this approach to build an antimicrobial reference set comprising compounds with known activity against the important bacterial pathogen *P. aeruginosa*. We rapidly elucidated the MOAs of three new molecules with anti-pseudomonal activity, and importantly, demonstrated the value of using hypersensitized strains, depleted for essential targets, to yield more robust transcriptional responses to perturbation. Using CRISPRi, we then constructed a transcriptionally repressed reference set and demonstrated proof-of-concept that gene responses to gene target depletion can mimic its chemical inhibition, affording MOA prediction when no chemical entities exist to serve as a reference. Empowered by PerSpecTM and mutant strains depleted in essential targets, this work enables an unbiased and readily scalable reference-based strategy for MOA elucidation to assist antibiotic development efforts in an era fraught with antimicrobial resistance.

60 **Significance Statement**

61 Target identification remains a time-consuming step in antibiotic discovery. Although the principle that molecules with
62 similar MOAs elicit similar transcriptional responses has long been established, the broad application of
63 transcriptomics-based MOA prediction remains experimentally cumbersome. We developed a highly scalable strategy
64 for MOA prediction in the important bacterial pathogen *P. aeruginosa* that accentuates perturbation-specific gene
65 responses to target inhibition by antibiotics or transcriptional repression by CRISPRi. Employing this strategy, we
66 rapidly elucidated the MOAs of three new molecules with anti-pseudomonal activity and provide proof-of-concept that
67 transcriptionally repressed gene targets can also serve as references for MOA prediction. This work paves the way for
68 transcriptomics-based MOA prediction at large-scale, supporting antibiotic development efforts in an era fraught with
69 resistance.

Main Text

Introduction

Pseudomonas aeruginosa and other gram-negative bacteria pose a major threat to human health due to the growing prevalence of antibiotic resistance in healthcare settings. Surging multi-drug-resistant bacterial infections threaten to outpace our ability to develop novel antibiotics[1-4]. Antibiotic discovery campaigns have failed to deliver new antibiotic classes, including extensive efforts in the post-genomic era. As recently reviewed, new strategies are being developed to address the looming antibiotic shortage through target-based approaches carried out in whole cells at scale[5]. Most of these novel approaches differ from standard target-based screening, in which the target is known by design, because they prioritize the identification of new molecules with whole-cell antibacterial activity. For example, in a method called PROSPECT (primary screening of strains to prioritize expanded chemistry and targets), using a ubiquitin degradation tag, barcoded strains of *Mycobacterium tuberculosis* were screened within a single pool, yielding tenfold more chemical candidates than traditional methods due to the ability to sample a wider range of genetic mutants within a single pool[6]. Meanwhile, the diversity of chemical matter that can be assembled into screening libraries is rapidly expanding. More sophisticated methods to extract and identify biosynthetic gene clusters from soil-dwelling bacteria, which can then be expressed in laboratory strains, are propelling the discovery of natural product antimicrobials[7]. Moreover, screening for natural products in bacteria that share similar requirements for antibiotics as humans, such as those in the gut microbiome of nematodes, led to the discovery of Darobactin, a first-in-class inhibitor of the outer membrane chaperone BamA[8, 9]. While accelerating the discovery of new chemical entities, these modern approaches also exacerbate the critical downstream challenge of mechanism-of-action (MOA) determination to enable candidate development. Currently, extensive biochemical and genetic efforts are required for target and MOA elucidation, which impose major bottlenecks in antibiotic discovery pipelines[5]. More streamlined MOA prediction tools, which ideally can be integrated early into antibiotic development pipelines, are needed to alleviate the bottleneck and guide hit prioritization, medicinal chemistry optimization, and eventual hit-to-lead transition.

Transcriptional profiling of chemical perturbations has long been used to explore the MOAs of antimicrobial agents, first described in yeast using microarray to delineate the MOA of the inhibitor dyclonine [10]. In bacteria, early work in *Bacillus subtilis* generated the first antimicrobial reference set based on gene expression using microarray[11],

99 followed by later work showing conditional mutants underexpressing essential gene targets mimic chemical inhibition
100 of their corresponding gene targets[12]. In the era of next-generation sequencing, genome-wide transcriptomics has
101 replaced microarray as the preferred method to generate affordable gene expression profiling. Over the past decade,
102 gene expression profiling of chemical, environmental or genetic stressors has been performed in a wider array of
103 bacteria, including *B. subtilis*, *M. tuberculosis*, *Staphylococcus aureus*, *Escherichia coli and P. aeruginosa* [11-17].
104 The most extensive expression profiling experiment in *P. aeruginosa* aimed to identify the genetic loci important for
105 antimicrobial resistance[17]. More recently, Espinoza et al. developed a machine learning method for transcriptomics-
106 based MOA prediction in *E. coli*, from which a putative protein synthesis inhibitor was identified based on the similarity
107 of its transcriptional signature compared to the reference set of forty-one antibiotics [15, 18]. This method involves a
108 feature selection algorithm, called *Clairvoyance*, which chooses a set of genes that maximizes its ability to predict the
109 MOA of the known compounds in the reference set.
110
111 Despite the growing affordability and accessibility of genome-wide expression profiling, its broad application in
112 transcriptomics-based MOA prediction remains limited by technical confounding that encumber experimental
113 throughput[19, 20]. Ideally, unbiased transcriptomics-based MOA prediction should be rooted in gene expression
114 responses specific to the given chemical or genetic perturbation. However, during perturbations, bacteria also change
115 the expression of a plethora of background genes, unrelated the perturbed pathway, which obfuscate the
116 perturbation-specific signals. For example, background gene responses relating to growth phase, cellular stress, or
117 late-stage cell death might be shared across a diverse panel of antimicrobial compounds. To minimize confounding,
118 expression profiling experiments require careful, low-throughput experimental designs, in which chemical or genetic
119 perturbations occur in test tubes with close monitoring of the bacterial optical density prior to RNA sampling[11, 12,
120 14, 15]. These experimental approaches, while necessary, are inherently unscalable and impede the expansion of
121 high-quality reference sets into new mechanistic classes or other clinically relevant bacterial pathogens.
122
123 To facilitate the broader applicability of gene expression profiling for MOA prediction, we developed Perturbation-
124 Specific Transcriptional Mapping (PerSpecTM): a reference-based strategy for MOA prediction that leverages its
125 large-throughput scale to accentuate changes in gene expression that are specific to target inhibition or transcriptional
126 repression. We optimized a treatment protocol in which RNA, sufficient in quality and quantify, could be recovered

127 from 60μL of mid-log bacteria under chemical or genetic perturbation in 384-well format. Across this large and diverse
128 population of samples, PerSpecTM leverages z-scoring as the primary metric for relative gene expression to highlight
129 perturbation-specific transcriptional changes, adapted from a strategy previously used in human cancer cell lines[21].
130 We applied PerSpecTM in the important bacterial pathogen *P. aeruginosa*, building two orthologous reference sets:
131 (1) an antimicrobial reference set compressing 37 antimicrobial compounds; and (2) a transcriptional repression
132 reference set comprising 15 strains, each exhibiting tunable transcriptional repression of an essential gene target
133 using CRISPRi. Querying against the antimicrobial reference set, we rapidly elucidated the MOA of three new
134 molecules with anti-pseudomonal activity, and importantly, demonstrated the value of using hypersensitized strains,
135 depleted for essential targets, to yield more robust transcriptional responses to perturbation. Using the transcriptional
136 repression reference set, we then demonstrated proof-of-concept that repressed gene target expression can
137 phenocopy its chemical inhibition, which could afford MOA prediction when no chemical entities exist to serve as a
138 reference. Empowered by PerSpecTM and mutant strains depleted in essential targets, this work enables an
139 unbiased and highly scalable reference-based strategy for MOA elucidation to assist antimicrobial discovery efforts in
140 the face of the looming shortage of antibiotics in healthcare systems worldwide.
141

Results

Developing Perturbation-Specific Transcriptional Mapping (PerSpecTM)

We set out to generate an antimicrobial reference set of chemical treatments of the *P. aeruginosa* laboratory strain UCBPP-PA14 (abbreviated hereafter as PA14) and related mutant strains. We ultimately sought to perform transcriptional profiling in large-throughput format, enabling the generation of hundreds-to-thousands of expression profiles. To enable such scale, we first optimized the assay conditions in 384-well format. Specifically, we determined the optimal cell density, incubation time, and compound concentration for which we could elicit robust transcriptional responses across many different compound treatments.

We first performed an expression profiling time trial on a smaller set of 21 antimicrobial compounds, depicted in bold in **Table 1**, which collectively targeted four distinct cellular mechanisms: DNA synthesis, protein synthesis, peptidoglycan synthesis, and membrane integrity. All expression profiling experiments were performed in 384-well format with 60uL per well at a bacterial inoculum of ~1 x 10^8 CFU/mL (OD_{600} of 0.1). Importantly, for the time trial, antimicrobial agents were dosed at 3-fold their minimum-inhibitory concentrations (MICs) determined by conventional broth microdilution at a much lower inoculum of ~5 x 10^5 CFU/mL[22]. All samples were incubated without shaking for 30, 60, 90, or 120 minutes before chemical lysis, RNA extraction, and RNA sequencing.

The resulting gene expression data were represented as z-scores, calculated for each gene per batch. The z-score provides a measure of gene expression after chemical (or genetic) perturbation relative to the median expression across the entire population of samples within a batch. Provided the batch consists of a diverse set of antimicrobial agents (or genetic perturbations) with multiple MOAs, z-scores highlight transcriptional changes that are unique to a given perturbation, and de-emphasize common gene responses across the population, such as those relating non-specifically to growth, cellular stress, or impending cell death. Thus, the relevant signals are accentuated in an unbiased manner, without needing to optimize or select genes based on *a priori* knowledge. The ability to calculate z-scores across a variety of treatments, rather than just assessing differential expression relative to negative control samples, was made possible by the large-throughput scale of our method. In addition, treatment conditions yielding “transcriptionally weak” responses were removed from the analysis, defined as the differential expression between

169 compound treatment and vehicle treatment wherein no gene had an adjusted p-value that was more significant than
170 the minimum adjusted p-value found when comparing PA14 in water versus DMSO control (i.e. $p_{adj} < 10^{-20}$).
171
172 We then determined the Pearson distances ($\sqrt{(1 - Pearson\ Correlation)}$) of the transcriptional signatures
173 (represented as a z-score value for each gene) between adjacent timepoints over the 120-minute time trial (**Fig. 1a**).
174 The results demonstrated that the transcriptional responses to treatment by inhibitors of DNA synthesis, protein
175 synthesis and membrane integrity plateaued on transcriptional endpoints by 90 minutes. The transcriptional
176 responses to treatment with peptidoglycan synthesis inhibitors were more heterogenous as reflected in their larger
177 Pearson distances, which never fully plateaued even by 120 minutes. We wondered whether the increased
178 transcriptional variability among peptidoglycan synthesis inhibitors related to the inoculum effect, a phenomenon in
179 which antibiotics lose significant activity at high bacterial densities. **Table 1** denotes the antimicrobial compounds
180 exhibiting strong inoculum effects (≥ 4-fold shift in MIC at the high bacterial density) with an asterisk, which comprise
181 all of the peptidoglycan synthesis inhibitors, except imipenem, due to upregulation of the beta lactamase AmpC at
182 high bacterial density[23]. Interestingly, the Pearson distances for imipenem across the time trial also plateau by 90
183 minutes, comparable to inhibitors of protein synthesis, DNA synthesis and membrane integrity. Taken together, these
184 data suggest an incubation time of 90 minutes ensures a stable transcriptional endpoint for most antimicrobial
185 compounds active at the experimental inoculum.
186
187 After establishing an optimal 90-minute intubation time, we sought to determine a general, optimal antimicrobial
188 concentration that elicits a strong transcriptional response. Here we used an expanded set of 39 antimicrobial
189 compounds, shown in **Table 1**, targeting the same four core cellular processes but through more diverse targets. We
190 added several compounds with known anti-pseudomonal activity, but not yet approved for human use. For example,
191 we included cell envelope-related compounds, such as inhibitors of LpxC, LptD, BamA and MreB that disrupt
192 lipopolysaccharide (LPS) biosynthesis, outer membrane protein assembly, and cell shape, respectively. We also
193 included antimicrobial compounds that are not known to act on *P. aeruginosa*, such as the antibiotic nitrofurantoin,
194 which works through a complex mechanism involving reactive oxygen species that interfere non-specifically with
195 protein synthesis, DNA synthesis and cell wall metabolism[24]. We also added: (1) the anti-bacterial agent
196 fosmidomycin, which inhibits Dxr to block isoprenoid synthesis; (2) the Hsp90 inhibitor radicicol, an antifungal agent

197 that likely interferes with gyrase-related proteins with a conserved Bergerat fold[25-27]; (3) the anti-staphylococcal
198 agent fusidic acid, an inhibitor of the elongation factor FusA1 that blocks translation[28]; (4) the antibiotic rifampicin,
199 which blocks transcription through the inhibition of the DNA-dependent RNA polymerase RpoB[29]; (5) the fatty acid
200 synthesis inhibitor thiolactomycin; and (6) the membrane pore former LL-37. Lastly, we included several anti-cancer
201 agents, such as cisplatin, doxorubicin and hydroxyurea, which exhibit antimicrobial activity by inhibiting DNA synthesis
202 through DNA alkylation, deoxyribonucleic acid depletion and DNA intercalation, respectively [30-36].
203
204 In the gene expression dose response experiment, unlike the time trial, all antimicrobial compounds were dosed at an
205 adjusted concentration to account for their MICs at high inoculum (i.e., 1 x 10^8 CFU/mL), thereby correcting for the
206 inoculum effect. Whenever possible, antimicrobial reference compounds were tested at 4-fold, 2-fold and 0.5-fold their
207 MICs determined in broth microdilution experiments at high inoculum. However, by design, not all compounds in the
208 gene expression dose response experiment exhibited anti-pseudomonal activity, allowing us to analyze transcriptional
209 response relative to compound activity. For compounds that were non-lethal, either dosed below their MICs or
210 strongly affected by the inoculum effect, we performed gene expression at their highest possible concentrations based
211 on solubility.
212
213 In general, almost all compounds dosed above their MICs (determined at high inoculum) demonstrated robust
214 transcriptional responses (**Fig. 1b**). In contrast, antimicrobials that were non-lethal yielded variable transcriptional
215 responses. In some cases, such as the DNA synthesis inhibitors and membrane disruptors, robust gene expression
216 signals were generated despite sublethal dosing, suggesting that bacteria are primed to detect non-lethal
217 perturbations of certain cellular processes. In the case of some of the beta lactam antibiotics, such as piperacillin,
218 ceftazidime, and aztreonam, robust transcriptional responses could not be elicited due to compound insolubility at
219 dosing necessary to overcome the inoculum effect (**SI Appendix, Fig. S1**). In fact, aztreonam treatment yielded a
220 transcriptionally weak response across all treatment conditions, and thus it was removed from the reference set
221 construction moving forward. Taken together, these results demonstrate that antimicrobial activity at high
222 experimental inoculum (i.e. 1 x 10^8 CFU/mL) is necessary to ensure a robust transcriptional response to all antibiotics
223 in this large-throughput assay format, even while some compounds are still able to generate robust signals despite
224 sublethal dosing.

225 **Transcriptional responses to antimicrobial compounds with similar MOAs are correlated**

226 As expected, our large-throughput gene expression dataset confirmed that lethal antimicrobial treatments elicited
227 transcriptional responses that correlated by MOA. The Pearson correlations of pairwise combinations of all 90-minute
228 treatments, at the highest dose, are shown as a similarity matrix in **Fig. 1c**. Compounds with limited anti-pseudomonal
229 activity, comprising doxorubicin, radicicol, hydroxyurea, piperacillin, fusidic acid, and rifampicin, had positive
230 correlation values with vehicle treatment. Treatments that were more correlated to vehicle control than any other
231 treatment (including those of the same compound at different doses or time points) were removed from the reference
232 set to ensure that only reliable signals be used for MOA prediction. This requirement removed fusidic acid from the
233 reference set. Of the remaining antimicrobial compounds, Pearson correlations were strongest between compounds
234 sharing the same MOA. Notably, stronger correlations were also observed within antibiotic subclasses. For example,
235 protein synthesis inhibitors separated into subclasses based on the strength of their correlations, grouping together
236 macrolides (clarithromycin and erythromycin), tetracyclines (tetracycline, minocycline and doxycycline) and
237 aminoglycosides (amikacin and gentamicin), while distinguishing them from the other subclasses. Likewise,
238 fluoroquinolones (ciprofloxacin and levofloxacin) formed a clear subgroup within the larger class of DNA synthesis
239 inhibitors. Finally, the transcriptional responses to LptD inhibitors (POL-7001 and POL-7080) and LpxC inhibitors (PF-
240 5081090 and PF-04753299), which block LPS synthesis and transport to the cell surface, respectively, tightly
241 correlated within the group of membrane integrity inhibitors. Meanwhile, despite the resolution obtained for
242 subclasses, the transcriptional responses to inhibitors of membrane integrity and inhibitors of peptidoglycan synthesis
243 also correlated with one another, suggesting a shared cellular response to defects in membrane disruption and
244 peptidoglycan synthesis.

245

246 Lastly, to verify that the transcriptional treatment responses correlated with known biology, we determined the most
247 upregulated and downregulated genes in response to three of the most well-studied antibiotic classes:
248 aminoglycosides, fluoroquinolones and polymyxins. **Table S1** shows the top 25 upregulated and downregulated
249 genes with known annotations in PA14 in response to treatment with aminoglycosides, fluoroquinolone and
250 polymyxins. Indeed, previously reported gene expression signatures were readily apparent for each antibiotic class,
251 providing a cross-check that the PerSpecTM approach indeed highlights changes gene expression directly related to
252 each specific chemical perturbation.

253

254 **Transcriptional responses to treatment of hypersusceptible strains correlate with wildtype treatments**

255 The requirement that compounds have potent wildtype activity to elicit a reliable, robust transcriptional response
256 poses a barrier to the early integration of transcriptomics-based MOA prediction for new compounds found to have
257 some but not highly potent antimicrobial activity. To address this concern, we sought to determine whether
258 hypersusceptible strains could be used in place of wildtype strains to obtain robust transcriptional signals for
259 compounds with limited wildtype activity. While an efflux pump mutant could serve as a hypersusceptible strain in
260 some cases, not all compounds are guaranteed to have increased activity against efflux-deficient strains. We thus
261 selected an alternative strategy that involved engineering hypersensitive mutants by depleting their cognate targets,
262 leveraging the concept of gene dosage in yeast, and later demonstrated in bacteria[6, 37-39].

263

264 We genetically engineered seven hypomorphic strains by depleting the essential gene target corresponding to a set of
265 known antimicrobial agents. Targets with their corresponding on-target antimicrobial included *lptD*-POL7080, *dxr*-
266 fosmidomycin, *murA*-fosfomycin, *gyrA*-ciprofloxacin, *lpxC*-PF-04753299, *dhfR*-trimethoprim, and *bamA*-MRL-494.
267 Most strains were constructed by inserting the target gene at the neutral *attB*-Tn7 site under the control of an artificial
268 promoter (either a constitutive weak promoter or an arabinose-inducible promoter; **Table S2**), followed by deletion of
269 the native gene. In the case of the *lptD* hypomorph, the native promoter was replaced with an arabinose-inducible
270 promoter controlling the entire operon containing *lptD*. The growth phenotypes of most engineered hypomorphs were
271 comparable to wildtype, except for the *gyrA*, *dxr* and *lptD* hypomorphs (**SI Appendix**, **Fig. S2**). We then measured the
272 MICs of compounds for these hypomorphs. The *lptD*, *murA*, and *bamA* hypomorphs were 15 – 20-fold more
273 susceptible to their corresponding gene target inhibitors, POL7080, fosfomycin, and MRL-494, respectively, whereas
274 the *dxr*, *gyrA*, *lpxC* and *dhfR* showed no significant difference from wildtype (**Table S2**).

275

276 We then measured the expression profile of each hypomorph treated with vehicle, one on-target compound (*i.e.,*
277 compound whose activity is due to inhibition of the corresponding target) and two off-target compounds (*i.e.,*
278 compound whose activity is not related to the corresponding target). Hypomorphic strains were incubated with
279 compounds at 2-fold their MICs (determined at high inoculum against each hypomorph), or vehicle, at a final cell
280 density of ~1 x 10^8 CFU/mL for 90 minutes before chemical lysis, RNA extraction, and subsequent library

281 construction. Z-scores were calculated across each hypomorph, rather than across all hypomorphs, to dampen
282 background transcriptional changes related to strain-specific genetic perturbations from the repressed gene target.
283 **Fig. 2** shows a combined uniform manifold approximation and projection (UMAP) showing all 90-minute treatments at
284 the highest dose per strain across the gene expression time trial, dose response and hypomorph experiments. The
285 changes in gene expression of hypomorphs to chemical perturbation correlated with wildtype treatment responses.
286 For example, treatment of the g*yrA*-hypomorph with its on-target inhibitor, ciprofloxacin, grouped with PA14 treatment
287 with ciprofloxacin, as did treatment of the *lpxC*-hypomorph with PF-04753299, *bamA*-hypomorph with MRL-494, *dhfR*-
288 hypomorph with trimethoprim, *murA*-hypomorph with fosfomycin, and *lptD*-hypomorph with POL7080. Meanwhile,
289 using this mutant-specific z-score approach, grouping of mutant and wildtype responses was agnostic of whether a
290 compound's molecular target related to the underlying genetic alteration in a strain, as exemplified by the treatment
291 response of the *gyrA*-hypomorph to doxycycline, a protein synthesis inhibitor unrelated to GyrA, grouping together
292 with PA14 response to doxycycline.
293
294 The correlation between responses of hypersusceptible mutants and wildtype, after adjusting experimental treatment
295 concentrations and computationally accounting for strain-specific transcriptional differences, enables the
296 characterization of expression responses of compounds without wildtype activity. Indeed, the transcriptional response
297 of the engineered *dxr*-hypomorph to fosmidomycin provides proof-of-concept for the utility of using genetically
298 depleted strains to facilitate MOA prediction. Fosmidomycin inhibits Dxr, an essential reductoisomerase in the 2-C-
299 methyl-D-erythritol 4-phosphate (MEP) pathway of isoprenoid precursor biosynthesis [40]. While fosmidomycin
300 exhibited an MIC of 12.5uM at low inoculum, it was inactive against PA14 at the high inoculum used for expression
301 profiling experiments, but delayed growth of the *dxr*-hypomorph (**SI Appendix**, **Fig. S3**). While the expression
302 response of PA14 to fosmidomycin treatment grouped near vehicle controls, the response of the *dxr*-hypomorph
303 grouped with inhibitors of membrane integrity, accurately reflecting the cellular mechanism targeted by fosmidomycin
304 **(Fig. 2).**
305
306 **MOA and target prediction using the antimicrobial reference set**
307 We next set out to evaluate the potential usefulness of the antimicrobial reference set as a tool for predicting the
308 MOAs and targets of novel chemical entities. We first calculated Pearson correlation values between each query and

309 reference treatment, where a treatment refers to a combination of a compound, concentration, time point and strain.
310 After removing any query treatments that are most correlated to vehicle control, the maximal correlation values
311 between the query to each target category are then averaged across all query treatments of the same query
312 compound to produce the final similarity score. Ultimately, the predicted target is determined by the highest overall
313 correlation to the query compound, similar to methods described previously [14]. The predicted MOA of each query
314 compound is then assigned as the known cellular pathway associated with the predicted target.
315
316 To evaluate the performance of this prediction method, we performed a leave-one-out cross validation (**SI Appendix,**
317 **Fig. S4**), wherein the expression profiles for each antimicrobial compound in the reference set were queried against
318 the expression profiles of all other compounds in the reference set. The similarity score analysis predicted correct
319 MOAs for 34 of the 37 antimicrobial compounds (92%), and correct target assignments for 22 of the compounds
320 (60%). Of note, 10 compounds (27%) were the only representatives of their respective target categories, and thus the
321 leave-one-out cross validation had no chance of correct target prediction. Removing those ten singleton compounds
322 from the query, the correct MOA was assigned for 25 out of 27 compounds (94%), and the correct target predicted for
323 22 of the compounds (81%).
324
325 To assess confidence in MOA and target predictions, we next obtained positive predictive values (PPVs) for the
326 prediction of both MOA and target, respectively. The PPV is the percent of compounds in the leave-one-out cross
327 validation whose prediction was correct for a similarity score value above a given threshold. The singleton compounds
328 were included in the calculation of PPV for a more conservative estimate of confidence in target prediction, since they
329 act as proxies for targets not represented by the reference set. Of note, the similarity score distributions are different
330 for different MOAs, and thus may merit different levels of confidence depending on the predicted MOA (**Fig. S4b**).
331 However, due to the limited number of compounds per category, the PPVs were calculated across the entire data set
332 for both MOA and target.
333
334 **External validation of the antimicrobial reference set**
335 We next queried the largest publicly available gene expression dataset in *P. aeruginosa[17]* against our antimicrobial
336 reference set to test whether this MOA prediction approach remained valid despite major differences in experimental

337 design. Indeed, the experimental design of the query gene response dataset was highly divergent from the methods
338 employed in our study. The study aimed to elucidate intrinsic mechanisms controlling antimicrobial resistance, and
339 gene expression experiments comprised duplicate treatments with fourteen antimicrobials (both antibiotics and
340 antiseptics) at sublethal dosing, comprising one fluoroquinolone (ciprofloxacin), three aminoglycosides (gentamicin,
341 neomycin and tobramycin), four peptidoglycan synthesis inhibitors (ampicillin, carbenicillin, cefoperazone and
342 aztreonam), one membrane disruptor (polymyxin B), and five antiseptics (benzalkonium chloride, peroxide, bleach,
343 silver nitrate and povidone-iodine). While the size and chemical diversity of the query dataset allowed us to represent
344 gene expression changes as z-scores, weak transcriptional responses from the sublethal dosing limited the
345 PerSpecTM approach. **Table 2** summarizes the MOA and target predictions of only the chemical treatments having
346 robust transcriptional responses. Of note, the transcriptional response to treatment with tobramycin, ampicillin,
347 carbenicillin, cefoperazone, aztreonam, povidone-iodine, and silver nitrate and were transcriptionally weak, and thus
348 excluded from the analysis. Neomycin also had a transcriptionally weak response but had a meaningful signal and is
349 included in the table. Meanwhile, benzalkonium chloride was most highly correlated to vehicle control and was
350 excluded from analysis.
351
352 Interestingly, the MOA and target were predicted correctly for most of the transcriptionally robust samples (top
353 predictions shown in **Table 2**; full data shown in **SI Appendix**, **Fig. S5**): gentamycin and neomycin were assigned as
354 protein synthesis inhibitors with the 30S ribosomal subunit as the predicted target, and ciprofloxacin was assigned as
355 a DNA synthesis inhibitor with GyrA/ParC as the predicted target. In the case of polymyxin B, the highest similarity
356 score implicated a membrane integrity disruptor with LptD as the potential target, but the similarity score was below
357 that of any compound in the leave-one-out cross validation, and thus could not be assigned a prediction with a known
358 confidence level. Although LptD had the highest similarity score of 0.26, LPS, the known target, was not far behind
359 with a similarity score of 0.23 (**SI Appendix**, **Fig. S5**). Interestingly, of the antiseptics, peroxide was assigned as a
360 DNA synthesis inhibitor with GyrA/ParC as the predicted target, although DNA was not far behind (**SI Appendix**, **Fig.
361 S5**). This finding is supported by the fact that peroxide has been shown to induce oxidative damage and cause
362 double-stranded DNA breaks[41], similar to cisplatin and fluoroquinolones. Meanwhile, bleach was predicted to be a
363 DNA synthesis inhibitor based on highest similarity to nitrofurantoin, a known inducer of ROS production in bacteria.
364 Interestingly, bleach also correlated to many of the inhibitors of membrane integrity, consistent with bleach imparting

generalized oxidative damage on both DNA and membranes[42]. These results suggest that external datasets, particularly those of sufficient size and diversity to permit z-score calculations, are amenable to target prediction using the PerSpecTM approach with our antimicrobial reference set.

Nevertheless, not all external datasets are of sufficient size and diversity to allow z-score calculations. To better validate our antimicrobial reference set using more limited external data, we queried a smaller external dataset[43] against our antimicrobial reference set, which included PA14 treated with DNA inhibitors (ciprofloxacin, levofloxacin, lomefloxacin, novobiocin or DMSO control). Once again, the experimental design of the query dataset diverged highly from our methods: bacteria were exposed to each compound in biological triplicate at sublethal dosing and grown overnight from an optical density of 0.05 to a final density of 0.6 during the exposure. The small size and limited diversity of compound MOAs across this external dataset precluded the use of z-scores across the batch of treatments. Instead, we compared perturbed expressional changes to negative controls by calculating z-scores relative to negative controls, which we termed nz-scores. As summarized in **Table 2** (full data shown in **SI Appendix**, **Fig. S6a**), lomefloxacin was accurately assigned as a DNA synthesis inhibitor with GyrA/ParC as the predicted target based on a similarity score of 0.30 (62% target PPV, 94% MOA PPV). Of note, PPVs for this external dataset were determined by performing the leave-one-out cross validation using nz-scores queried against the z-scores of the reference set. Likewise, novobiocin from this external dataset was assigned as a DNA synthesis inhibitor with GyrB as the predicted target based on a similarity score of 0.26 (58% target PPV, 92% MOA PPV) **(Table 2;** full data shown in **SI Appendix**, **Fig. S6b**). Despite the highly divergent gene expression methodology, these correlation analyses accurately yield accurate MOA and target predictions using our approach. Taken together, these results provide proof-of-concept that external gene expression data can be queried against our reference set to yield helpful mechanistic predictions, and we suspect the confidence of these predictions will grow stronger if care is taken to better reproduce our experimental methods at lethal compound dosing, thereby ensuring robust transcriptional responses from which perturbation-specific gene expression changes can be readily identified with the PerSpecTM method.

393 **MOA and target determination of new screening hits using the antimicrobial reference set**

394 We next sought to test whether PerSpecTM could predict MOAs of new molecules. To this end, we focused on three
395 small molecules we had previously identified using a multiplexed chemical genetic based strategy termed
396 miniPROSPECT[6, 44]. All three compounds were discovered based on their specific activity against certain
397 hypomorphic strains: PA-0918 against *lptD*-hypomorph; PA-69180 against *oprL*-hypomorph; and PA-5750 against
398 *lptE*-hypomorph (**Table 3).** In the case of PA-0918 and PA-5750, we performed expression profiling using PA14
399 because both compounds were active against wildtype. PA-69180 had no wildtype activity, however, and therefore we
400 performed gene expression profiling with the *oprL*-hypomorph, which encodes for the reduced expression of the
401 essential cell wall scaffolding protein OprL.

402

403 PA-0918 was assigned as an inhibitor of membrane integrity with LPS as the predicted target based on a similarity
404 score of 0.74 (100% target PPV, 100% MOA PPV) (**Table 2**; full data shown in **SI Appendix**, **Fig. S7a**). PA-0918
405 most highly correlated with colistin, a polymyxin antibiotic known to target LPS along the outer membrane of Gram-
406 negative bacteria [45-47]. To test whether PA-0918 shares a similar mechanism-of-action with colistin as a direct
407 membrane disruptor, we utilized an ethidium bromide uptake assay, in which membrane disruption is detected by
408 increased flux of ethidium bromide into the cells. We found that PA-0918 phenocopies colistin's ability to promote
409 rapid uptake of ethidium bromide into cells (**SI Appendix**, **Fig. 8**), consistent with PA-0918 disrupting the outer
410 membrane to increase permeability.

411

412 Using the *oprL*-hypomorph, PA-69180 was predicted as a peptidoglycan synthesis inhibitor with MreB, the major Rod
413 protein responsible for the rod shape of gram-negative bacteria, as the predicted target based on a similarity score of
414 0.38 (68% target PPV, 94% MOA PPV) (**Table 2**; full data shown in **SI Appendix**, **Fig. S7b**). To test whether PA-
415 69180 could be an MreB inhibitor, we used a mecillinam rescue assay described previously [48], which leverages the
416 fact that overexpression of the cell-division protein FtsZ in *E. coli* (strain MG1655/FtsZup) renders the Rod system
417 non-essential, thus conferring resistance to MreB inhibition. Meanwhile, MreB inhibition in this strain reverses the
418 lethal effects of the penicillin binding protein 2 inhibitor mecillinam, which otherwise causes a toxic malfunctioning of
419 the Rod machinery leading to cell death. We found that PA-69180 phenocopies the known MreB inhibitor A22, with
420 MG1655/FtsZup being both resistant to PA-69180 killing and resistant to mecillinam killing upon the addition of PA-

421 69180 (**Table S3**). We also generated resistant mutants to PA-69180 in the efflux-deficient strain PAO397 and
422 performed whole-genome sequencing to reveal single nucleotide polymorphisms (SNPs) driving point mutations in
423 MreB that conferred resistance (**Table S4**). Two mutants, PAO397-r1 and PAO397-r3, had mutations at distinct amino
424 acid positions adjacent to the ATP binding pocket of MreB that conferred high-level resistance to PA-69180 and
425 cross-resistance to A22 based on 32–64-fold shifts in MIC relative to the parent PAO397 strain, thereby confirming
426 MreB as the target (**Table S5**).
427
428 Finally, PA-5750 was assigned as a DNA synthesis inhibitor with GyrA/ParC as the predicted target based on a
429 similarity score of 0.56 (88% target PPV, 96% MOA PPV) (**Table 2**; full data shown in **SI Appendix**, **Fig. S7c**). Based
430 on this prediction, we immediately moved to testing PA-5750's ability to both intercalate DNA or inhibit DNA gyrase.
431 To probe for DNA intercalation, we utilized the human topoisomerase I DNA unwinding assay, which identifies
432 molecules that intercalate relaxed DNA, thereby altering the linking number to stimulate supercoiling activity of
433 topoisomerase I. There was no evidence of DNA intercalation by PA-5750, or the negative control ciprofloxacin,
434 based on the absence of supercoiled DNA, in contrast to the known DNA intercalator m-amsacrine (m-AMSA) (**SI**
435 **Appendix**, **Fig. S9a**). In a complementary assay, in which DNA intercalators displace ethidium bromide from purified
436 plasmid, we showed that m-AMSA exhibited a dose-dependent displacement of ethidium bromide from purified
437 plasmid, whereas PA-5750 and ciprofloxacin did not substantially displace ethidium bromide (**SI Appendix**, **Fig. S9b**).
438 Having ruled out PA-5750 as a DNA intercalator, we next tested whether PA-5750 inhibited *E. coli* DNA gyrase's
439 ability to supercoil DNA. Indeed, we found that PA-5750 reduced the amount of supercoiled plasmid generated by
440 DNA gyrase in a manner similar to known gyrase inhibitors ciprofloxacin and novobiocin (**SI Appendix**, **Fig. S9c**),
441 while the R enantiomer of PA-5750, which has no antibacterial activity, showed no gyrase inhibition. Lastly, based on
442 the observation that gyrase overexpression leads to ciprofloxacin hypersusceptibility through increased double-
443 stranded DNA breaks[49], we sought to test the impact of gyrase overexpression on PA-5750 activity. We engineered
444 a PAO1 strain that overexpressed both *gyrA* and *gyrB* episomally under the control of rhamnose. Indeed,
445 overexpression of gyrase led to an increase in susceptibility to PA-5750 (**SI Appendix**, **Fig. S9d**). Taken together,
446 these results support the prediction of PA-5750 as targeting gyrase, and overall, validate the ability of this expression-
447 based approach to MOA predictions.
448

449 **Assessing the transcriptional signatures of hypomorphic strains**

450 With the well-validated reference set in hand, we sought to test whether the transcriptional signals of hypomorphic
451 strains correlate with chemical inhibition of their gene products. This is an important question to ascertain because our
452 transcriptomics-based MOA prediction strategy relies on a reference set built exclusively of compounds that
453 chemically inhibit gene products in essential cellular pathways. However, there are other attractive gene targets
454 without known inhibitors, which would show up as “blind spots” in MOA prediction analyses. If the transcriptional
455 repression of a gene product phenocopies its chemical inhibition, then using the transcriptional responses of
456 hypomorphic strains could be a powerful approach for predicting the transcriptional profile of gene targets without
457 known inhibitors. If feasible, a comprehensive set of hypomorphic strains could in theory provide the blueprint for
458 every targetable pathway, facilitating a more complete reference set for MOA prediction of screening hits.

459

460 To this end, we reanalyzed the hypomorph gene expression data to investigate whether background transcriptional
461 response in hypomorphic strains phenocopied wildtype treatment with on-target control compound. In this new
462 analysis, z-score calculations were instead carried out across all genetic mutants, rather than within each strain, to
463 include strain-specific transcriptional differences in our analyses. Pearson correlations were stronger between genetic
464 mutants and untreated PA14 rather than between the genetic mutants and PA14 treated with on-target control
465 compounds (**SI Appendix**, **Fig. S10**), indicating that transcriptional signatures of hypomorphic strains could not
466 reliably predict the transcriptional profile of chemical inhibition of the target gene of interest.

467

468 **Assessing the transcriptional signatures of CRISPRi strains**

469 We wondered whether the transcriptional responses of genetic hypomorphs were dampened due to the lack of acute
470 perturbation in these systems. When hypomorphic strains were controlled by weakened promoters, we postulated that
471 transcriptional changes might to dissipate over time as cells adapt gradually to restrictive gene expression. We
472 therefore shifted to a genetic system designed to induce gene repression more rapidly to better mimic the effect of
473 chemical inhibition by small molecules. We specifically turned to CRISPRi technology to create an inducible gene
474 repression system, in which arabinose could be added to downregulate gene expression at the transcriptional level in
475 a dose-dependent manner. This new approach would allow stable cell growth in the absence of arabinose, followed
476 by more sudden perturbation through the transcriptional repression of target genes by arabinose exposure at the very

477 beginning of the gene expression experiments. We constructed eighteen CRISPRi strains encoding arabinose-
478 induced dCas9 at the neutral *attB*-Tn7 site alongside constitutively expressing guide RNAs targeting no gene (so-
479 called CRISPRi-*C26*), *dhfR*, *dxr*, *gyrA*, *gyrB*, *leuS*, *lptA*, *lptD*, *lptE*, *lpxC*, *mreB*, *murA*, *parC*, *pbpA*, *ssb*, *rplJ*, *rpoB* and
480 *rpsL*, summarized in **Table S6**. In each CRISPRi strain, the addition of arabinose drove dCas9 expression and
481 subsequent transcriptional repression of the target gene of interest. We characterized the arabinose-dependent
482 growth phenotypes of each CRISPRi strain (**SI Appendix**, **Fig. S11**). The CRISPRi-C26 control strain showed no
483 significant growth delay over an arabinose range of 0 – 0.5% (w/v), indicating no appreciable dCas9-mediated toxicity
484 in PA14. CRISPRi-*dhfR*, CRISPRi-*lptA*, CRISPRi-*lpxC*, CRISPRi-*mreB*, CRISPRi-*murA* and CRISPRi-*parC* all
485 demonstrated severe arabinose dose-dependent growth delays, whereas CRISPRi-*dxr*, CRISPRi-*gyrA*, CRISPRi-
486 *gyrA-2,* CRISPRi-*gyrB*, CRISPRi-*leuS*, CRISPRi-*lptD*, CRISPRi-*lptE*, CRISPRi-*rplJ*, CRISPRi-*rpoB* and CRISPRi-*rpsL*
487 exhibited more moderate growth delays at higher arabinose concentrations. By comparison, CRISPRi-*pbpA* and
488 CRISPRi-*ssb* showed no significant growth delay.
489
490 We next performed high-throughput gene expression profiling of all CRISPRi strains at 360 minutes in 0%, 0.125%,
491 and 0.5% arabinose before chemical lysis, RNA extraction, and subsequent library construction. Z-scores were
492 calculated across all strains and arabinose doses, thereby accentuating strain-specific transcriptional changes. As
493 summarized in **Table S7**, CRISPRi strains in general demonstrated an arabinose-dependent decrease in target
494 mRNA levels at 360 minutes compared to the CRISPRi-*C26* control strain, ranging from 1.5 – 174-fold in 0.125% or
495 0.5% (w/v) arabinose for conditions yielding p-values below 0.05. Notably, gene expression changes were not
496 statistically significant across both arabinose conditions (i.e. 0.5% and 0.125%) for CRISPRi-*lptE*, and CRISPRi-*ssb*
497 and CRISPRi-*rpoB*.
498
499 Next, we assessed whether the transcriptional profiles of the CRISPRi strains could potentially serve as a reference
500 set from which we could predict targets of unknown compounds. We utilized the same MOA prediction method as in
501 the previous sections, using z-scores of the CRISPRi strains as the reference set (across both doses of arabinose
502 above 0% at 360 minutes), and queried the transcriptional responses of 90-minute antibiotic treatments, comprising
503 an on-target agent for each CRISPRi strain. All compound treatments were against wildtype PA14, except for
504 fosmidomycin, which was used against the *dxr*-hypomorph strain since it was not active against PA14. We again

505 removed treatment conditions yielding “transcriptionally weak” responses, defined as the differential expression
506 between arabinose treatment and control guide C26 wherein no gene had an adjusted p-value that was more
507 significant than the minimum adjusted p-value of PA14 in water versus DMSO control (i.e. $p_{adj} < 10^{-20}$). Applying this
508 filter, CRISPRi-*dhfR*, CRISPRi-*gyrA-2*, CRISPRi-*leuS*, and CRISPRi-*lptE* were removed from analysis on account of
509 their weak transcriptional responses.
510
511 As shown in **Fig. 3**, the transcriptional responses of antibiotic treatments generally correlated to transcriptional
512 responses of their associated CRISPRi strains. These results demonstrate accurate target predictions in many cases.
513 For example, ciprofloxacin treatment correlated strongest with CRISPRi-*parC,* followed by CRISPRi-*gyrA*, accurately
514 mapping to its known target of GyrA/ParC. Similarly, the GyrB inhibitor novobiocin correlated strongest to CRISPRi-
515 *gyrB*; the LpxC inhibitor PF-04753299 mapped to CRISPRi-*lpxC*, the LPS transport inhibitor POL7080 mapped to
516 CRISPRi-*lptA*, the MurA inhibitor mapped to CRISPRi-*murA*, and the aminoglycoside amikacin mapped to CRISPRi-
517 *rpsL*. In cases of incorrect target prediction, the MOAs of the query compounds were still accurately predicted for all
518 but two of the compounds. For example, all protein synthesis inhibitors (amikacin, clarithromycin, and rifampicin)
519 correlated strongest with the three CRISPRi strains designed to interfere with protein synthesis, CRISPRi-*rpsL*,
520 CRISPRi-*rplJ* and CRISPRi-*rpoB*. Similarly, fosmidomycin was correctly predicted to disrupt membrane integrity. Of
521 the two compounds with misassigned MOAs, the peptidoglycan synthesis inhibitors A22 and imipenem, both were
522 predicted to be membrane disruptors and still correlated to a lesser extent to CRISPRi strains disrupting
523 peptidoglycan synthesis. Moreover, inhibitors of membrane integrity and peptidoglycan synthesis tended to correlate
524 across mechanistic classes in general, suggesting a shared transcriptional response to disruption of in either cellular
525 process. We wonder whether this observation may relate to the recent discovery that MurA stimulates LpxC through
526 direct biochemical interaction[50], linking the regulation of peptidoglycan biosynthesis with that of membrane integrity.
527 Taken together, these data demonstrate that the transcriptional response of chemical inhibition of a given gene target
528 correlates with CRISPRi-induced repression in gene expression, providing proof-of-concept that CRISPRi strains
529 could be integrated in chemical screening pipelines to facilitate MOA prediction of gene targets without available
530 chemical inhibitors.
531
532

533 **Discussion**

534 Target identification is central to any antibiotic discovery pipeline by building confidence in hits and guiding medicinal
535 chemistry efforts towards chemical optimization. The advancement of more sophisticated approaches to antibiotic
536 discovery has greatly expanded the chemical screening space and increased the number of primary hits discovered
537 from target-based screens in whole cells. While exciting, these novel platforms further exacerbate the bottleneck that
538 target identification already imposes on antibiotic development efforts. Moreover, the risk of rediscovering similar
539 compounds across screens, particularly those hindered by toxicities or resistance vulnerabilities, could be mitigated
540 by integrating MOA prediction tools earlier in screening pipelines to better prioritize hits.

541

542 Transcriptional-based approaches for target identification are particularly beneficial because they concurrently yield
543 valuable insights into the biology of how target disruption leads to killing. Other groups have long-established
544 expression profiling as a valuable tool for MOA prediction in bacterial pathogens[11-16]. To date, however,
545 background changes in gene expression – those relating to growth, general stress, or cell death – drown out
546 perturbation-specific gene responses, necessitating experimental designs that control for background gene
547 expression and limit the throughput and broad applicability of this approach. The novelty of PerSpecTM lies in its
548 scale: low sample volumes in 384-well format afford an unprecedented diversity of chemical or transcriptional
549 perturbations within a single experimental batch. Paired with the calculation of z-scores across this diverse sample
550 set, PerSpecTM highlights perturbation-specific changes in gene expression that drive accurate MOA prediction
551 without the need for complex methods or *a priori* knowledge to select subsets of genes, or rigorous experimental
552 control of non-specific changes in background gene expression. Importantly, antimicrobial activity was necessary to
553 ensure a robust transcriptional response to chemical treatments, which poses a barrier for MOA prediction of new
554 compounds that lack wildtype activity. While an efflux pump mutant could serve as a hypersusceptible strain for some
555 compounds, not all compounds exhibit increased activity in such strains. Thus, we engineered hypomorphic strains
556 that are hypersensitized to inhibitors by depleting their cognate targets. Importantly, for compounds lacking wildtype
557 activity, we showed that hypomorphic strains could be incorporated into PerSpecTM to facilitate MOA prediction
558 because antimicrobial compounds grouped by MOA agnostic of genetic strain. We subsequently validated our
559 antimicrobial reference set with accurate MOA prediction of both internal and external datasets.

560

561 Our three internal screening hits – PA-0918, PA-69180 and PA-5750 – highlight the power of MOA determination
562 early in the antibiotic discovery process. All three compounds originated directly from chemical libraries, and therefore
563 had not yet undergone extensive structure-activity-relationship studies. We rapidly identified PA-0918 as a membrane
564 disruptor, PA-69180 as an MreB inhibitor, and PA-5750 as an inhibitor of DNA gyrase. The case of PA-69180, which
565 lacked significant wildtype activity, highlights the advantage of leveraging the *oprL*-hypomorph to generate a robust
566 transcriptional signal. The case of PA-5750, which was discovered based on its activity against the *lptE*-hypomorph,
567 adeptly highlights the advantage of integrating MOA prediction tools early in the discovery process. Based on its
568 activity against the *lptE*-hypomorph, which has a deficit in the LPS transport system, we expected PA-5750 to act on a
569 cellular pathway relating to LPS biosynthesis, transport, or membrane biology in general. However, based on the
570 results of PerSpecTM, we instead identified PA-5750 as a gyrase inhibitor, which ultimately guided us away from
571 efforts towards chemical optimization at such an early stage after its discovery. Early target elucidation of PA-5750
572 also demonstrates how PerSpecTM can help address the major liability that antimicrobial activity against hypomorphs
573 might relate to changes in membrane integrity that enhance xenobiotic uptake, rather than directly to the repressed
574 gene target. Taken together, these data support the earlier integration of genome-scale transcriptional profiling in
575 antibiotic discovery pipelines to facilitate MOA prediction of chemical screening hits, which can help guide hit
576 prioritization ahead of chemical optimization and eventual the hit-to-lead transition.
577
578 Referenced-based MOA prediction by expression profiling is inherently limited by the diversity of the reference set,
579 which poses a barrier to discovering inhibitors of novel genetic pathways without known chemical inhibitors. We
580 therefore explored an orthologous approach by constructing a transcriptionally repressed reference set using
581 CRISPRi technology. We demonstrated that chemical inhibition of a series of gene targets correlated strongest with
582 their corresponding CRISPRi knockdown strains. Importantly, our results suggest that the least fit CRISPRi strains,
583 such as CRISPRi-*parC*, CRISPRi-*lptA*, yielded the strongest Pearson correlations to chemical inhibition of their
584 corresponding gene targets. Thus, any CRISPRi-based MOA prediction approach will rely on the fitness level across
585 the CRISPRi strains in the reference set. In our case, we constructed a small set of CRISPRi strains without
586 significant focus on the fitness level of each strain. In future iterations of this approach, we believe first employing a
587 chemical-genetic platform to quantify bacterial fitness of a larger library of CRISPRi strains, as reported previously in
588 *M. tuberculosis*[51], would yield a stronger CRISPRi reference set and generate more robust correlations for MOA

predictions. Nevertheless, these data provide proof-of-concept that a dedicated CRISPRi reference set could facilitate accurate MOA prediction, including for gene targets without known chemical inhibitors. In theory, a library of CRISPRi strains could be used to define the transcriptional map of all essential genes of a certain bacterium, serving as a reference set for screening hits with unelucidated targets, or new targets without known small molecule inhibitors.

Despite the upside to transcriptomic-based MOA prediction platforms, certain downsides warrant consideration. First and foremost, a significant amount of time, effort and funding is necessary to build a reference set for a given bacterial pathogen. While we show the reference set for *P. aeruginosa* is useful for MOA prediction in other pseudomonas strains, new reference sets will need to be built from the ground up for MOA prediction platforms in other important bacteria, such as *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and other high-priority Gram-negative bacterial pathogens. Moreover, transcriptional sequencing still requires a level of knowledge, experience and access that prevents data sharing across groups of scientists. In addition, transcriptional profiling at scale in 384-well format adds further cost to an already expensive endeavor, especially because PerSpecTM requires a diverse set of samples within any plate for z-scores to highlight perturbation-specific changes in gene expression. At a minimum, this would require a query compound to be tested along with vehicle control and at least three other compounds, each one representing a different MOA (DNA synthesis, Protein Synthesis, and either Peptidoglycan Synthesis or Membrane Integrity), as we demonstrated with the hypomorph data. For data sets with low diversity in represented MOAs, we employed nz-scores to validate our chemical reference set against external data, which requires only vehicle control as the comparator. Though not as robust as z-scores, we think this approach will make our data more accessible to other groups, who could use nz-scores and query unknown compounds, alongside a vehicle control, if they are unable to include additional diverse antibiotics. By sharing our PerSpecTM approach, and associated reference sets, we hope to lower this barrier by allowing other groups to integrate their own gene expression data into our pipeline.

Lastly, while gene expression profiling relies on transcriptional responses across a reference set of chemical perturbations, other genomic-based strategies are equally valid as MOA prediction that also provide insights into chemical-genetic interactions. Strategies aimed at modulating gene target expression can impact the sensitivity to small molecule inhibitors, as overexpressed and underexpressed targets will decrease and increase sensitivity, respectively [39, 49]. Indeed, transposon libraries were used to combine overexpression and underexpression

617 approaches in a single system, in which an outward-facing promoter within the transposon drove, suppressed or
618 abrogated gene expression [52]. In an approach called phenomic profiling, gene deletion libraries or transposon
619 libraries have been deployed to facilitate MOA prediction by mapping gene interactions in response to chemical
620 treatments, allowing for network mapping predicting MOA [17, 53, 54]. As we look to the future, CRISPRi libraries
621 could be used to generate the transcriptional responses to knockdown any essential gene product, even those without
622 a known chemical inhibitor, thereby creating a more expansive transcriptional landscape that could serve as a
623 reference for future antibiotic discovery initiatives.
624

625 **Materials and Methods**

626 **Bacterial strain construction**

627 All *P. aeruginosa* strains used for gene expression experiments were derived from the UCBPP-PA14 lab strain as
628 described previously[55]. The DNA gyrase overexpression strain was derived from PAO1[56]. **Table S8**, **Table S9**
629 and **Table S10** describe all plasmids, strains and primers used in this study. Detailed methods describing all strain
630 construction are available in the **Additional Methods** of **SI Appendix**.

631

632 **MIC determination and growth kinetics**

633 All MIC and growth kinetic experiments were conducted in biological triplicate in 384-well microplates (Thermo Fisher
634 Scientific, Waltham MA) using the broth microdilution method previously described[22]. All arabinose-inducible
635 hypomorphic strains were grown initially in the presence of 0.25% (v/v) arabinose in LB, and subsequently sub-
636 cultured without arabinose. Growth curves of hypomorphic strains were performed without arabinose by back-diluting
637 mid-log cultures to $5x10^5$ CFU/mL bacteria (OD_{600} 1.0 = 10^9 CFU/mL based on plate counts). Growth curves for all
638 CRISPRi strains were performed at various doses of arabinose, the inducer of dCas9. For MIC assays at high
639 inoculum, mid-log bacterial cultures were diluted to 1 x 10^8 CFU/mL bacteria (OD_{600} 1.0 = 10^9 CFU/mL based on plate
640 counts). Antimicrobial compounds were serially diluted in either DMSO or water to a final concentration of 0.5% (v/v).
641 For MIC experiments, microplates were incubated in a humidity chamber without shaking for 16 hours at 37°C, after
642 which time the OD_{600} was measured in the Spark Multimode Reader (Tecan, Männedorf Switzerland). For growth
643 kinetics, Microplates were incubated in a large humidity cassette in the Spark Multimode Reader (Tecan, Männedorf
644 Switzerland) for 24 hours at 37°C, and OD_{600} measurements were recorded after 30-seconds of shaking every hour.
645 Data were plotted and analyzed using GraphPad Prism10 (GraphPad Software, Boston MA) to determine growth
646 kinetic curves or MICs, the latter defined as the minimal compound concentrations required for complete inhibition of
647 bacterial growth.

648

649 **Gene expression experiments, sequencing, and analysis**

650 Gene expression experiments were carried out in biological triplicate in 384-well microplates (Thermo Fisher
651 Scientific, Waltham MA). Bacterial cultures were grown in LB with shaking (37°C, 250rpm) to mid-log phase.
652 Meanwhile, stock solutions of compounds were dissolved fresh in DMSO or water, and 30μL of compound solutions

653 were mixed with 30μL of mid-log culture to yield $1x10^8$ CFU/mL bacteria and compounds in 0.5% (v/v) DMSO or water
654 in LB. Cells were treated at 37°C without shaking in a humidity chamber. In the case of the gene expression time
655 trial, cells were treated for 30, 60, 90, and 120 minutes. In the case of the CRISPRi strains, cells were treated for 360
656 minutes. In all other experiments, cells were treated for 90 minutes. At the end of the incubation period, samples were
657 then mixed with 30μL of 3X RNAgem Blue Buffer (Zygem, Charlottesville VA), chemically lysed by incubation in a
658 thermocycler at 75°C for 10 minutes, flash frozen and stored at -80°C.
659

660 **Generation of gene expression libraries**

661 Total RNA was extracted using the Direct-zol kit (Zymo Research, Irvin CA), and RNA quality and quantity were
662 analyzed using the RNA ScreenTape with the 2200 TapeStation (Agilent, Santa Clara CA). Illumina cDNA libraries
663 were generated using a modified version of the RNAtag-seq protocol[57]. Briefly, 250ng of extracted RNA was
664 fragmented, dephosphorylated, and ligated to DNA adapters carrying 5'-AN_8-3' barcodes of known sequence with a 5'
665 phosphate and a 3' blocking group. Barcoded RNAs were pooled and depleted of rRNA using the Pan-Bacteria
666 riboPOOL depletion kit (siTOOLs Biotech, Galen Laboratories). Pools of barcoded RNAs were converted to Illumina
667 cDNA libraries in 2 main steps: (i) reverse transcription of the RNA using a primer designed to the constant region of
668 the barcoded adaptor with addition of an adapter to the 3' end of the cDNA by template switching using SMARTScribe
669 (Clontech) as described[58]; (ii) PCR amplification using primers whose 5' ends target the constant regions of the 3' or
670 5' adaptors and whose 3' ends contain the full Illumina P5 or P7 sequences. cDNA libraries were sequenced on the
671 Illumina [NovaSeq SP 100] platform to generate paired end reads.
672

673 **Computational analysis of gene expression profiling**

674 *Processing raw reads*

675 Sequencing reads from each sample in a pool were demultiplexed based on their associated barcode sequence using
676 custom scripts, allowing up to 1 mismatch in the barcode. Barcode sequences were removed from the first read as
677 were terminal G's from the second read that may have been added by SMARTScribe during template
678 switching. Sequencing reads were aligned to the UCBPP-PA14 (NC_0088463.1) reference sequence using BWA[59],
679 assigned genomic features using custom scripts, and visualized using GenomeView[60]. Samples that had low read

680 counts, where over 80% of the genes had zero counts, were filtered out of the data set. In addition, any genes that
681 had zero counts in 50% or more of the samples in a sequencing batch were removed.
682
683 *Assessing robustness of transcriptional response*
684 To assess the level of differential gene expression induced by each treatment condition, DESeq2[61] was used to
685 calculate the $\log_2$ fold change and adjusted p-values (with target false discovery rate, or FDR, set to 0.05) for each
686 gene relative to control. However, even small changes to the environment can lead to changes in gene expression
687 that may reach statistical significance, although not biologically interesting. We used the comparison of PA14 gene
688 expression between DMSO and water as a baseline for setting a threshold to separate "weak" from "robust"
689 responses. Specifically, for a treatment condition to be considered as having a robust transcriptional response, we
690 require that at least one gene be different from the negative control with an adjusted p-value that is smaller than 10^{-20}.
691 Such robust conditions were included in the reference set, while weak ones were excluded. Although some weaker
692 signals may reflect meaningful biological changes, we wanted to only include robust signals in the reference set to
693 predict mechanisms of unknown compounds more reliably. To further ensure that only reliable transcriptional
694 signatures were used to predict MOA, we additionally removed any treatment conditions whose transcriptional
695 response correlated more highly to negative control conditions than any other condition in the data set, including other
696 time points or doses of the same compound.
697
698 *Calculation of moderated z-scores and nz-scores*
699 Read counts were transformed using the variance stabilizing transformation from the DESeq2 package (v1.28.1) in R
700 (4.0.0)[61]. Then, to identify gene expression changes of interest, moderated z-scores were calculated as described
701 previously[21]. More specifically, robust z-scores (using medians and median absolute deviations) for each gene were
702 calculated per batch, where each batch is defined as all the treatments on the same 384-well plate that share a strain
703 and time point. In cases where there was not a diverse set of mechanisms represented by the compounds in the
704 batch, expression levels were standardized using the median and median absolute deviations of only the negative
705 control samples from each batch, rather than all the treatments in the batch. We termed these values "nz-scores" (z-
706 scores relative to negative controls). The data sets that used nz-scores in this manuscript were the external data set
707 of DNA synthesis inhibitors [43] and the screening hit PA-69180 that was used to treat the oprL-hypomorph. All other

708 data involved a diverse set of compounds within each batch, such that z-scores could be calculated. Next, replicate
709 data were combined using a weighted average, where the weights were determined by the Spearman correlation
710 between replicate transcriptional profiles, as described previously[21]. Finally, extreme z-score values were clipped
711 such that fewer than 1% of the values were affected. For the reference set, z-score values lower than -10 were set to -
712 10, and those higher than 10 were set to 10, so that extreme values would not spuriously impact downstream
713 correlation values. After calculation of all z-score values, transcriptional data were visualized using a UMAP[62],
714 implemented by the R package umap (v0.2.10.0).
715
716 *Selection of marker genes to validate transcriptional responses with known biology*
717 Within each antibiotic class, the z-score gene responses to 90-minute compound treatment were sorted for strength
718 (i.e. absolute value of z-score) and consistency (i.e. same sign of z-score across all samples). If the z-scores for a
719 given gene had disparate signs within the antibiotic class, that gene was removed from analysis for that class. Top up-
720 and down-regulated genes were assessed for validation with known biology of the targets.
721
722 *Prediction of compound targets and leave-one-out cross validation*
723 The reference set of transcriptional profiles was used to predict the targets of unknown compounds by using Pearson
724 correlation values. Depending on the composition of each query data set, z-score or nz-scores were calculated.
725 Individual query compound treatments (e.g. multiple doses or time points) were correlated to the reference set z-score
726 values. A similarity score was then generated between the query compound and each target category using all
727 available treatments of the query compound, and all available treatments of the target category in the reference set.
728 For each query treatment, the maximal Pearson correlation to all the treatments in the given target category was
729 recorded. Any query treatment that was most highly correlated to vehicle control was removed. Then, if there were
730 multiple remaining treatments for the given query compound, the average of their recorded maximal correlation values
731 was calculated to be the similarity score for each target category. The target category with the highest similarity score
732 then was taken to be the predicted target of the query compound, similar to the method described previously[8]. Each
733 target category is a component of a more general MOA category, so the MOA prediction for the query compound
734 follows directly from target prediction.
735

736 The leave-one-out cross validation of the method was performed in the manner described above, wherein each
737 compound in the reference set was taken to be a query compound. When querying a given compound, all other
738 instances of the same compound (e.g. different doses, time points, or strains) were removed from the reference set.
739 Target predictions were made for each compound, and corresponding MOA predictions were assigned. Furthermore,
740 the positive predictive values (PPVs), i.e. the percent of compound targets predicted correctly at each similarity score
741 threshold, were recorded during the leave-one-out cross validation to be used as a measure of confidence for
742 predictions on unknown compounds. Note that target categories comprised of only one compound (27% of
743 compounds), which could never be predicted correctly in the leave-one-out cross validation, were maintained in the
744 calculation of PPV to obtain a conservative estimate. PPV was calculated both at the level of specific target prediction,
745 and at the level of general MOA prediction. Also, the leave-one-out cross validation was performed for querying both
746 z-scores and nz-scores, separately, so that PPVs could be assigned for queries using z-scores and nz-scores
747 appropriately. PPVs were calculated using the pROC library (v1.16.2) in R. Predictions with similarity scores below
748 the minimum similarity score in the leave-one-out cross validations were assigned a PPV of NA, since no confidence
749 could be assessed due to being out of the range of our reference data.
750
751 *Assessing transcriptional similarity of hypomorphic strains to wildtype treated with matched compounds*
752 To assess the similarity of transcriptional profiles of hypomorphic strains to those of wildtype treated with a matched
753 compound, the definition of the batch was further restricted to treatments on the same 384-well plate sharing a time
754 point, thereby allowing different strains to be included in the same batch and revealing strain-specific transcriptional
755 signals. Pearson correlation values were then calculated between these z-scores and those from wildtype treated with
756 a compound targeting the same gene, as well as to those from wildtype with vehicle, all from the same 384-well plate.
757
758 *Using transcriptional profiles of CRISPRi strains to predict compound mechanisms*
759 Transcriptional data from the CRISPRi strains were processed in the same manner as those from compound
760 treatments. Z-scores were calculated for each gene across all CRISPRi samples at all doses of arabinose. Differential
761 gene expression between each sample and the control strain (CRISPRi-C26) at matched doses of arabinose was
762 used to assess the strength of transcriptional response. The same p-value threshold as before was used to filter out
763 weak signals. Then, predictions of compound mechanism and target were performed in the same manner as

764 described earlier, except using the CRISPRi transcriptional profiles as the reference data set. Pearson correlation
765 values were calculated between the z-scores of the CRISPRi strains and those of the wildtype strain treated with
766 different compounds targeting the same genes as the CRISPRi constructs. The exception was fosmidomycin where
767 the *dxr*-hypomorph strain was used instead of wildtype since the compound was not active against wildtype PA14. A
768 similarity score between transcriptional profiles of each compound (query) and each CRISPRi strain (reference set)
769 was calculated in the same manner as described above. The transcriptional data used as the query were the relevant
770 90 minute treatments from the antimicrobial reference data set.
771
772 *Scripts and data accessibility*
773 Scripts for calculating z-scores, nz-scores and predicting compound targets were written in R and stored in github
774 (https://github.com/broadinstitute/psa_rnaseq_manuscript_rproject). Other researchers may utilize the reference data
775 set to predict targets or affected cellular mechanisms using transcriptional data from compounds with unknown
776 mechanisms. All raw sequencing data and z-score values can be found on the Gene Expression Omibus (GEO
777 accession GSE251671, https://www.ncbi.nlm.nih.gov/geo/)
778
779 **Biochemical characterization of screening hits**
780 Resistance selection experiments, as well as assays quantifying ethidium bromide uptake and displacement,
781 mecillinam rescue, DNA intercalation, and DNA gyrase activity, are described in detail in the **Additional Methods** of
782 the **SI Appendix**.
783

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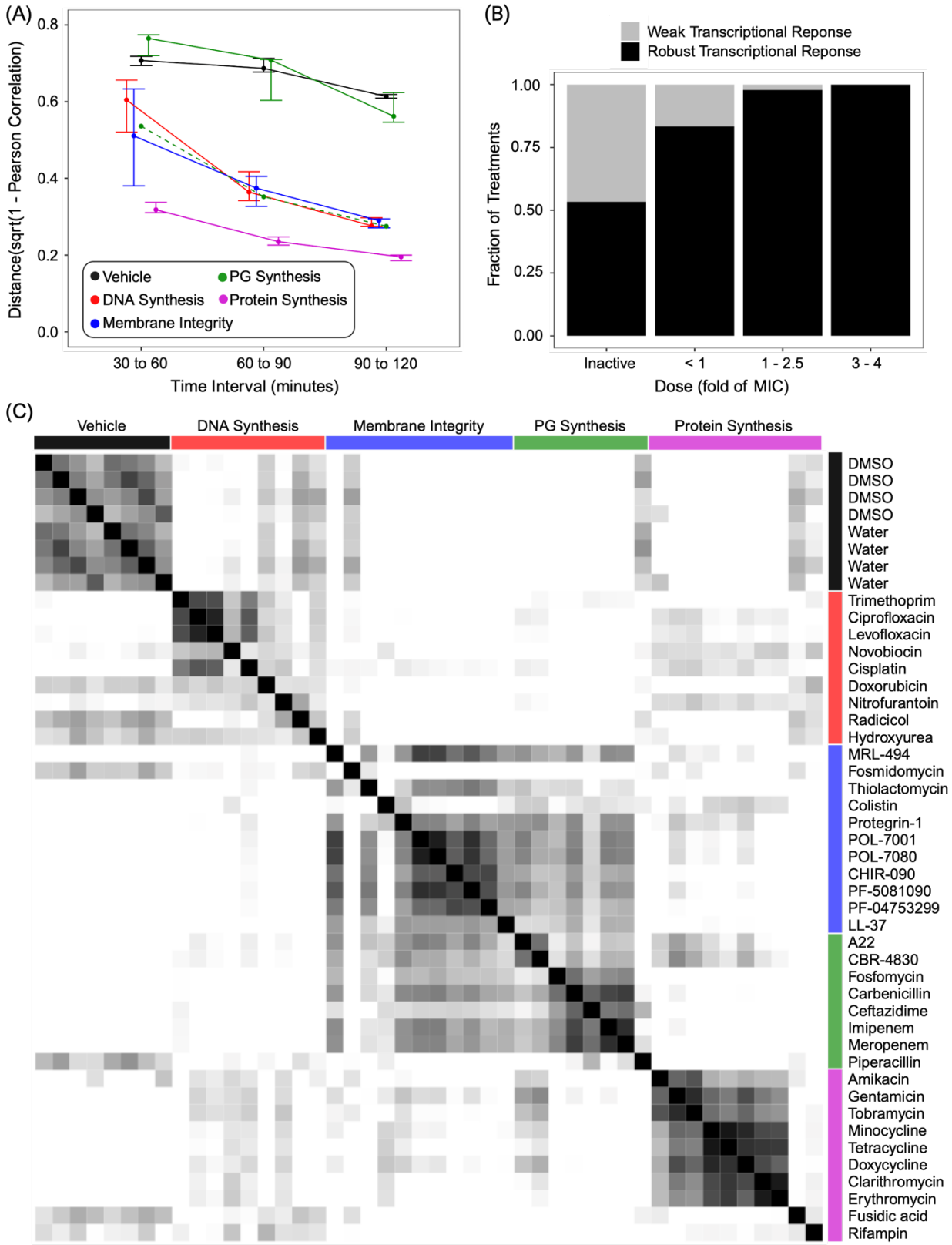
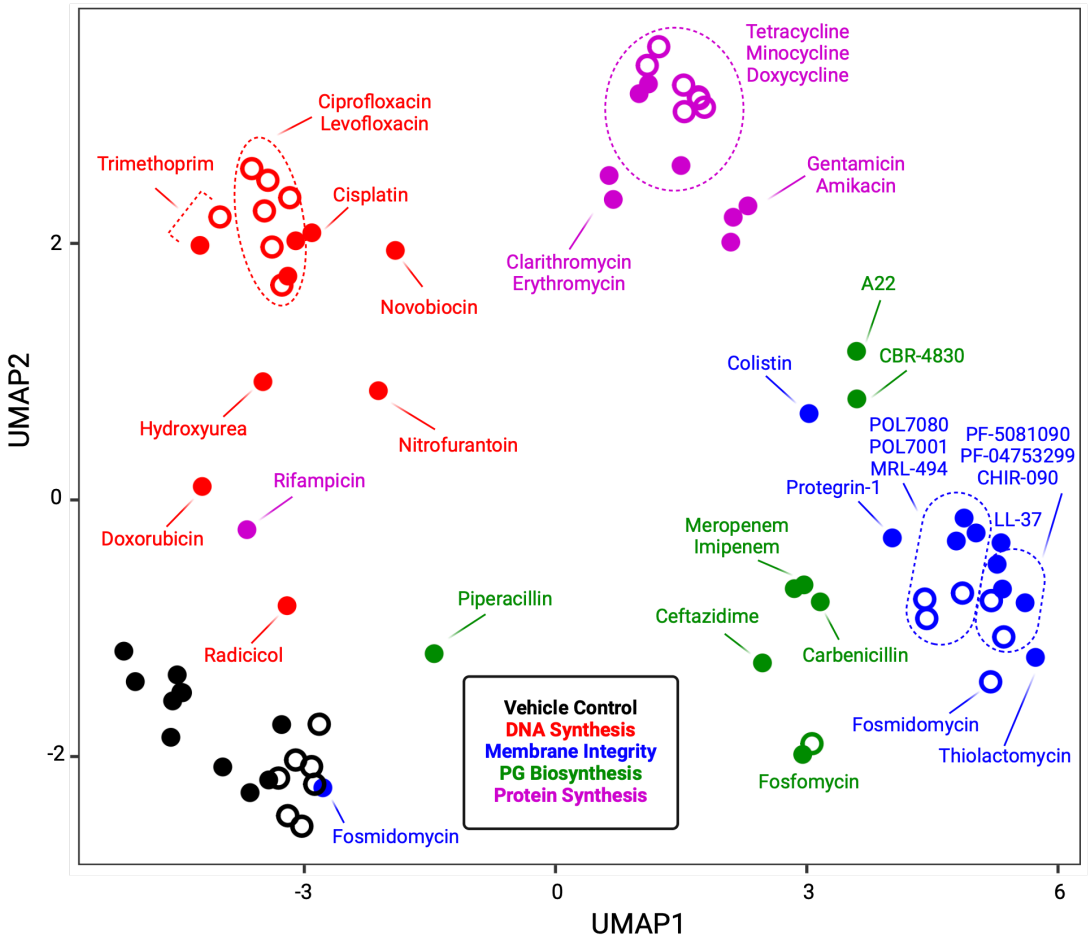
900 **Figures and Tables**



901

902 **Fig. 1.** Gene expression time trial and dose response. (A) Pearson distance between adjacent time points plateau to a
903 stable endpoint for inhibitors of DNA synthesis, membrane integrity, and protein synthesis. Vehicle and inhibitors of
904 peptidoglycan synthesis demonstrate more variability throughout the time course. Imipenem (dotted green line), a
905 peptidoglycan synthesis inhibitor which does not exhibit a significant inoculum effect, also plateau to a stable
906 transcriptional endpoint by 90 minutes. (B) The fraction of all 90-minute treatments having a robust transcriptional
907 response as a function of dose relative to the MIC. Note that a robust transcriptional signature is defined by the
908 differential expression between compound treatment and vehicle control, where at least one gene has an adjusted p-
909 value that is more significant than the minimum adjusted p-value found when comparing PA14 in water versus DMSO
910 control (i.e. $p_{adj} < 10^{-20}$). (C) Heat map shows the Pearson correlations of all 90-minute treatments at the highest dose
911 from both the gene expression time trial and dose response experiments. The rows and columns are sorted based on
912 the MOA of the compounds. All correlation values below zero were set to zero for visualization purposes.

913



914

915 **Fig. 2.** Treatments of hypersusceptible strains correspond to treatments of wildtype P. aeruginosa. UMAP
916 visualization comprising 90-minute treatments from the gene expression time trial, dose response, and hypomorph
917 experiments. Data includes PA14 and hypomorphic strains treated with vehicle (black) or 39 antimicrobial compounds
918 inhibiting DNA synthesis (red), membrane integrity (blue), peptidoglycan synthesis (green), and protein synthesis
919 (magenta). Closed circles indicate PA14 treatments, whereas open circles indicate treatment of a hypomorphic strain.
920 Antimicrobial compounds with robust activity at the experimental inoculum ($1x10^8$ CFU/mL) group by treatment
921 mechanism agnostic of genetic strain. Protein synthesis inhibitors form sub-groups that separate tetracyclines,
922 macrolides and aminoglycosides. Except for novobiocin, compounds with limited activity PA14, such as
923 fosmidomycin, piperacillin, rifampicin, doxorubicin and hydroxyurea, cluster closer to vehicle.

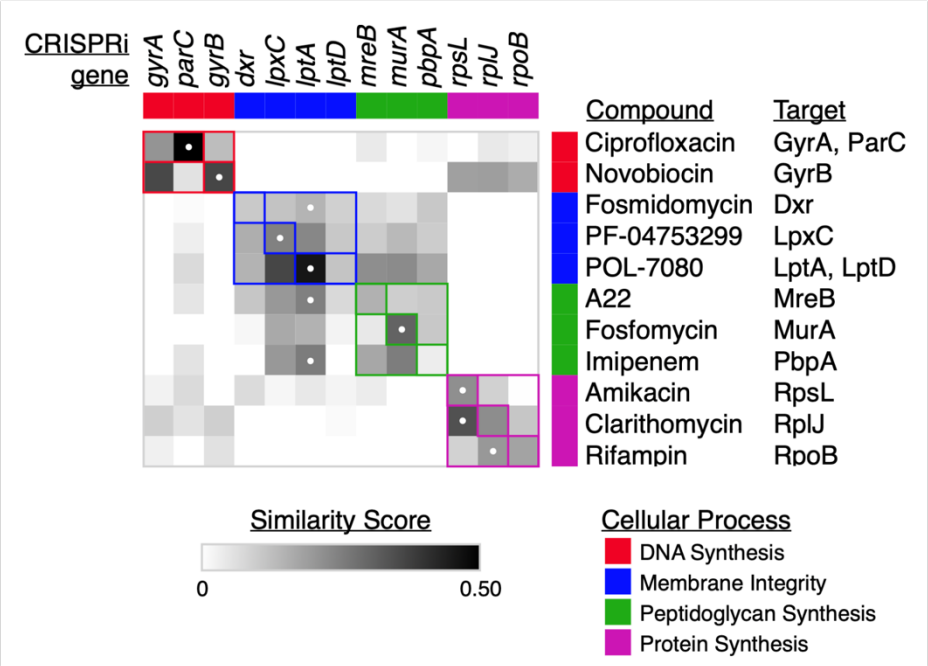


Fig. 3. Similarity scores of CRISPRi reference set to corresponding antimicrobial treatments. Heatmap intensity indicates similarity scores of 90-minute antibiotic treatments when queried against the panel of CRISPRi strains with 0.125% or 0.5% arabinose, the inducer of dCas9 expression. Compound treatments were of PA14 wildtype in all cases except fosmidomycin, which was used to treat the *dxr*-hypomorph, since it did not cause a growth delay of PA14 at the given inoculum. White dots indicate the maximal similarity score for each row. Colored boxes indicate the corresponding target gene(s) or mechanism for each antimicrobial compound.

931 **TABLES:**

Table 1: Summary of the 39 antimicrobial compounds included in the reference set. Antimicrobial compounds used in gene expression time trial are indicated in bold. Antibiotics exhibiting greater or equal to 4-fold shift in MIC at high inoculum are indicated by an asterisk.

MOA	Antibiotic	Class	Target	MIC (μM) Low Inoculum	MIC (μM) High Inoculum
DNA Synthesis	**Ciprofloxacin**	**Fluoroquinolone**	**GyrA/ParC**	**0.25**	**0.25**
	Levofloxacin	**Fluoroquinolone**	**GyrA/ParC**	**1.5**	**1.5**
	Novobiocin	**Novobiocin**	**GyrB**	**>800**	**>800**
	Trimethoprim	**Anti-metabolite**	**DhfR**	**100**	**200**
	Cisplatin	Alkylator	DNA	100	100
	Hydroxyurea	Anti-metabolite	Ribonucleotide reductase	>3200	>3200
	Doxorubicin	Anthracyclines	DNA intercalator	>800	>800
	Radicicol	Macrocycle	HtpG (Hsp90 homolog)	>400	>400
	Nitrofurantoin	Nitrofuran	Protein/DNA damage	>800	>800
Peptidoglycan Synthesis	**Meropenem***	**Carbapenem**	**PBPs**	**1.5**	**6.25**
	Imipenem	**Carbapenem**	**PBPs**	**1.5**	**3.0**
	Ceftazidime*	**Cephalosporin**	**PBPs**	**12.5**	**400**
	Piperacillin*	**Beta Lactam**	**PBPs**	**6.25**	**>400**
	Carbenicillin*	**Beta Lactam**	**PBPs**	**200**	**3200**
	Aztreonam*	**Monobactam**	**PBPs**	**6.25**	**>400**
	Fosfomycin*	**PEP Synthesis**	**MurA**	**250**	**1600**
Protein Synthesis	**Tetracycline**	**Tetracycline**	**30S Ribosome**	**50**	**50**
	Minocycline	**Tetracycline**	**30S Ribosome**	**12.5**	**12.5**
	Erythromycin	**Macrolide**	**50S Ribosome**	**100**	**100**
	Clarithromycin	**Macrolide**	**50S Ribosome**	**100**	**100**
	Amikacin	**Aminoglycoside**	**30S Ribosome**	**1.5**	**3**
	Gentamicin	**Aminoglycoside**	**30S Ribosome**	**3**	**3**
	Doxycycline	Tetracycline	30S Ribosome	50	50
	Tobramycin*	Aminoglycoside	30S Ribosome	1.56	6.25
	Rifampicin	Ansamycins	RpoB	>400	>400
	Fusidic acid	Fusidane	FusA1 (EF-G)	>400	>400
Membrane Integrity	**Colistin***	**Polymyxin**	**Membrane**	**0.78**	**6.25**
	Protegrin-1*	**AMP**	**Membrane**	**2**	**8**
	PF-5081090	**LPS Synthesis**	**LpxC**	**0.4**	**0.8**
	PF-04753299	**LPS Synthesis**	**LpxC**	**0.4**	**0.8**
	LL-37*	AMP	Membrane	12.5	>50
	Fosmidomycin*	Isoprenoid biosynthesis	Dxr	12.5	>400
	POL7001*	LPS Transport	LptD	0.0625	0.500
	POL7080*	LPS Transport	LptD	0.0625	0.125
	CHIR-090*	LPS Synthesis	LpxC	0.78	3.125
	A22*	Actin homolog	MreB	12.5	400
	CBR-4830*	Actin homolog	MreB	15.63	62.5
	MRL-494	OMP folding	BamA	25	25
	Thiolactomycin	Fatty Acid Synthesis	KasA	>400	>400

932

Table 2: Target and MOA prediction of antimicrobial compounds from external and internal datasets using the PerSpecTM approach.

Compound	Predicted Target	Predicted MOA	Similarity Score	Target / MOA PPV	Closest Reference
External Validation using Murray et al. (2015) [17]					
Peroxide	GyrA/ParC	DNA synthesis	0.51	81% / 96%	Ciprofloxacin
Gentamicin	30S RS	Protein synthesis	0.48	79% / 96%	Tobramycin
Ciprofloxacin	GyrA/ParC	DNA synthesis	0.33	61% / 94%	Levofloxacin
Neomycin	30S RS	Protein synthesis	0.33	61% / 94%	Amikacin
Bleach	DNA	DNA synthesis	0.26	NA / NA	Nitrofurantoin
Polymyxin B	LptD	Membrane Integrity	0.26	NA / NA	POL7080
External Validation using Franke et al. (2021) [43]					
Lomefloxacin	GyrA/ParC	DNA synthesis	0.30	62% / 94%	Ciprofloxacin
Ciprofloxacin	GyrA/ParC	DNA synthesis	0.29	62% / 94%	Ciprofloxacin
Levofloxacin	GyrA/ParC	DNA synthesis	0.29	62% / 94%	Ciprofloxacin
Novobiocin	GyrB	DNA synthesis	0.26	58% / 92%	Novobiocin
Internal Validation using screening hits					
PA-0918	LPS	Membrane Integrity	0.74	100% / 100%	Colistin
PA-69180	MreB	Peptidoglycan Synthesis	0.38	68% / 94%	A22
PA-5750	GyrA/ParC	DNA synthesis	0.56	88% / 96%	Ciprofloxacin

933

Table 3: Chemical structures and MIC data for screening hits PA-0918, PA-69180 and PA-5750. The fold decrease in MIC relative to PA14 is shown in parentheses.

Screening Hit	Screening Strain	Chemical Structure	MIC (μM) PA14	MIC (μM) Screening Strain	MIC (μM) PAO397
PA-0918	*lptD*-hypomorph		250	125 (2)	31.2 (8)
PA-69180	*oprL*-hypomorph		>250	31.2 (>8)	15.6 (>16)
PA-5750	*lptE*-hypomorph		250	125 (2)	<15.6 (>16)

934