

1 **Metabolic connections between folate and peptidoglycan pathways in *Pseudomonas***
2 ***aeruginosa* inform rational design of a dual-action inhibitor**

3 Luke N. Yaeger¹, David Sychantha¹, Princeton Luong¹, Shahrokh Shekarriz¹, Océane
4 Goncalves², Annamaria Dobrin¹, Michael R. Ranieri¹, Ryan P. Lamers¹, Hanjeong Harvey¹,
5 George C. diCenzo³, Michael Surette¹, Jean-Philippe Côté², Jakob Magolan¹, Lori L. Burrows¹

6

7 ¹Department of Biochemistry and Biomedical Sciences, and the Michael G. DeGrootte Institute
8 for Infectious Disease Research, McMaster University, Hamilton, Ontario, Canada

9 ²Département de Biologie, Université de Sherbrooke, Sherbrooke, Québec, Canada

10 ³Department of Biology, Queen's University, Kingston, Ontario, Canada

11

12

13

14

15

16

17

18

19

20

21

22

23

24

25

26 **Abstract**

27 Peptidoglycan is an important bacterial macromolecule that confers cell shape and
28 structural integrity, and a key antibiotic target. The synthesis and turnover of peptidoglycan are
29 carefully coordinated with other cellular processes and pathways. Although there are
30 established connections between peptidoglycan and DNA replication or outer membrane
31 biosynthesis, connections between peptidoglycan and folate metabolism are comparatively
32 unexplored. Folate is an essential cofactor for bacterial growth and required for the synthesis of
33 many important metabolites. Here we show that inhibition of folate synthesis in the important
34 Gram-negative pathogen *Pseudomonas aeruginosa* has downstream effects on peptidoglycan
35 metabolism and integrity. Folate inhibitors reduced expression of the AmpC β -lactamase
36 through perturbation of peptidoglycan recycling, potentiating the activity of β -lactams normally
37 cleaved by that resistance enzyme. Folate inhibitors also synergized with fosfomycin, which
38 inhibits MurA - the first committed step in peptidoglycan synthesis - resulting in dose-dependent
39 formation of round cells that underwent explosive lysis. The insights from this work were used to
40 design a dual-active inhibitor that overcomes NDM-1-mediated meropenem resistance and
41 synergizes with the folate inhibitor, trimethoprim. This work shows that folate and peptidoglycan
42 metabolism are intimately connected and offers new opportunities to exploit this relationship in
43 strategies to overcome antibiotic resistance in Gram-negative pathogens.

44

45 **Introduction**

46 High levels of antibiotic resistance in the opportunistic and nosocomial pathogen
47 *Pseudomonas aeruginosa* can limit treatment options and impact the development of new
48 therapeutics¹. Combination therapy is among the strategies that can be used to restore the
49 efficacy of current antibiotics against resistant strains². For example, β -lactam and β -lactamase
50 inhibitor combinations extended the spectrum of cell-wall targeting β -lactams to strains
51 producing antibiotic-degrading enzymes³. Some combinations go beyond unidirectional
52 potentiation, achieving drug-drug synergy through their mutual potentiation. The classic
53 antibiotic combination of trimethoprim (TMP) and sulfamethoxazole (SUL) inhibits separate
54 steps in folate biosynthesis, and together the two drugs are more potent than the single agents⁴.
55 In another example, single-drug inhibition of multiple targets is thought to explain the
56 effectiveness of fluoroquinolones and β -lactams⁵, which essentially synergize with themselves
57 by inhibiting more than one interdependent target.

58 TMP and SUL block the production of tetrahydrofolate (THF), an essential cofactor in
59 one-carbon metabolism for all forms of life. Humans rely on their diet for folate acquisition, while
60 many bacteria must synthesize folate *de novo* from GTP and chorismate, making the folate
61 biosynthetic pathway an attractive target for antibiotics⁶. Sulfonamide antibiotics, including SUL,
62 inhibit dihydropteroate synthase (FolP) by displacing its substrate, para-aminobenzoic acid
63 (PABA). Some sulfonamides then react with the other FolP substrate, dihydropterin
64 pyrophosphate, to form a dead-end metabolite⁷. FolP is two steps upstream of the TMP target,
65 dihydrofolate reductase (FolA), which catalyzes the formation of THF from dihydrofolate (DHF).
66 THF and its derivatives act as cofactors and one-carbon donors to generate key metabolites
67 including purines, methionine and S-adenosyl methionine, thymidylate, glycine, and serine⁸. The
68 large number of THF-dependent metabolites suggests that treatment with folate inhibitors can
69 have far-reaching effects on cellular physiology. The best-studied effect of folate inhibition is
70 decreased pools of thymidylate, a metabolite required for DNA synthesis⁹. By inhibiting DNA
71 synthesis, TMP treatment can induce the SOS response and expression of Sula, which inhibits
72 assembly of the FtsZ ring that scaffolds the cell division machinery¹⁰.

73 A careful review of the literature reveals many clues supporting connections between the
74 folate and PG pathways. Early studies of anti-folates revealed that induction of the SOS-
75 response in folate-depleted cells induced filamentation, suggesting impacts on cell wall
76 metabolism^{11,12}. Assembly of the divisome is further regulated by levels of S-adenosyl
77 methionine (SAM), a folate-dependent metabolite¹³. Antifolate-induced morphological changes
78 extend beyond filamentation, as cell-wall deficient cells can also arise following folate
79 inhibition¹⁴. Further linking the folate and cell wall pathways, Richards and Xing reported
80 accumulation of Lipid II in *Enterobacter cloacae* following TMP and sulfadiazine treatment,
81 suggesting a blockage in PG assembly post-precursor biosynthesis¹⁵. A screen for
82 *Acinetobacter baumannii* mutants that were significantly less fit during growth with TMP-SUL
83 uncovered mutants with disruptions in peptidoglycan recycling¹⁶. An attempt to generate
84 antibiotic mechanism-of-action signatures using *B. subtilis* led to misannotation of TMP and SUL
85 as cell wall antibiotics, suggesting their impacts on physiology were similar to those of cell wall-
86 targeting drugs¹⁷. Synergy between antifolates and cell wall-active antibiotics was reported in
87 multiple studies of drug-drug interactions in *Escherichia coli*, including TMP with vancomycin¹⁸,
88 oxacillin¹⁹, fosmidomycin²⁰, and mecillinam²¹, and a patent that includes a combination therapy
89 of TMP-SUL and fosfomycin has been issued²². Despite these intriguing links, the
90 mechanism(s) of synergy between anti-folates and cell wall-active drugs remains unclear.

91 As in most Gram-negative bacteria, the peptidoglycan (PG) cell wall of *P. aeruginosa* is
92 composed of repeating disaccharide-pentapeptide subunits that are crosslinked to form a mesh-
93 like structure. PG metabolism can be broadly divided into subunit synthesis, assembly, and
94 turnover/recycling^{23–25}. MurA performs the first committed step, transferring an enolpyruvyl
95 group to uridine diphosphate N-acetyl glucosamine (UDP-GlcNAc). MurB-F, MraY, and MurG
96 then catalyze the formation of Lipid II, a GlcNAc-N-acetyl muramic acid (MurNAc) disaccharide
97 with a MurNAc-linked pentapeptide stem (L-alanine [L-Ala], γ -D-glutamate [D-Glu], meso-
98 diaminopimelate [mDAP], and two D-alanine residues [D-Ala]), which is anchored in the inner
99 membrane by undecaprenol diphosphate. MurJ flips Lipid II into the periplasm where
100 transglycosylases attach the GlcNAc of Lipid II to the MurNAc of a growing PG chain. D,D-
101 transpeptidases crosslink PG glycan strands via amide bonds between the 4th (D-ala) and 3rd
102 (mDAP) amino acids of different peptide stems. L,D-transpeptidases crosslink two mDAP
103 residues on different peptide stems, although these 3-3 crosslinks are less abundant than 4-3
104 crosslinks²⁶. Mature PG can be cleaved by amidases, endopeptidases, and lytic
105 transglycosylases to allow for cell growth and division, as well as recycling of PG components.
106 Amidases separate the peptide stem from the glycan backbone, endopeptidases cleave the
107 inter-peptide stem amide bond, and lytic transglycosylases cleave the GlcNAc-MurNAc bond
108 and, for terminal subunits, release a GlcNAc-1,6-anhydroMurNAc (anhMurNAc) peptide. This
109 fragment is taken up, broken down, and its substituents re-enter the PG synthesis pathway. PG
110 metabolism has a high energetic cost, draws upon many different metabolite pools, and carries
111 out the final, irreversible step of the cell cycle; therefore, it is carefully coordinated with many
112 aspects of cell physiology. In this work we use bioinformatics, microscopy, and chemical
113 genetics to characterize the connections between folate and PG metabolism, and leverage our
114 findings in the design of a dual inhibitor that overcomes Gram-negative meropenem resistance.

115

116 **Results**

117 We previously discovered that a *P. aeruginosa oprF* mutant was hypersensitive to TMP,
118 suggesting a possible link between folate and PG metabolism²⁷. OprF is a major outer
119 membrane porin with a C-terminal PG-binding domain that anchors the outer membrane to the
120 cell wall²⁸. The hypersusceptibility of the *oprF* mutant, combined with the results of a detailed
121 literature survey that revealed multiple points of intersection between cell-wall and folate
122 biosynthetic pathways, prompted further investigation. First, we noted several striking structural
123 similarities between a subset of folate and PG enzymes that further strengthen the potential for

124 integration of the pathways. For example, both FolC, the folylpolyglutamate synthase, and
125 MurC-F belong to the Mur ligase family (**Fig 1a**)²⁹. MurA is structurally related to AroA (involved
126 in chorismate biosynthesis), and both use the same reaction mechanism for the addition of
127 enolpyruvate³⁰. PabC, which catalyzes the production of PABA, is structurally homologous to a
128 D-amino acid aminotransferase^{31,32} that produces D-Glu and D-Ala for the PG stem peptide.
129 Finally, *P. aeruginosa* Cpg2 is a periplasmic carboxypeptidase that cleaves folate to produce
130 glutamyl and pteroyl groups³³; it resembles DapE, which catalyzes production of mDAP³⁴.
131 Beyond these structural similarities, there is intriguing synteny between a subset of folate and
132 PG genes (**Fig 1b**). Notably, *folP* is adjacent to *glmM*, encoding a phosphoglucosamine mutase
133 that feeds glucosamine 1-phosphate into PG synthesis. The SUL resistance gene *sul2* (a
134 resistant *folP* allele) is commonly transferred on resistance plasmids with *glmM*³⁵.

135 Synteny can indicate coevolution between genes or pathways³⁶. We investigated the
136 extent of the *folP*-*glmM* relationship by measuring the proximity of the two genes across a
137 collection of over 40,000 representative prokaryotic genomes. The results were binned into
138 distances of 0-100, 100-1k, 1k-10k, 10k-100k, and over 100k base pairs. Synteny between *folP*
139 and *glmM* was largely constrained to the phylum *Proteobacteria* (*Pseudomonadota*), but not
140 present in all proteobacteria (**Fig 1c**). Plotting the distance between *folP* and *glmM* on a
141 phylogenetic tree of 938 complete proteobacterial genomes showed that the synteny is
142 generally conserved for all but the families *Xanthomonadaceae*, *Sphingomonadaceae*, and
143 *Rhizobiaceae* (**Fig 1d**). This finding corroborates and expands upon a previous report that
144 *Xanthomonas campestris* is distinct among species of the class *Gammaproteobacteria*, in that
145 *glmM* and *folP* are encoded separately³⁷. Some *Rhizobiaceae* species lack a *folP* homologue
146 and presumably import folate, like some lactobacilli^{38,39}. The benefits of organizing *glmM* and
147 *folP* together as a transcriptional unit remain unclear, as do the conditions that permit their
148 uncoupling; however, this analysis demonstrates an evolutionary relationship between these
149 genes and prompted additional studies.

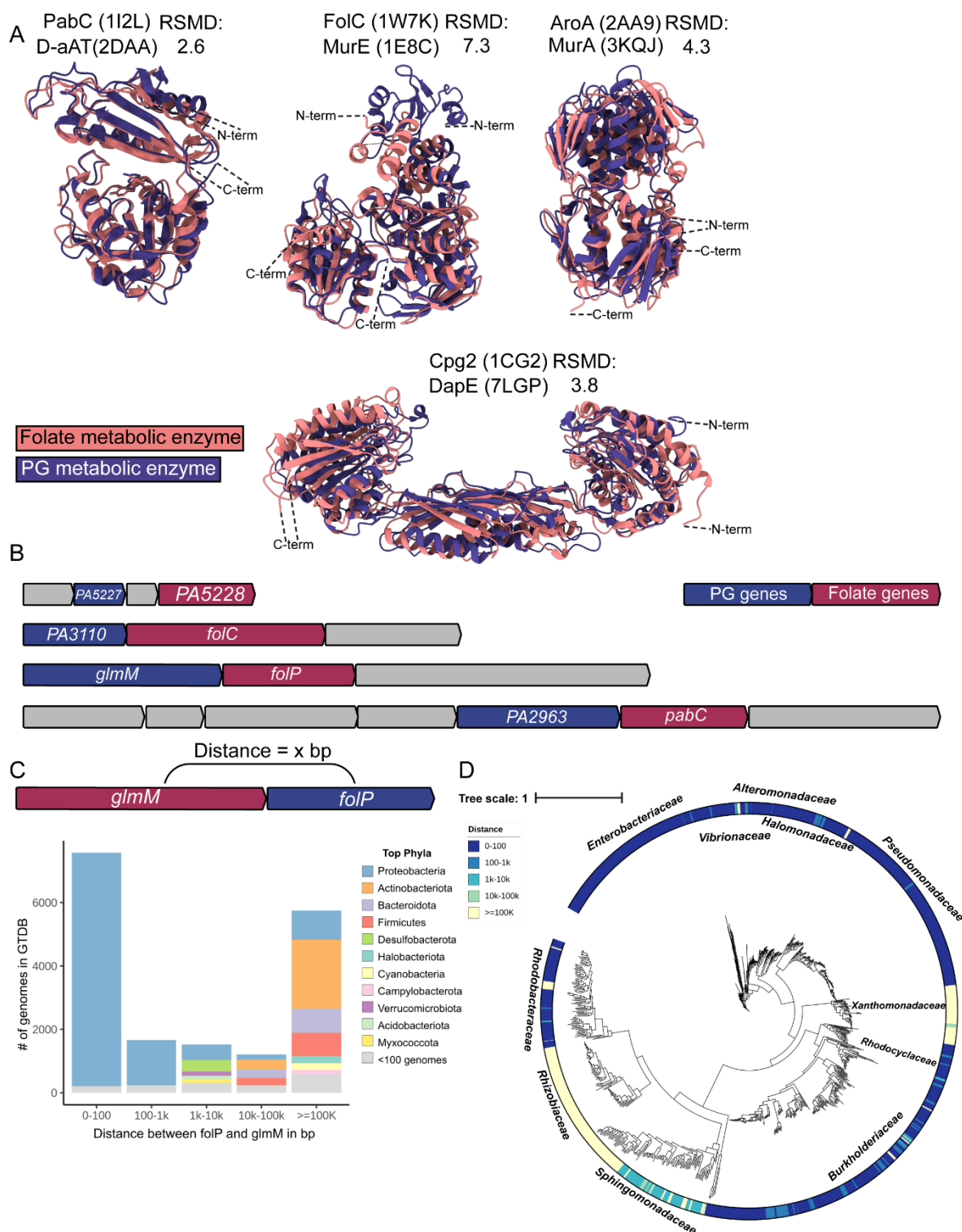


Figure 1. Folate-PG structural and synteny relationships. **A)** A comparison of structural similarity between folate (orange) and PG (blue) metabolic enzymes. The structures were overlaid using the Matchmaker function in ChimeraX. Above each pair of structures are the protein names and PDB codes, where the top name and code corresponds to the folate protein, while the bottom name and code correspond to the PG protein. **B)** An illustration of the *P. aeruginosa* PAO1 operons containing

folate and PG-related genes (shown in red and blue, respectively). Other genes within the predicted operons are shown in gray. The unnamed genes are labelled with their *P. aeruginosa* PAO1 locus tag. PA5227 encodes a ZapA homologue, PA5228 encodes a Fau homologue, PA3110 encodes a DedD homologue, and PA2963 encodes an MltG homologue. **C**) The distance in base pairs between the *glmM* and *folP* genes was binned into discrete distances shown on the X-axis, and the number of genomes that fall into each bin are shown on the Y-axis. The legend shows the colour corresponding to each phyla. **D**) An unrooted species phylogenetic tree overlaid with the distance between *folP* and *glmM* mapped across representative genomes of the phylum *Proteobacteria*. The names of major families of the phylum *Proteobacteria* are labelled next to the corresponding genomes. A scale bar and legend are shown in the top left, and the scale bar represents the average number of amino acid substitutions per site. The distance between *folP* and *glmM* is represented by a colour scale, where dark blue indicates a smaller intergenic distance and light yellow indicates a larger intergenic distance.

150

151 *Folate inhibitor phenotypes resemble those caused by cell wall-targeting antibiotics*

152 Following up on our discovery that a *P. aeruginosa oprF* mutant is hypersensitive to
153 TMP²⁷, we found that this mutant is also hypersensitive to SUL (**Fig 2a**). We next tested the
154 ability of TMP to compromise envelope integrity using the classic method of exposing cells to
155 hyper- or hypo-osmotic stresses. Changing the NaCl concentration of the LB medium revealed
156 that the minimal inhibitory concentration (MIC) of TMP was ~8 x lower in high NaCl conditions
157 (**Fig 2b**). To determine if this phenotype was specific to folate inhibition, rather than a general
158 effect of disrupting DNA synthesis, we used the DNA gyrase inhibitor ciprofloxacin (CIP) as a
159 control. The MIC of CIP was reduced ~2x in response to the same high NaCl stress (**Fig 2c**),
160 suggesting that folate inhibition impacts the cell envelope beyond simply inhibiting DNA
161 synthesis. We reasoned that if TMP exposure affected the cell envelope, it might alter bacterial
162 morphology. PG synthesis inhibitors can have drastic impacts on cell shape and lead to lysis⁴⁰⁻
163 ⁴². We grew *P. aeruginosa* with increasing concentrations of TMP and examined its morphology
164 using confocal microscopy. TMP induced the formation of round cells that were reminiscent of
165 PG-less L-forms⁴³ (**Fig 2d**), suggesting that inhibition of folate synthesis leads to loss of rod
166 shape in a subpopulation of *P. aeruginosa* cells. These round cells eventually undergo
167 explosive cell lysis (**Extended Data Movie 1**). Previous reports of TMP-induced changes in cell
168 morphology suggested increased filamentation; however, that work was primarily done in *E.*
169 *coli*⁴².

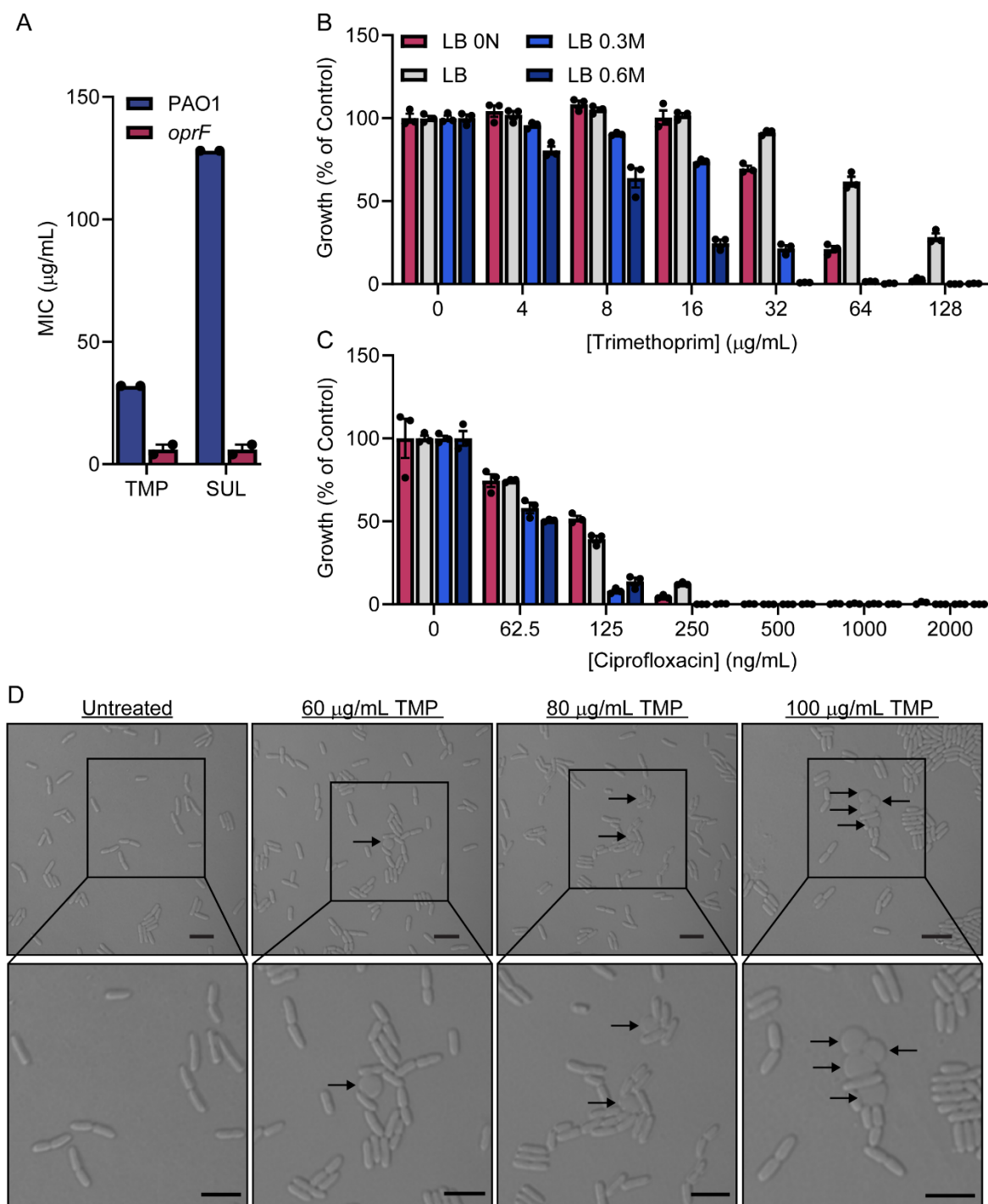


Figure 2. Folate inhibition affects cell envelope integrity. **A)** MICs were measured with a liquid dose-response assay. The antibiotics tested are shown on the X-axis, and the MIC is shown on the Y-axis. The wild type and *oprF* mutant are coloured blue and red, respectively, and individual MICs from two biological replicates are plotted as black circles. The data points for each biological replicate are averaged from three technical replicates. The error bars show the standard error of the mean. **B and C)** A dose-response showing the effect of **B)** TMP or **C)** CIP on PAO1 growth in different osmolarity strength LB. The range of TMP concentrations are shown on the X-axis and

growth at each concentration is shown as a percent of the vehicle-treated control and plotted on the Y-axis. Each colour represents different growth media, with the molar concentration of NaCl shown beside the rectangles in the legend (LB 0N = LB with no NaCl). The experiment was repeated in biological duplicates and data from a representative biological replicate are shown, where the bars represent the mean of a technical triplicate, the black circles represent individual data points, and the error bars represent the standard error of the mean. **D)** Micrographs of PAO1 cells treated with increasing TMP concentrations (indicated above each image). A 5-micron scale bar is shown in the bottom right corner of each image. The arrows point to round cells. Close-ups of each image are shown below the original image. Sample preparation and imaging was performed in biological triplicates and images were sampled from at least three separate locations on the agarose pad. Representative micrographs are shown.

170

171 *A chemical-genetic screen uncovers specific interactions of PG inhibitors with TMP*

172 Many PG synthesis genes are essential, but sublethal doses of a chemical inhibitor can
173 titrate the activity of these critical enzymes without killing the cell. Therefore, we used a
174 chemical-genetic approach to probe the possible mechanism of antifolate-induced envelope
175 perturbation. Using checkerboard assays, we first screened a collection of PG inhibitors that
176 target different steps of PG metabolism for interactions with TMP. Of the compounds tested (**Fig**
177 **3a**), fosfomycin (FOS) and cefoxitin (FOX), which target MurA and multiple penicillin binding
178 proteins (PBPs), respectively (**Fig 3b**), potentiated TMP (**Fig 3c**). This interaction is a general
179 feature of folate inhibition, as SUL also potentiated FOS and FOX (**Fig 3c**). However, FOS and
180 FOX failed to synergize with ciprofloxacin, suggesting that the synergistic effects are not due to
181 inhibition of DNA synthesis (**Extended Data Fig 1a**). Overexpression of FoaA, the target of TMP,
182 increased the concentration required for potentiation (**Extended Data Fig 1b**), indicating the
183 primary mechanism of action, rather than off-target effects, drives the interaction. While
184 antifolates and FOS/FOX may synergize by increasing cell permeability (as is the case with
185 potentiation of many antibiotics by polymyxin B), we do not believe this to be the case because:
186 1) We did not see potentiation of TMP/SUL by polymyxin B (**Extended Data Fig 1c**); 2)
187 Polymyxin B does not potentiate FOS (**Extended Data Fig 1d**); 3) The lack of interaction
188 between TMP and most cell wall-targeting antibiotics (**Fig 3a**) supports more specific
189 mechanisms of potentiation; and 4) no TMP potentiation was observed in a *glpT* mutant that
190 blocks FOS from crossing the inner membrane⁴⁴ (**Extended Data Fig 1e**).

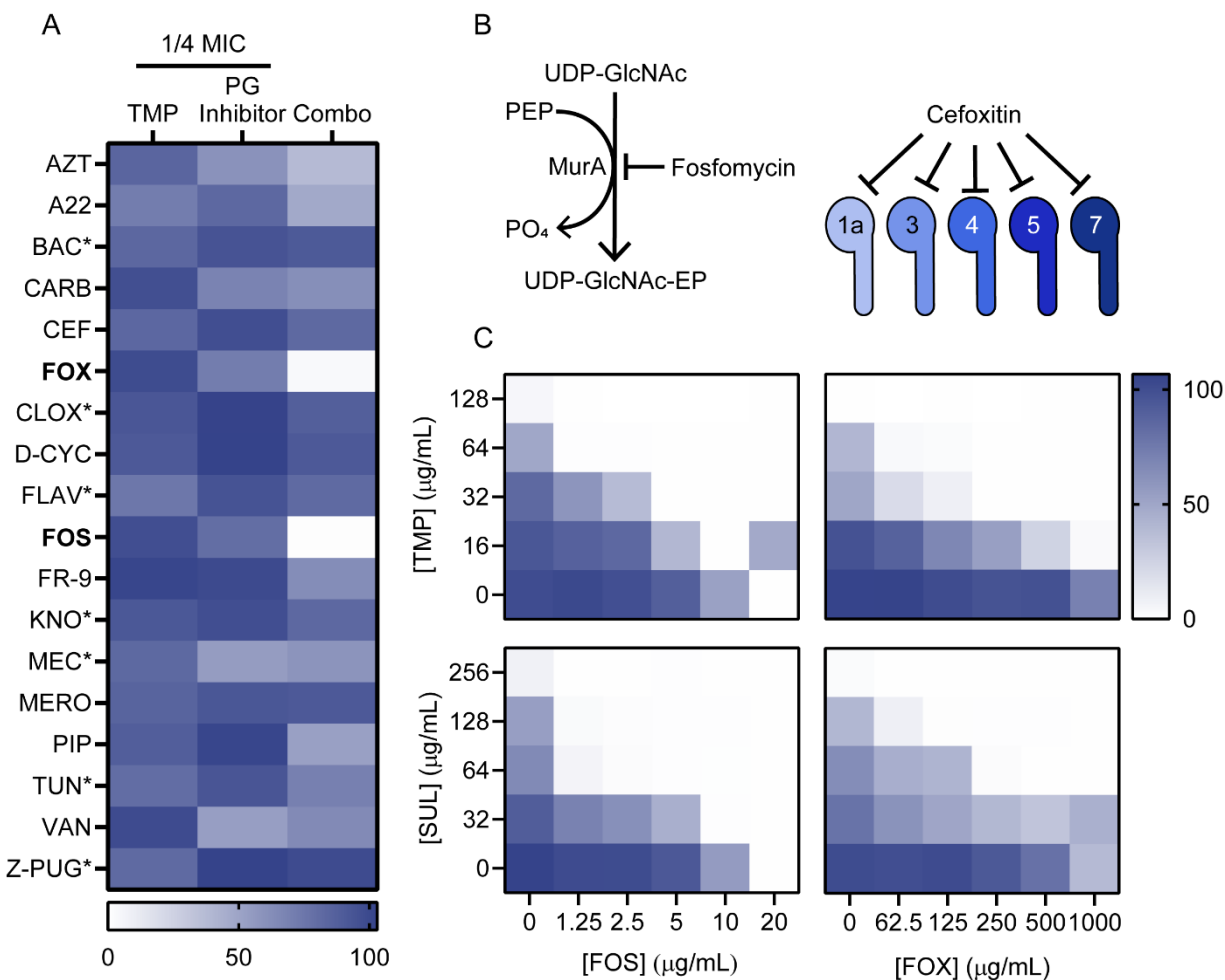


Figure 3. An interaction screen reveals specific antifolate/PG inhibitor potentiation. A) The heatmap in **A)** summarizes the data from 8x8 checkerboard assays with TMP and the antibiotics listed to the left of the heatmap. The abbreviations are as follows: AZT=aztreonam, A22=A22, BAC=bacitracin, CARB=carbenicillin, CEF=cefexime, FOX=cefoxitin, CLOX=cloxacillin, D-CYC=D-cycloserine, FLAV=flavomycin, FOS=fosfomycin, FR-9=FR-900098, KNO=kanosamine, MEC=mecillinam, MERO=meropenem, PIP=piperacillin, TUN=tunicamycin, VAN=vancomycin, Z-PUG=Z-PUGNAc. Asterisks indicate antibiotics that did not reach an MIC at the highest concentration tested. Bold font indicates inhibitors that demonstrated synergy. Growth is shown as a percent of the vehicle control and corresponds to the scale bar at the bottom of the heat map. The left column of the heatmap shows growth with 1/4 MIC TMP alone. The middle column shows growth after treatment with 1/4 MIC of the PG inhibitor alone. The right column shows growth after treatment with 1/4 MIC of both TMP and the PG inhibitor. **B)** The left diagram shows the reaction catalyzed by MurA, which is inhibited by fosfomycin. PEP=phosphoenolpyruvate, PO₄=phosphate, EP=enolpyruvate. The right diagram shows the PBPs inhibited by cefoxitin, according to Ropy *et al.*⁴⁵. **C)** Heatmaps from 8x8 checkerboards condensed to 5x6 checkerboards. The antibiotics and the respective concentration ranges used are shown on the bottom or left heatmap axes. Growth is shown as a percent of the vehicle control and corresponds to the scale bar on the top right heatmap. Checkerboard assays were repeated in biological triplicates and representative heatmaps from one replicate are shown.

192 *TMP potentiates FOX by suppressing the AmpR/AmpC response*

193 FOX, like other β -lactams, can inhibit multiple PBPs⁴⁵. We next sought to understand
194 why TMP specifically potentiated FOX, but not the other β -lactams in our panel (**Fig 3a**). *P.*
195 *aeruginosa* is typically insensitive to FOX, which inhibits PBPs 4 (DacB) and 5 (DacC), D,D-
196 carboxypeptidases that cleave the terminal D-Ala residue from pentapeptide stems to limit the
197 extent of cross-linking. Inhibiting these two PBPs increases the pool of GlcNAc-anhMurNAc-
198 pentapeptides that are recycled via the inner membrane transporter, AmpG. These products
199 bind the regulator AmpR to induce expression of AmpC, a β -lactamase that degrades FOX⁴⁶
200 (**Fig 4a**). Of the β -lactams we tested, only FOX is both a substrate and inducer of AmpC,
201 suggesting that antifolates might impact the AmpR/AmpC pathway. To test this idea, we chose
202 additional representatives of three types of β -lactams: AmpC inducer and substrate, AmpC non-
203 inducer but substrate, and AmpC inducer but non-substrate. **Figure 4b** shows the fractional
204 inhibitory concentrations (FICI) derived from checkerboard assays of β -lactams from each type
205 in combination with TMP. A pair of compounds with an FICI of 0.5 or less is considered
206 synergistic. Supporting our hypothesis, only those β -lactams that are both inducers and
207 substrates of AmpC synergized with TMP. We reasoned that if AmpC and AmpR were required
208 for the TMP-FOX interaction, then TMP should not further reduce the FOX MIC of *ampR* or
209 *ampC* mutants. Indeed, TMP and FOX failed to synergize in *ampC* or *ampR* mutants (**Fig 4c**).

210 Genetic inactivation of PBPs 4 and 5 via point mutation of their catalytic Ser to Ala
211 mimics antibiotic inhibition and activates the AmpR/AmpC pathway⁴⁵. Using a PBP4 S72A,
212 PBP5 S64A double mutant (*dacBC***), we tested whether genetic activation of the AmpR/AmpC
213 response was sufficient for TMP potentiation of cefotaxime (TAX), a non-inducer but substrate
214 of AmpC. As expected, the double mutant was more resistant than the wild type to TAX and we
215 observed TMP potentiation, indicating that inactivating PBP4/5 is sufficient (**Fig 4d**). Further,
216 potentiation was lost in a *dacBC** ampG* triple mutant, suggesting a reliance on AmpG for
217 transport of the inducer GlcNAc-anhMurNAc-pentapeptide into the cytoplasm (**Fig 4d**). A
218 catalytically-inactive S90A point mutant of AmpC also lacked potentiation (**Extended Data Fig**
219 **2a**), even though its expression was highly induced by FOX (**Extended Data Fig 2b**), showing
220 that AmpC activity is required for synergy with TMP.

221 If TMP potentiated FOX activity by preventing *ampC* induction, then transcription from
222 the *ampC* promoter should be reduced. We cloned the *ampC* promoter upstream of a *lux*
223 cassette to create a luminescent transcriptional reporter. As expected, there was almost no
224 luminescence in the untreated condition and very high luminescence in the FOX-treated

225 condition, where *ampC* is induced (**Fig 4e**). Adding TMP in addition to FOX led to a dose-
226 dependent reduction in luminescence. Decreased *ampC* promoter activity implied that AmpC
227 activity in whole cells should also be reduced. We grew cells with FOX alone and in combination
228 with increasing concentrations of TMP, then measured AmpC activity using nitrocefin hydrolysis
229 as a readout. Nitrocefin is a chromogenic β -lactamase substrate commonly used to measure
230 activity⁴⁷. As predicted, we saw a dose-dependent decrease in nitrocefin hydrolysis with TMP
231 relative to the FOX-only control (**Fig 4f**). To rule out the possibility that TMP was a direct
232 inhibitor of AmpC activity, we performed the nitrocefin assay *in vitro* with purified AmpC and
233 TMP, and saw no inhibition (**Extended Data Fig 3**).

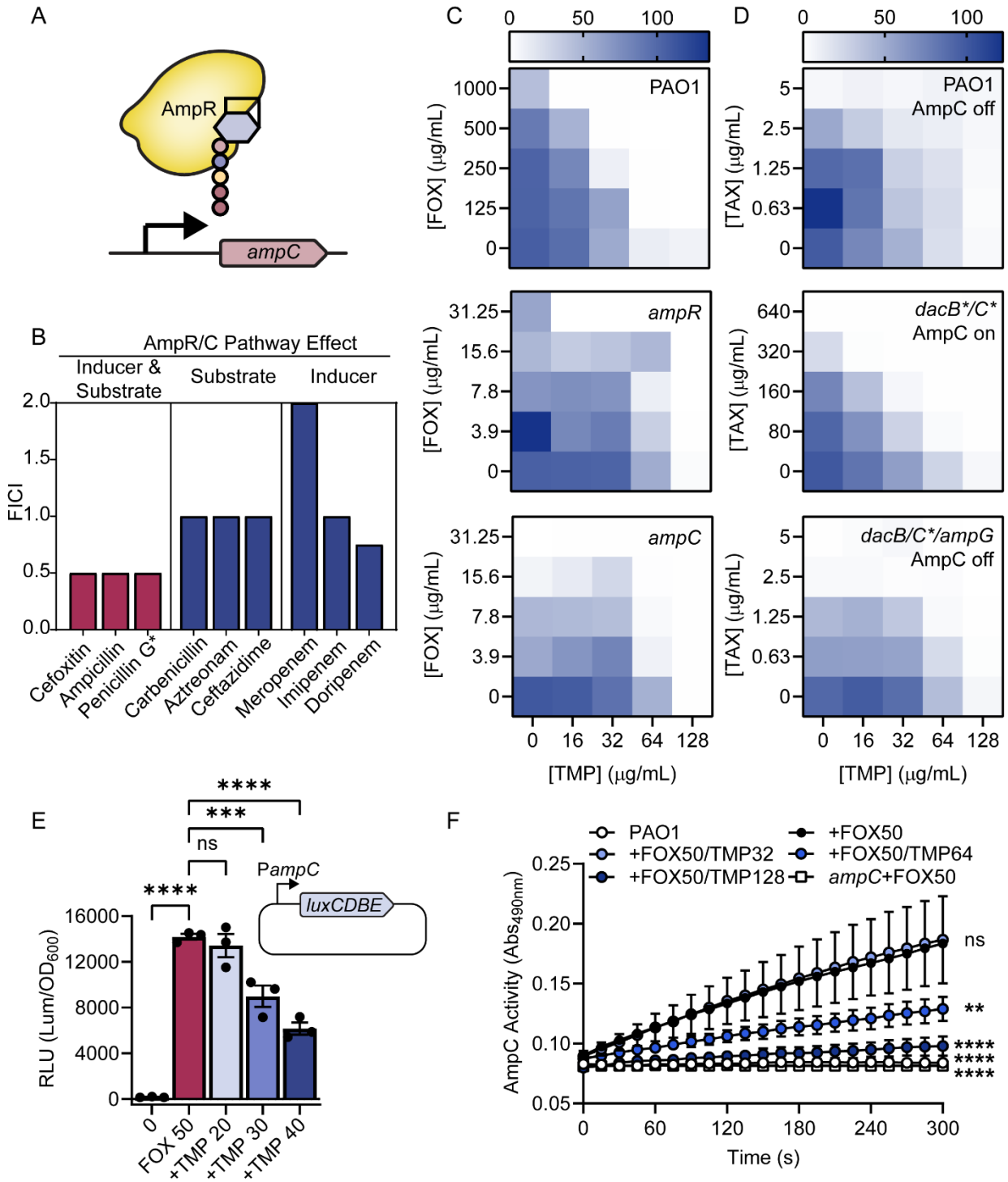


Figure 4. TMP potentiates β -lactams by impairing induction of *ampC* expression. **A)** A schematic showing activation of *ampC* transcription by AmpR bound to anhydromuramic acid (anhMurNAc)-pentapeptide. **B)** A summary of the fractional inhibitory concentrations (FICI) from 8x8 checkerboard assays of TMP and the antibiotics indicated below each bar. An asterisk indicates that an MIC was not reached for that antibiotic alone. The category of β -lactam is based on whether each β -lactam induces and/or is a substrate for AmpC. The mean FICI was calculated from two biological replicates of 8x8 checkerboard

assays. **C)** Heatmaps showing 5x5 checkerboards that condense data from 8x8 checkerboards. The intensity of the blue colour corresponds to the growth as a percent of the vehicle control shown in the legend above the top heatmap. The TMP concentrations are listed at the bottom and FOX concentrations on the left. Note that the FOX concentration range is lower for the *ampC* and *ampR* mutants (middle and bottom) as these mutants are more sensitive. The strain is listed in top right corner of the corresponding heatmap. Checkerboards were performed in biological duplicate, and heatmaps are shown for a representative replicate. **D)** Heatmaps showing 5x5 checkerboards that condense data from 8x8 checkerboards. The intensity of the blue colour corresponds to the growth as a percent of the vehicle control. TMP concentrations are listed at the bottom and TAX concentrations on the left. The TAX concentration range is higher for the *dacBC*** mutant as this mutant is more resistant. The strain and status of the AmpC pathway are listed in the top right corner of the corresponding heatmap. Checkerboards were performed in biological duplicate, and heatmaps are shown for a representative replicate. **E)** The level of *ampC* promoter-driven relative luminescence (RLU, calculated as arbitrary luminescence units divided by the matched OD₆₀₀ growth value) across the different treatment conditions. A schematic showing the plasmid construct containing the *ampC* promoter upstream of the *luxCDBE* genes is shown above the graph. “0” indicates no antibiotic added, FOX50=50 µg/mL cefoxitin, +TMP 20/30/40= 20, 30, or 40 µg/mL trimethoprim added in addition to 50 µg/mL cefoxitin. The bars represent the mean RLU, the individual data points are shown as black circles, and error bars are the standard error of the mean. Technical triplicates were performed for two biological replicates, and a representative replicate is shown. A one-way ANOVA followed by a Dunnett’s multiple comparisons test was performed to compare all the data to the FOX50 condition. ns=not significant, ***=p<0.001, ****=p<0.0001. **F)** Nitrocefin hydrolysis (measured as absorbance at 490 nm) over time. The symbols show the mean of two data points from technical duplicates, the error bars show the standard error of the mean, and the lines connect each symbol over time. The circles show the wild-type strain treated as in the legend. The square symbols indicate the negative control *ampC* treated with cefoxitin. Experiments were performed in technical and biological duplicate, and data from a representative biological replicate are shown.

234

235 *Determinants of the TMP-FOS interaction*

236 With the mechanism of TMP-FOX potentiation clarified, we next addressed the
237 mechanism of TMP-FOS potentiation. Unlike β-lactams, FOS inactivates a single cytoplasmic
238 target (MurA), which can be modelled *in silico*. Flux through folate and PG synthesis pathways
239 can be modelled using genome-scale metabolic reconstruction (GEMs) and predicted with
240 constraint-based flux balance analysis (FBA) using COBRA⁴⁸. We ran an established *P.*
241 *aeruginosa* GEM through the COBRA FBA to model drug-drug interactions, to determine if
242 TMP/SUL potentiation of FOS could be predicted from known *P. aeruginosa* physiology⁴⁹. The
243 model predicted TMP-SUL potentiation (**Extended Data Fig 4a**), but not the TMP/SUL-FOS
244 interaction (**Extended Data Figs 4b and c**), suggesting that the latter stems from an aspect of
245 cell physiology – possibly PG recycling – not adequately modelled by FBA.

246 We next looked for genetic interactions with TMP-FOS. First, we found that FOS could
247 not potentiate TMP or SUL against the hypersensitive *oprF* mutant, suggesting that loss of *oprF*

248 and FOS treatment have overlapping effects on antifolate susceptibility (**Extended Data Fig 5**).
249 However, loss of potentiation in the *oprF* background was not particularly informative because
250 OprF has a number of predicted roles⁵⁰. To identify additional mutants with changes in TMP-
251 FOS potentiation, we screened a PA14 transposon mutant library⁵¹ in 1536-colony density on
252 four agar conditions: no drug, sub-MIC TMP, sub-MIC FOS, or a synergistically lethal
253 combination of the two (**Figs 5a and Extended Data 6a**). This approach allowed us to identify
254 mutants with altered susceptibility to TMP or FOS alone, or mutants such as *oprF* in which
255 TMP-FOS interaction was lost. A subset of mutants grew on the combination plate, suggesting
256 loss of synergy (**Extended Data Fig 6b**). Mutants resistant to only TMP or FOS also grew on
257 the TMP+FOS plates, and we identified those with known changes in susceptibility to TMP or
258 FOS (*anmK*⁵², *oprM*⁵³, *folE2*⁵⁴, *glpT*⁴⁴, **Fig 5b**), providing internal validation of the screen. A
259 number of purine biosynthesis mutants, including *purN*, *purF*, *purL*, *purC*, and *purD*, had
260 increased sensitivity to the TMP/FOS combination. We cherry-picked eight purine biosynthesis
261 mutants, including the five above, from the transposon library and performed follow-up
262 checkerboards. Some mutants displayed a small increase in TMP sensitivity, which was
263 expected given that purine biosynthesis depends on folate (**Extended Data Fig 7**). Notably,
264 *purC* and *purM* mutants were 8 and 4 times more sensitive to TMP, while the *purM* mutant was
265 ~4 times more resistant to FOS. As well, *purD* and *purN* mutants were slightly more sensitive to
266 the TMP/FOS combination. Although it is still unclear how disruption of purine biosynthesis
267 impacts FOS susceptibility, these data suggest a possible mechanism underlying the TMP-FOS
268 interaction.

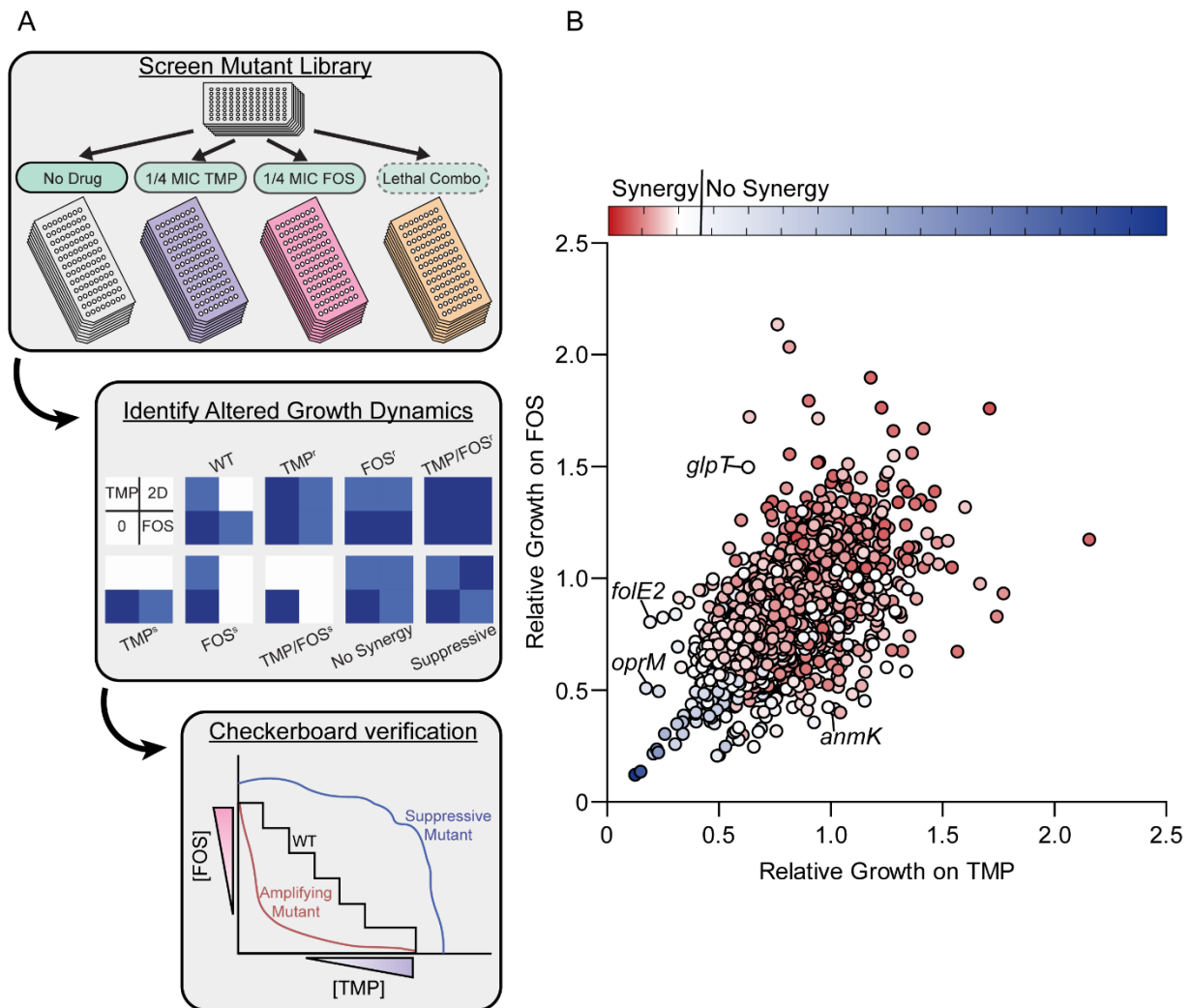


Figure 5. A genome-wide screen of TMP-FOS interaction determinants. A) A schematic outlining the mutant library screen, analysis, and follow-up workflow. The top panel shows the four solid medium conditions on which the library was pinned. The middle panel shows the predicted outcomes of mutants in the screen. “2D” indicates the two-drug treated condition, while “s” and “r” indicate hypersensitive or resistant, respectively. Blue represents growth. The bottom panel shows the predicted outcomes for the wild-type strain and different mutants on a checkerboard assay. **B)** A scatter plot showing the results of the PA14 library screen. Normalized growth relative to the untreated control for the TMP (X-axis) or FOS (Y-axis)-only conditions are plotted on the axes. Red and blue indicate synergy or no synergy, respectively, while colour intensity indicates the degree of synergy. Data points for select mutants that internally validate the screen are labelled.

269

270 Disruption of PG recycling by TMP

271 Aberrant PG recycling is a common determinant of sensitivity to both FOS and FOX in *P.*
 272 *aeruginosa*⁵⁵, consistent with our observation that TMP effects on AmpC expression were
 273 AmpG-dependent. To explore this further, we reasoned that treatment with TMP might change

274 the abundance or proportions of soluble PG recycling fragments, and used LC-MS to measure
275 abundance of select soluble PG species. In TMP-treated samples, we observed significantly
276 increased GlcNAc-anhMurNAc and decreased anhMurNAc abundance (**Figure 6**), suggesting a
277 possible block in PG recycling. Disaccharides lacking the stem peptide are products of
278 amidases, either periplasmic or cytoplasmic, and a substrate for the NagZ β -*N*-
279 acetylglucosaminidase. AmpD amidase activity is negatively correlated with AmpC induction⁵⁶,
280 while NagZ activity is required for FOS resistance⁵⁷, so accumulation of this disaccharide is
281 consistent with known factors that increase FOX and FOS sensitivity.

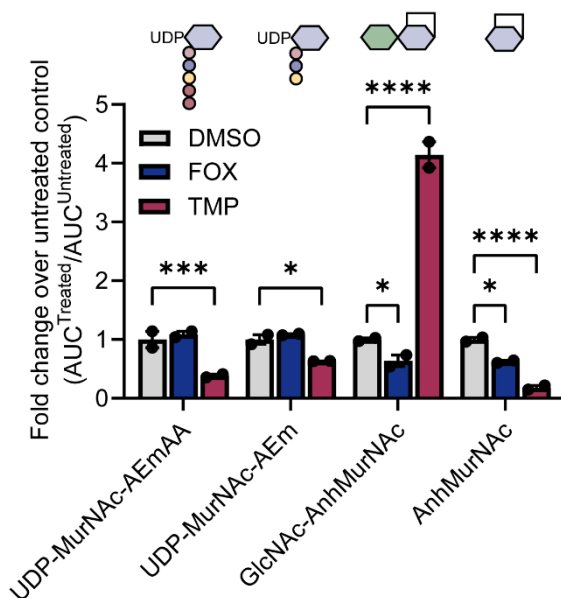


Figure 6. TMP treatment drives accumulation of the GlcNAc-anhMurNAc PG recycling intermediate. The abundance of different soluble PG metabolites was measured by LC-MS and quantified by integrating the peak of the extracted ion chromatogram corresponding to each species' M/Z. The PG metabolites are listed on the X-axis and a cartoon of each is shown above the corresponding bar. AEmAA = the five amino acids of the stem peptide. The Y-axis shows the integrated peak value or area under the curve (AUC) for each condition and species divided by AUC of the DMSO control sample for the matched species, and the data are represented as the fold-change relative to the control. Therefore, each DMSO condition has a mean fold change of 1. Two biological replicates were performed, each with two technical replicates. One representative biological replicate is shown, and each individual data point is shown as a black circle. The bars indicate the mean of the technical replicates, and the error bars show the standard of the mean. The grey, blue, and red bars correspond to DMSO, FOX (50 μ g/mL), and TMP (64 μ g/mL) treated samples, respectively. A two-way ANOVA followed by Dunnett's multiple comparisons test was performed to compare the FOX and TMP treated conditions to the DMSO control. *= $p < 0.05$, ***= $p < 0.001$, ****= $p < 0.0001$.

282

283 *A novel dual inhibitor overcomes meropenem resistance by targeting FolP and NDM-1*

284 Having established at least two ways that anti-folates may impact susceptibility to cell
285 wall-targeting antibiotics, we leveraged the folate-PG relationship for rational design of a novel
286 dual-function small molecule inhibitor. The recently developed metallo- β -lactamase (MBL)
287 inhibitor ANT-2681 has some structural resemblance to sulfathiazole⁵⁸, a sulfonamide antibiotic
288 that inhibits folate synthesis (**Fig 7a**)⁵⁹. ANT-2681 contains the core 2-sulfanilamidothiazole of
289 sulfathiazole with some additional substituents, including a carboxylate moiety that is required
290 for MBL inhibition via coordination to active-site zinc ions. The key structural feature of
291 sulfathiazole is the sulfanilamide that competes with *para*-aminobenzoic acid (PABA) for the
292 FolP-binding pocket⁵⁹. An X-ray crystal structure of sulfathiazole-bound FolP showed that the
293 variable ring of sulfonamides sits outside the binding pocket and thus could likely tolerate
294 substitution on the thiazole moiety⁵⁹.

295 Leveraging this information, we synthesized MLLB-2201, a carboxylate-containing
296 sulfonamide with potential dual activity against FolP and MBLs (**Fig 7a**). Gratifyingly, MLLB-
297 2201 was a low micromolar inhibitor of the MBLs NDM-1 and VIM-2, with IC₅₀ values of 1.8 and
298 14.5 μ M, respectively (**Fig 7b**). Despite having a lower IC₅₀ than ANT-2681⁵⁸, MLLB-2201 still
299 restored meropenem activity against an *E. coli* strain expressing NDM-1, whereas sulfathiazole
300 could not (**Fig S8a**). Although treatment with MLLB-2201 alone resulted in minimal growth
301 inhibition, it synergized with TMP against the Gram-positive pathogen, methicillin-resistant
302 *Staphylococcus aureus* (**Fig S8b**), suggesting some FolP inhibition. Finally, when MLLB-2201
303 was tested in combination with TMP and meropenem against the *E. coli* strain expressing NDM-
304 1, we observed three-way synergy (**Fig 7c**). MLLB-2201 (16 μ g/mL) in combination with TMP
305 (31.25 ng/mL) and meropenem (4 μ g/mL) inhibited growth of this strain, representing 16-fold
306 and 64-fold reductions in the single-antibiotic MICs.

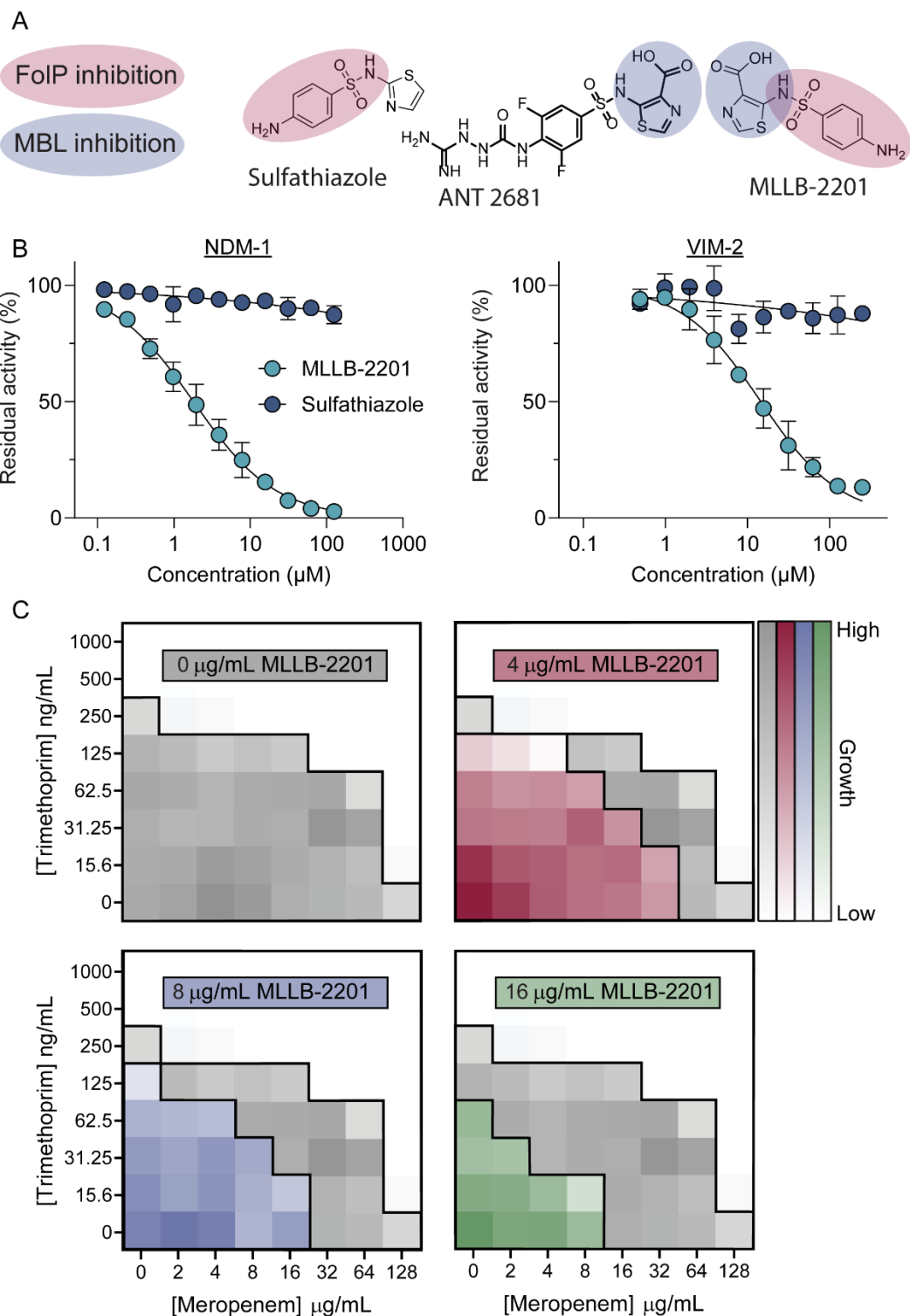


Figure 7. A dual-active inhibitor of metallo- β -lactamases overcomes resistance to meropenem via triple synergy. A) Chemical warheads responsible for FolP and metallo- β -lactamase inhibition are highlighted in red and blue, respectively. **B)** Effects of MLLB-2201 (light blue circles) and sulfathiazole (dark blue circles) treatment on NDM-1 (left) and VIM-2 (right) hydrolysis of meropenem. Increasing concentrations of each inhibitor are plotted on

the X-axis, while the residual enzyme activity is plotted on the Y-axis as a percent of the uninhibited enzyme activity. Two biological replicates were performed, each with two technical replicates. A representative biological replicate is shown, with the mean of the technical replicates plotted as a coloured circle and the standard error of the mean plotted as error bars. To calculate IC₅₀ values, curves were fitted to each data series and are shown as a black line. **C)** Three-way checkerboards showing the effects of trimethoprim, meropenem, and MLLB-2201 on growth of an *E. coli* strain expressing NDM-1. The concentrations of trimethoprim and meropenem are consistent across each checkerboard and the values are shown on the outer left and bottom axes, respectively. The concentration of the 3rd compound, MLLB-2201, increases from left to right and top to bottom, and is indicated within each checkerboard. The growth values for the three triple-combination checkerboards are overlaid in coloured squares on top of the trimethoprim and meropenem-only checkerboard data to illustrate the effects of MLLB-2201 addition. The intensity of each colour indicates the relative growth where white represents no growth and full colour saturation represents full growth. Checkerboard assays were repeated in biological duplicates and data from a representative replicate are shown.

307

308 **Discussion**

309 Tetrahydrofolate plays a central role in one-carbon metabolism and the synthesis of
310 critical metabolites, including thymidylate, purines, methionine, and glycine/serine
311 interconversion. Given the importance of these metabolites for growth, disrupting folate
312 biosynthesis is an effective strategy to kill pathogenic bacteria. The widespread dependence on
313 folate metabolism for growth resulted in many interesting reports of potential connections
314 between folate metabolism and broader cell physiology; however, the many consequences of
315 folate inhibition that drive these observations also makes them challenging to study. Using
316 bioinformatics, microscopy, and chemical genetics, we characterized the relationship between
317 folate and PG metabolism in the important pathogen, *P. aeruginosa*. Our data point to a
318 requirement for folate metabolism in maintaining cell envelope integrity as well as for full
319 induction of *ampC* expression through the AmpR pathway.

320 Early hypotheses about connections between folate and PG metabolism date back
321 about 50 years¹¹, and likely stemmed from observation of morphological changes that followed
322 SulA-mediated inhibition of cell division by induction of the SOS response. Investigation of the
323 mechanism of mutual (rather than unidirectional) potentiation between TMP and SUL led to a
324 model where these antibiotics were proposed to increase one another's entry into the cell⁶⁰.
325 Their synergistic mechanism is now known to arise from early folate pathway inhibition by a
326 substrate accumulation feedback loop⁶¹, however data from the group that originally proposed
327 increased compound uptake showed a striking effect of folate inhibition on the abundance of
328 lipid II in *E. cloacae*¹⁵. Thus, while the initial model of mutual of potentiation was incorrect, the

329 data supporting that model still revealed relevant effects of folate inhibition on the cell wall,
330 which we further characterized here. This work also provides potential insight into the
331 synergistic mechanisms of an antifolate-fosfomycin combination that was recently patented²².

332 Our data suggest that folate inhibition affects PG recycling. Using inactive point mutants
333 of PBP4 and PBP5, we showed that overproduction of the sentinel anhMurNAc-pentapeptide
334 recycling product that triggers AmpR-mediated expression of *ampC* led to TMP potentiation of
335 non-inducing β -lactams. This potentiation also required AmpG, the muropeptide permease, and
336 AmpR, suggesting that the sentinel product must enter the cytoplasm and activate the pathway
337 by the canonical mechanism. Disrupting PG recycling in *P. aeruginosa* is also an effective
338 strategy to potentiate FOS, as the recycling pathway in *P. aeruginosa* bypasses MurA^{62,63}.
339 Antifolate-mediated disruption of PG recycling explains why TMP uniquely potentiated FOX and
340 FOS in our initial PG-inhibitor interaction screen. Accordingly, we found that TMP significantly
341 increased the abundance of GlcNAc-anhMurNAc and decreased the abundance of anhMurNAc.
342 This finding supports the idea that TMP treatment disrupts PG recycling. Accumulation of
343 stemless PG glycans is indicative of amidase activity, and the increase in GlcNAc-anhMurNAc
344 coupled with a decrease in anhMurNAc suggests a possible bottleneck at the NagZ recycling
345 step, either from reduced NagZ activity or increased production of stemless disaccharides.
346 Alternatively, the lytic transglycosylase RlpA specifically cleaves stemless PG and liberates it for
347 recycling⁶⁴. Therefore, an increase in RlpA activity could also generate an excess of GlcNAc-
348 anhMurNAc.

349 Despite defining the interaction of antifolates with specific PG inhibitors, we have yet to
350 precisely identify which effects occurring downstream of folate inhibition are responsible for
351 compromising PG metabolism. Our screen of the PA14 transposon library pointed to impacts on
352 purine biosynthesis. The screen also identified mutants that were hypersensitive to TMP or
353 FOS. Examination of purine mutants confirmed that *purC* and *purM* are hypersensitive to TMP,
354 suggesting that small molecule inhibitors of these steps in purine synthesis could sensitize *P.*
355 *aeruginosa* to the widely used TMP-SUL combination, cotrimoxazole. Previous work in *B.*
356 *subtilis* showed that depleting purine but not thymidylate production (both folate dependent
357 metabolites) caused a large decrease in PG turnover^{65,66}. This observation aligns with the
358 results of our mutant screen and the finding that DNA-synthesis inhibition is insufficient for FOS
359 or FOX potentiation. It is unclear how the cells might sense purine depletion and concomitantly
360 decrease the rate of PG turnover. Changes in the purine-dependent signalling nucleotide c-di-
361 AMP affect PG synthesis in some bacteria⁶⁷, but this second messenger has not yet been

362 reported in *P. aeruginosa*. To uncover more factors involved in regulating PG turnover, the
363 *dacBC*** mutant that has constitutively elevated *ampC* expression could be used. Mutations that
364 decrease *ampC* promoter activity in that background are likely to be compromised in PG
365 recycling. Such a screen could uncover new factors affecting PG turnover, and point to novel
366 ways to increase sensitivity of *P. aeruginosa* to AmpC-inducing β -lactams. Here we identified
367 TMP as one such inhibitor that indirectly reduces degradation of β -lactams by AmpC, opening a
368 new avenue for possible β -lactam adjuvant development.

369 Building upon the findings that folate inhibitors can potentiate β -lactams in *P.*
370 *aeruginosa*, we designed MLLB-2201 as a dual inhibitor of metallo- β -lactamases and FolP, the
371 target of sulfonamides. In theory, this strategy could achieve three-way potentiation with a β -
372 lactam and TMP against *P. aeruginosa*. 1) TMP and MLLB-2201 synergize by inhibiting different
373 steps in folate metabolism, similar to TMP-SUL. 2) MLLB-2201 blocks β -lactam degradation by
374 metallo- β -lactamases. 3) TMP and MLLB-2201 decrease *ampC* induction, thus reducing β -
375 lactam hydrolysis by AmpC. While we achieved some of these effects, *P. aeruginosa* remained
376 largely unaffected by MLLB-2201 alone. A medicinal chemistry effort is currently underway to
377 increase the potency of MLLB-2201, with a particular focus on improving FolP inhibition and
378 anti-*Pseudomonas* activity. Together, this work demonstrates the potential for new, dual-target
379 metallo- β -lactamase inhibitors with antibiotic and adjuvant properties.

380 Folate metabolism is vital to multiple aspects of bacterial physiology and there is an
381 increasing recognition of the extent to which PG metabolism is coordinated with other cellular
382 processes. For example, tailoring of PG peptide stems by D,D-carboxypeptidases, the targets of
383 FOX, regulates activity of the Bam β -barrel assembly complex⁶⁸. MurA and LpxC catalyze the
384 first committed steps of PG and lipopolysaccharide (LPS) biosynthesis, respectively, and in *P.*
385 *aeruginosa*, the FOS target MurA activates LpxC through a physical interaction that ensures the
386 balanced consumption of UDP-GlcNAc by each pathway⁶⁹. To coordinate positioning of the
387 daughter chromosomes during cell division, SlmA interacts with and prevents polymerization of
388 FtsZ⁷⁰. Our data suggest that the folate and PG pathways are more intimately connected than
389 previously appreciated, and that targeting their intersection could open new avenues for drug
390 development.

391

392

393

394 **Materials and Methods**

395 **Bacterial strains and growth conditions**

396 All strains (**Table S1**) were stored at -80°C in 15% glycerol stocks that were used to
397 inoculate overnight cultures. Overnight cultures were grown in 3 mL of lysogeny broth (LB,
398 Lennox, Bioshop) at 37°C while shaking at 200 RPM. A 1:100 dilution of overnight cultures into
399 fresh LB media was performed for subcultures. Antibiotics for plasmid maintenance were added
400 when appropriate at the following concentrations: gentamicin at 15 µg/mL (Gent15) for *E. coli* or
401 30 µg/mL (Gent30) for *P. aeruginosa*; ampicillin at 100 µg/mL (Amp100) for *E. coli*; carbenicillin
402 at 200 µg/mL (Carb200) for *P. aeruginosa*; 50 µg/mL kanamycin (Kan50) for *E. coli*.

403 **Plasmid and strain construction**

404 pMS403 was constructed from pMS402Gm. pMS402Gm was created by digesting
405 pMS402 and pPS856 with PstI, then isolating the gentamicin resistance cassette from the
406 pPS856 digest reaction and using T4 ligase to insert the gentamicin cassette into pMS402.
407 Next, pMS402Gm was digested at a BsiWI site within the *DHFRII* gene, the overhangs were
408 filled in using the Klenow fragment polymerase, and the resulting blunt ends were rejoined by
409 ligation with T4 ligase and transformed into *E. coli* DH5α. Transformants were selected on
410 Gent15 and tested for trimethoprim sensitivity to determine *DHFRII* inactivation, then confirmed
411 by sequencing.

412 pMS403(*PampC*) was created by PCR amplifying the *ampC* promoter with *PampC* Fwd
413 and *PampC* Rvs (all primers are listed in **Table S1**), then digesting both pMS403 and the
414 purified PCR product with BamHI and XbaI, and ligating the two purified digest products with T4
415 ligase. The ligation product was transformed into *E. coli* DH5α and plasmids isolated from
416 Gent15 resistant colonies were verified for the correct insert with sequencing.

417 pUCP20 (*DHFRII*) was created by PCR amplifying the *DHFRII* gene from the pMS402
418 backbone with *DHFRII* Fwd and *DHFRII* Rvs primers. Next, pUCP20 and the purified PCR
419 product were digested with EcoRI and HindIII and the purified digest products were ligated
420 together with T4 ligase, then transformed into *E. coli* DH5α. Plasmids from Amp100 resistant
421 colonies were isolated and sequenced to verify the correct insert.

422 pEX18Gm (*ampC*), pEX18Gm (*ampC* S90A), and pEX18Gm (*ampG*) were constructed
423 by digesting the gBlock inserts in pUC57 (Genscript) with EcoRI and HindIII (or SacI for *ampG*).
424 The inserts were gel purified and ligated using T4 ligase into empty pEX18Gm that was

425 digested with EcoRI and HindIII/SacI. The ligations were transformed into *E. coli* DH5 α and
426 transformants were selected on Gent15 plates. Plasmids isolated from the transformants were
427 sequenced to verify the correct insertion.

428 pEX18Gm (*ampR*) was constructed by amplifying 500 bp regions of chromosomal DNA
429 from PAO1 that flank the 5' and 3' ends of *ampR* using two PCR reactions containing the
430 *dampR* Up Fwd and *dampR* Up Rvs primers, or the *dampR* Dwn Fwd and *dampR* Dwn Rvs
431 primers. The purified PCR products from these reactions were combined and used as templates
432 for overlap extension PCR with the *dampR* Up Fwd and *dampR* Dwn Rvs primers, and the
433 purified PCR product was digested with BamHI and HindIII. The purified digest product was
434 ligated into linearized pEX18Gm (digested with BamHI and HindIII) using T4 ligase. The
435 ligations were transformed into *E. coli* DH5 α and transformants were selected on Gent15 plates.
436 Plasmids isolated from the transformants were sequenced to verify the correct insertion.

437 pMS403 and pUCP20-based plasmids were introduced to *P. aeruginosa* by
438 electroporation. pEX18Gm-based plasmids were first introduced into *E. coli* SM10 by
439 electroporation, then *E. coli* SM10 was used to transfer the plasmid into *P. aeruginosa* by
440 conjugation. *P. aeruginosa* cells containing the plasmid were selected for on *Pseudomonas*
441 isolation agar (PIA) containing 100 μ g/mL of gentamicin to select for the first recombination
442 event. Colonies from the PIA Gent100 plates were streaked onto LB 5% sucrose no NaCl agar
443 plates and incubated at 30°C to select for a second recombination event. Colonies from the LB
444 sucrose plates were patched onto LB and LB Gent30 plates. Patches that grew on LB but not
445 LB Gent30 plates were tested for loss of intrinsic ampicillin resistance, which indicates loss of
446 AmpC activity. Mutants were confirmed with PCR.

447 Strains containing the *dacB*^{*}/*dacC*^{*} catalytically inactive point mutations were
448 constructed by site-directed mutagenesis of PAO1 wild-type alleles. The *dacB*^{*} primer included
449 a TCG→GCG mutation that converted the serine 72 codon to an alanine, while the *dacC*^{*}
450 primer included an AGC→GCG mutation that converted the serine 64 codon to an alanine.
451 These mutant alleles were crossed into the chromosome of PAO1 using allelic exchange with
452 the pEX18Gm plasmid. Mutants were confirmed by sequencing mutant alleles amplified with
453 PCR.

454 **Determination of minimal inhibitory concentration**

455 Minimum inhibitory concentration (MIC) assays were performed by passaging overnight
456 cultures into 3 mL of 10:90 LB (1:9 ratio of LB to phosphate buffered saline) and growing to an

457 OD₆₀₀ of ~0.1-0.3. Cultures were normalized to an OD₆₀₀ of 0.1 and diluted 1:500 into 10:90 LB.
458 Two microliters of the indicated antibiotic were added to the wells in rows A-F of a 96-well, and
459 2 µL of the vehicle control was added to the wells in rows G and H. Antibiotics were added at
460 75x the final concentration. After the antibiotics were added, 148 µL of the diluted, normalized
461 culture was added to the wells in rows A-G. Row H served as a sterility control and 148 µL of
462 sterile 10:90 was added to these wells. Plates were incubated at 37°C while shaking for 18
463 hours. Growth (OD₆₀₀) was measured using a plate reader (Multiskan Go, Thermo Fisher
464 Scientific).

465 **Checkerboard assays**

466 Overnight cultures were diluted 1:100 into 3 mL of LB and grown to ~0.1-0.3 OD₆₀₀. For
467 PA14 transposon mutants, overnight cultures were made using LB Gent30. Subcultures were
468 normalized to 0.1 OD₆₀₀ and diluted 1:500 in fresh LB. Serial dilutions of 1 µL of one antibiotic
469 was added in decreasing concentrations across rows A-H for columns 3-10, while the second
470 antibiotic was added in decreasing concentrations across columns 3-10 for rows A-H. Two
471 microliters of the respective vehicle control were added to columns 1, 2, 11, and 12. The 1:500
472 diluted cultures were added to the wells to a final volume of 150 µL. Sterility wells were filled to
473 150 µL with sterile LB. Plates were incubated at 37°C for 18 h while shaking at 200 rpm. Growth
474 (OD₆₀₀) was measured using a plate reader (Multiskan Go, Thermo Fisher Scientific).

475 **Bioinformatic analysis**

476 We established a local blast database by compiling a comprehensive genome dataset
477 from the GTDB, encompassing a total of 47,894 genomes as of June 2023. Subsequently, we
478 conducted tblastn searches on this local GTDB database⁷¹, comparing two protein sequences,
479 *folP* and *glmM*, from PAO1. To identify the best matches, we analyzed the tabular blast output
480 in R v4.2.0, selecting top hits based on their percent identity. We also removed duplicate hits
481 within contigs and calculated the genomic distance between the starting point of *glmM* and the
482 ending point of *folP*.

483 To test whether there is a potential phylogenetic association between *folP* and *glmM*, we
484 focused on a specific subset of proteobacterial genomes from our database. Specifically, we
485 targeted proteobacterial families with a representation of 100 or more genomes labeled as
486 complete in the RefSeq database. This subset, encompassing a total of 939 genomes, was then
487 used to construct a phylogenetic tree. This tree was generated through sequence alignment of
488 GTDB markers and subsequent approximately maximum likelihood tree construction using

489 FastTree 2⁷². The results were visually represented using the ggplot package in R v4.2.0, and
490 the phylogenetic tree was further enhanced and visualized using iTOL⁷³.

491 **Luminescent *ampC* promoter-reporter assay**

492 Overnight cultures of PAO1 containing pMS402(Empty) or pMS402(P*ampC*) were made
493 by inoculating 3 mL of LB Gent30 from frozen stocks and were incubated at 37 °C with shaking.
494 Subcultures were made by transferring 120 µL of the overnights into 3 mL of LB Gent30 and
495 incubated at 37°C with shaking for ~2 hours until an OD₆₀₀ of ~0.1-0.3 was reached. Cultures
496 were normalized to an OD₆₀₀ of 0.1, then diluted 1:500 in fresh LB Gent30. Assays were
497 prepared in white-walled 96-well plates with clear bottoms (Corning). Two microlitres of dilutions
498 of trimethoprim were added across rows A-E at the indicated concentrations. Cefoxitin was
499 added to the wells in rows A-F at a final concentration of 50 µg/mL. A DMSO vehicle control was
500 added to wells in rows G and H. Afterwards, 148 µL of the diluted culture was added to each
501 well of the plate, except row H, which served as a sterility control and received 148 µL of LB
502 Gent 30. The plate was incubated at 37°C with continuous double orbital shaking for 16 hours in
503 a Synergy Neo (Biotek) plate reader. Growth (OD₆₀₀) and luminescence (luminescence fiber)
504 measurements were taken every 15 minutes and promoter activity at 8 hours was graphed.
505 Relative luminescence units (RLU) were calculated by dividing each well's luminescence value
506 by its growth at the corresponding time point.

507 **Determination of β-lactamase activity**

508 Whole cell AmpC activity was determined as previously described with minor
509 modifications⁷⁴. Briefly, overnight cultures were grown in LB and subcultures were made by
510 diluting the overnight culture 1:25 into fresh LB, then incubated for 2 hours at 37°C while
511 shaking. Cells were then passaged again into fresh media containing 50 µg/mL of cefoxitin or a
512 DMSO vehicle control, and trimethoprim at the indicated concentrations. The cultures were then
513 incubated again for ~2-3 hours until cells reached OD₆₀₀ 0.4-0.6, then the cultures were
514 normalized to 0.3 OD₆₀₀ and 1 mL of normalized culture was centrifuged at 21 000 x g for 1
515 minute. The supernatant was decanted, and the cell pellet was washed with 1 mL of 50 mM
516 sodium phosphate buffer (pH 7.4), then resuspended in 1 mL of the sodium phosphate buffer.
517 Cells placed on ice then lysed with sonication by a microtip (Sonicator 2000, Microsonix) using
518 three 10 second pulses with 10 second pauses between pulses. The cell debris was pelleted by
519 centrifugation at 21 000 x g for 5 minutes, and 500 µL of supernatant was moved to a fresh
520 Eppendorf tube. Five microliters of nitrocefin were added to wells of a clear flat-bottom 96 well

521 plate for a final concentration of 50 μ M, then 195 μ L of the clarified cell lysate was added to
522 each well. Absorbance at 490 nm was read with a spectrophotometer (Multiskan Go, Thermo
523 Fisher) immediately and for every 15 seconds thereafter until the linear range of the uninhibited
524 positive control was exceeded.

525 *In vitro* AmpC β -lactamase activity was measured with purified commercial AmpC
526 (Sigma Aldrich) from *P. aeruginosa*. AmpC was diluted to 400 nM (final concentration) across a
527 serial dilution of the indicated compound in 50 mM sodium phosphate buffer (pH 7.4), then
528 added to the wells of a 96 well plate containing nitrocefin at 50 μ M (final concentration) to a final
529 volume of 200 μ L. Then, absorbance at 490 nm was read immediately and for every 15 seconds
530 after for 10 minutes by a spectrophotometer (Multiskan Go, Thermo Fisher). The data within the
531 time points for the linear range of the no compound AmpC control were used for analysis.

532 *In vitro* metallo β -lactamase activity was measure using NDM-1 (5 nM) or VIM-2 (50 nM)
533 incubated in reaction buffer (25 mM HEPES-NaOH, 10 μ M ZnSO₄, pH 7.5) containing varying
534 amounts of inhibitor (500 – 1 μ M) and incubated for 5 min at room temperature. Residual
535 enzyme activity was determined by measuring β -lactam hydrolysis spectrophotometrically at
536 300 nm by adding a saturating amount of meropenem (500 μ M) to the reaction mixtures
537 containing enzyme and inhibitor. β -Lactamase assays were performed in a clear flat-bottom 96-
538 well plate at 25°C with a final assay volume of 200 μ L and monitored with a BioTek Synergy H1
539 microplate reader over 10 min. All reactions were performed in duplicate unless otherwise
540 stated.

541 **PA14 library screen**

542 Rectangular plates with 25 mL of LB 1.5% agar were poured that contained no antibiotic,
543 32 μ g/mL fosfomycin, 32 μ g/mL trimethoprim, or both antibiotics, and allowed to dry overnight.
544 An ordered PA14 transposon mutant library was pinned from source plates in 1536 colony
545 density onto the rectangular agar plates using a ROTOR HDA robotic colony replicator (Singer
546 Instruments). The screen was performed in duplicates using different source plates. Plates were
547 incubated for 18 h at 37°C, then imaged using a Phenobooth imaging system (Singer
548 Instruments) using the transmissive light mode. Images were further processed in FIJI (Image J)
549 as described previously⁷⁵. Briefly, the light absorbed by each colony was converted into an
550 integrated density value. Integrated densities were then normalized for plate position effects and
551 the relative growth was determined by comparing to the untreated control.

552 **Soluble muropeptide extraction and analysis**

553 Soluble muropeptides were prepared according to Weaver *et al.* (2022) with minor
554 modifications⁷⁶. Briefly, 50 mL flasks of LB were inoculated with 0.5 mL of an overnight culture.
555 Flasks were prepared in technical duplicate with antibiotics added at the indicated
556 concentrations. Cultures were incubated at 37°C while shaking until an OD₆₀₀ of ~0.4 was
557 reached. Then, flasks were immediately placed on ice, cultures were normalized to an OD₆₀₀ of
558 0.3, and 40 mL were transferred to pre-chilled 50 mL falcon tubes. The normalized cultures
559 were then centrifuged at 5030 x g and 4°C for 20 minutes on an Avanti J-26 XPI (Beckman-
560 Coulter) centrifuge (JS 5.3 rotor). The supernatant was decanted, and cell pellets were
561 resuspended in 1 mL ice cold 0.9% NaCl, washed twice with 1 mL 0.9% NaCl, and finally
562 resuspended in 1 mL of sterile nuclease free water. The Eppendorf tubes containing the
563 resuspended cells were boiled for 30 minutes to lyse the cells and centrifuged for 15 minutes at
564 21 000 x g in a benchtop centrifuge to pellet the cell debris. The supernatant was then passed
565 through a 0.2 µm filter and frozen at -80°C. Samples were concentrated as needed under
566 vacuum using a lyophilizer (Virtis). Lyophilized samples were dissolved in sterile nuclease free
567 water and the pH was adjusted to ~3 using formic acid. After adjusting the pH, samples were
568 centrifuged for 10 minutes at 21 000 x g in a benchtop centrifuge to pellet any precipitate.

569 Ten microliters of each sample were injected into an LC/Q-TOF (Agilent 6546) and
570 separated using an Eclipse Plus C18 column (Agilent, 95 Å pore size, 2.1x100 mm, 1.8 µm) at
571 50°C. Separating of PG species was achieved using a linear gradient of buffer A (water+0.1%
572 formic acid) to buffer B (acetonitrile+0.1% formic acid) over a 56 minute run time with a 0.4
573 mL/minute flow rate. The Q-TOF instrument was run in negative ionization mode with the
574 following parameters: 4000 V capillary voltage, 300°C source temperature, 300°C sheath gas
575 temperature, and a scan range of 100-1700 *m/z*. Data acquisition and analysis was performed
576 using the Agilent MassHunter qualitative analysis (v10.0) software. Extracted ion
577 chromatograms were manually curated using theoretical muropeptide masses. The selected
578 muropeptides were based on *m/z* values of 595.6639 for UDP-MurNAc-AEmAA (doubly
579 charged species), 524.6263 for UDP-MurNAc-AEm (doubly charged species), 477.1726 for
580 GlcNAc-anhMurNAc (singly charged species), and 274.0932 for anhMurNAc (singly charged
581 species).

582 **Western blots**

583 Quantification of total AmpC protein levels by western blot was performed exactly as
584 described in Lamers *et al.*⁷⁷. Samples were prepared identically, with the strains and conditions

585 described in the figure caption. The non-specific band shown in the image of the blot was used
586 as a loading control.

587 **Flux balance analysis**

588 The iPAO1 genome scale metabolic model developed by Zhu *et al.*⁴⁹ was imported into
589 Matlab R2020a (MathWorks) and the Cobra toolbox (Version 3.0)⁴⁸ was used to perform FBA
590 with a Gurobi mathematical solver (Version 9.0.2). FBA-Div was used to simulate antibiotic
591 treatment⁷⁸, where the substrates of inhibited reactions are diverted to a waste reaction. The
592 iPAO1 model contains an irreversible and reversible reaction for FolP; therefore, to inhibit both
593 reactions, the irreversible reaction (rxn02201) was removed from the model and flux through the
594 reversible reaction (rxn02200) was reduced, and the substrate (dihydropteroate) was diverted to
595 a waste reaction. To simulate a dilution range of antibiotic treatment, the optimal flux for each
596 reaction was determined under no reaction inhibition, then the flux rate was simulated at 20%
597 intervals from 0-100% of the optimal flux rate. The calculated biomass production rate at the
598 steady state for each interval was determined in single and double reaction inhibitions to
599 generate growth values that were used to create a checkerboard. Rich media growth conditions
600 with an abundance of nutrients was assumed for the metabolic modelling.

601 **Microscopy**

602 Overnight cultures were diluted 1:100 in LB with or without the trimethoprim at the
603 indicated concentrations and incubated until an OD₆₀₀ of ~0.4. All cultures were normalized to
604 an OD₆₀₀ of 0.3, then 1 mL of each culture was pelleted, washed with 1 mL of sterile PBS, then
605 resuspended in 100 µL of sterile PBS. Four microliters of the resuspended cells were spotted on
606 1.5% M9+glucose agarose pads on glass slides and covered with coverslips. Cells were imaged
607 with a Nikon Ti-2 Eclipse inverted confocal microscope using a 60X oil immersion objective lens.
608 Identical settings were used for all micrographs captured.

609 **Synthesis of MLLB-2201**

610 See supplemental information.

611 **Construction of graphs, structures, and statistical analysis**

612 All graphs and checkerboards were created using GraphPad (Prism, Version 10), and
613 statistical analyses were performed using GraphPad as well. The graph in Figure 1C was
614 created using R and the tree in Figure 1D was created using iTOL. The structures shown in

615 Figure 1A were downloaded from the protein data bank and modelled in ChimeraX (Version
616 1.4)⁷⁹.

617

618 **Acknowledgements**

619 This work was funded by Natural Sciences and Engineering Research Council of Canada
620 (www.nserc-crsng.gc.ca) grant RGPIN-2021-04237 to LLB and by infrastructure funding from
621 Canada Foundation for Innovation and the Ontario Research Fund (ORF-RE09-047). LLB holds
622 a Tier I Canada Research Chair in Microbe-Surface Interactions (CRC-2021-00103). LNY holds
623 an NSERC PGS-D award. OG holds a Chercheur Boursier Junior 1 Fellowship, from the Fonds
624 de Recherche du Québec-Santé.

625

626 **References**

- 627 1. Yaeger, L. N., Coles, V. E., Chan, D. C. K. & Burrows, L. L. How to kill *Pseudomonas*—
628 emerging therapies for a challenging pathogen. *Ann. N. Y. Acad. Sci.* **1496**, 59–81
629 (2021).
- 630 2. Tyers, M. & Wright, G. D. Drug combinations: a strategy to extend the life of antibiotics in
631 the 21st century. *Nat. Rev. Microbiol.* **17**, 141–155 (2019).
- 632 3. Bush, K. & Bradford, P. A. Interplay between β -lactamases and new β -lactamase
633 inhibitors. *Nat. Rev. Microbiol.* **17**, 295–306 (2019).
- 634 4. Estrada, A., Wright, D. L. & Anderson, A. C. Antibacterial antifolates: From development
635 through resistance to the next generation. *Cold Spring Harb. Perspect. Med.* **6**, (2016).
- 636 5. East, S. P. & Silver, L. L. Multitarget ligands in antibacterial research: Progress and
637 opportunities. *Expert Opin. Drug Discov.* **8**, 143–156 (2013).
- 638 6. Kordus, S. L. & Baughn, A. D. Revitalizing antifolates through understanding mechanisms
639 that govern susceptibility and resistance. *MedChemComm* **10**, 880–895 (2019).
- 640 7. Roland, S., Ferone, R., Harvey, R. J., Styles, V. L. & Morrison, R. W. The characteristics
641 and significance of sulfonamides as substrates for *Escherichia coli* dihydropteroate
642 synthase. *J. Biol. Chem.* **254**, 10337–10345 (1979).
- 643 8. Kwon, Y. K. *et al.* A domino effect in antifolate drug action in *Escherichia coli*. *Nat. Chem.*

- 644 *Biol.* **4**, 602–608 (2008).
- 645 9. Pritha Rao, T. V. & Kuzminov, A. Exopolysaccharide defects cause hyper-thymineless
646 death in *Escherichia coli* via massive loss of chromosomal DNA and cell lysis. *Proc. Natl.*
647 *Acad. Sci. U. S. A.* **117**, 33549–33560 (2020).
- 648 10. Fonville, N. C., Bates, D., Hastings, P. J., Hanawalt, P. C. & Rosenberg, S. M. Role of
649 RecA and the SOS response in thymineless death in *Escherichia coli*. *PLoS Genet.* **6**,
650 e1000865 (2010).
- 651 11. Dulaney, E. L. & Marx, L. M. A folic acid linked system in bacterial cell wall synthesis? *J.*
652 *Antibiot. (Tokyo)*. **24**, 713–714 (1971).
- 653 12. Daschner, F. Inhibition of cell wall synthesis by sulfonamides and trimethoprim.
654 *Chemotherapy* **22**, 12–18 (1976).
- 655 13. Wang, S., Arends, S. J. R., Weiss, D. S. & Newman, E. B. A deficiency in S-
656 adenosylmethionine synthetase interrupts assembly of the septal ring in *Escherichia coli*
657 K-12. *Mol. Microbiol.* **58**, 791–799 (2005).
- 658 14. Lazenby, J. J., Li, E. S. & Whitchurch, C. B. Cell wall deficiency – an alternate bacterial
659 lifestyle? *Microbiol. (United Kingdom)* **168**, (2022).
- 660 15. Richards, R. M. E. & Xing, D. K. L. Separation and Quantification of Murein and
661 Precursors from *Enterobacter cloacae* after Treatment with Trimethoprim and
662 Sulphadiazine. *J. Pharm. Pharmacol.* **46**, 690–696 (1994).
- 663 16. Geisinger, E. *et al.* Antibiotic susceptibility signatures identify potential antimicrobial
664 targets in the *Acinetobacter baumannii* cell envelope. *Nat. Commun.* **11**, 1–16 (2020).
- 665 17. Brazas, M. D. & Hancock, R. E. W. Using microarray gene signatures to elucidate
666 mechanisms of antibiotic action and resistance. *Drug Discovery Today* **10**, 1245–1252
667 (2005).
- 668 18. Zhou, A. *et al.* Synergistic interactions of vancomycin with different antibiotics against
669 *Escherichia coli*: Trimethoprim and nitrofurantoin display strong synergies with
670 vancomycin against wild-type *E. coli*. *Antimicrob. Agents Chemother.* **59**, 276–281
671 (2015).
- 672 19. Chandrasekaran, S. *et al.* Chemogenomics and orthology-based design of antibiotic

- 673 combination therapies. *Mol. Syst. Biol.* **12**, 872 (2016).
- 674 20. El Zahed, S. S. & Brown, E. D. Chemical-Chemical Combinations Map Uncharted
675 Interactions in *Escherichia coli* under Nutrient Stress. *iScience* **2**, 168–181 (2018).
- 676 21. Chevereau, G. & Bollenbach, T. Systematic discovery of drug interaction mechanisms.
677 *Mol. Syst. Biol.* **11**, 807 (2015).
- 678 22. Pace, J. L., Wiles, M. E., Adams, S. M. & Hussey, E. Combination therapy effective
679 against microorganisms, including drug resistant microorganisms. (2015).
- 680 23. Egan, A. J. F. & Vollmer, W. The physiology of bacterial cell division. *Ann. N. Y. Acad.*
681 *Sci.* **1277**, 8–28 (2013).
- 682 24. Typas, A., Banzhaf, M., Gross, C. A. & Vollmer, W. From the regulation of peptidoglycan
683 synthesis to bacterial growth and morphology. *Nat Rev Microbiol* **10**, 123–136 (2012).
- 684 25. Johnson, J. W., Fisher, J. F. & Mobashery, S. Bacterial cell-wall recycling. *Ann. N. Y.*
685 *Acad. Sci.* **1277**, 54–75 (2013).
- 686 26. Aliashkevich, A. & Cava, F. LD-transpeptidases: the great unknown among the
687 peptidoglycan cross-linkers. *FEBS J.* **289**, 4718–4730 (2022).
- 688 27. Yaeger, L. N. *et al.* A genetic screen identifies a role for *oprF* in *Pseudomonas*
689 *aeruginosa* biofilm stimulation by subinhibitory antibiotics. *bioRxiv* (2023).
- 690 28. Cassin, E. K. & Tseng, B. S. Pushing beyond the envelope: The potential roles of OprF in
691 *Pseudomonas aeruginosa* biofilm formation and pathogenicity. *J. Bacteriol.* **201**, (2019).
- 692 29. Sheng, Y. *et al.* Structural and functional similarities in the ADP-forming amide bond
693 ligase superfamily: Implications for a substrate-induced conformational change in
694 folylpolyglutamate synthetase. *J. Mol. Biol.* **302**, 425–438 (2000).
- 695 30. Eschenburg, S., Kabsch, W., Healy, M. L. & Schonbrunn, E. A new view of the
696 mechanisms of UDP-N-acetylglucosamine enolpyruvyl transferase (MurA) and 5-
697 enolpyruvylshikimate-3-phosphate synthase (AroA) derived from X-ray structures of their
698 tetrahedral reaction intermediate states. *J. Biol. Chem.* **278**, 49215–49222 (2003).
- 699 31. Jhee, K. H. *et al.* Stereochemistry of the transamination reaction catalyzed by
700 aminodeoxychorismate lyase from *Escherichia coli*: Close relationship between fold type
701 and stereochemistry. *J. Biochem.* **128**, 679–686 (2000).

- 702 32. Peisach, D., Chipman, D. M., Van Ophem, P. W., Manning, J. M. & Ringe, D. D-
703 Cycloserine inactivation of D-amino acid aminotransferase leads to a stable noncovalent
704 protein complex with an aromatic cycloserine-PLP derivative. *J. Am. Chem. Soc.* **120**,
705 2268–2274 (1998).
- 706 33. Rowsell, S. *et al.* Crystal structure of carboxypeptidase G2, a bacterial enzyme with
707 applications in cancer therapy. *Structure* **5**, 337–347 (1997).
- 708 34. Kochert, M. *et al.* Atomic-Resolution 1.3 Å Crystal Structure, Inhibition by Sulfate, and
709 Molecular Dynamics of the Bacterial Enzyme DapE. *Biochemistry* **60**, 908–917 (2021).
- 710 35. Sánchez-Osuna, M., Cortés, P., Barbé, J. & Erill, I. Origin of the mobile di-hydro-pterolate
711 synthase gene determining sulfonamide resistance in clinical isolates. *Front. Microbiol.*
712 **10**, 3332 (2019).
- 713 36. Watanabe, H., Mori, H., Itoh, T. & Gojobori, T. Genome plasticity as a paradigm of
714 eubacteria evolution. *J. Mol. Evol.* **44**, (1997).
- 715 37. Tavares, I. M., Leitão, J. H. & Sá-Correia, I. Chromosomal organization and transcription
716 analysis of genes in the vicinity of *Pseudomonas aeruginosa glmM* gene encoding
717 phosphoglucosamine mutase. *Biochem. Biophys. Res. Commun.* **302**, 363–371 (2003).
- 718 38. Fagen, J. R. *et al.* Comparative genomics of cultured and uncultured strains suggests
719 genes essential for free-living growth of *Liberibacter*. *PLoS One* **9**, (2014).
- 720 39. Henderson, G. B., Zevely, E. M. & Huennekens, F. M. Mechanism of folate transport in
721 *Lactobacillus casei*: Evidence for a component shared with the thiamine and biotin
722 transport systems. *J. Bacteriol.* **137**, 1308–1314 (1979).
- 723 40. Gardner, A. D. Morphological effects of penicillin on bacteria. *Nature* **146**, 837–838
724 (1940).
- 725 41. Yao, Z., Kahne, D. & Kishony, R. Distinct Single-Cell Morphological Dynamics under
726 Beta-Lactam Antibiotics. *Mol. Cell* **48**, 705–712 (2012).
- 727 42. Cushnie, T. P. T., O'Driscoll, N. H. & Lamb, A. J. Morphological and ultrastructural
728 changes in bacterial cells as an indicator of antibacterial mechanism of action. *Cellular*
729 *and Molecular Life Sciences* **73**, 4471–4492 (2016).
- 730 43. Mercier, R., Kawai, Y. & Errington, J. General principles for the formation and proliferation

- 731 of a wall-free (L-form) state in bacteria. *Elife* **3**, (2014).
- 732 44. Castañeda-García, A., Rodríguez-Rojas, A., Guelfo, J. R. & Blázquez, J. The glycerol-3-
733 phosphate permease GlpT is the only fosfomycin transporter in *Pseudomonas*
734 *aeruginosa*. *J. Bacteriol.* **191**, 6968–6974 (2009).
- 735 45. Ropy, A. *et al.* Role of *Pseudomonas aeruginosa* low-molecular-mass penicillin-binding
736 proteins in AmpC expression, β -lactam resistance, and peptidoglycan structure.
737 *Antimicrob. Agents Chemother.* **59**, 3925–3934 (2015).
- 738 46. Moya, B. *et al.* B-Lactam Resistance Response Triggered By Inactivation of a
739 Nonessential Penicillin-Binding Protein. *PLoS Pathog.* **5**, (2009).
- 740 47. Uri, J. V, Actor, P. & Weisbach, J. A. A rapid and simple method for detection of β -
741 lactamase inhibitors. *J. Antibiot.* **31**, 789–791 (1978).
- 742 48. Heirendt, L. *et al.* Creation and analysis of biochemical constraint-based models using
743 the COBRA Toolbox v.3.0. *Nat. Protoc.* **14**, 639–702 (2019).
- 744 49. Zhu, Y. *et al.* Genome-scale metabolic modeling of responses to polymyxins in
745 *Pseudomonas aeruginosa*. *Gigascience* **7**, (2018).
- 746 50. Chevalier, S. *et al.* Structure, function and regulation of *Pseudomonas aeruginosa* porins.
747 *FEMS Microbiol Rev* **41**, 698–722 (2017).
- 748 51. Liberati, N. T. *et al.* An ordered, nonredundant library of *Pseudomonas aeruginosa* strain
749 PA14 transposon insertion mutants. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 2833–2838
750 (2006).
- 751 52. Gisin, J., Schneider, A., Nägele, B., Borisova, M. & Mayer, C. A cell wall recycling
752 shortcut that bypasses peptidoglycan de novo biosynthesis. *Nat. Chem. Biol.* **9**, 491–493
753 (2013).
- 754 53. Köhler, T. *et al.* Multidrug efflux in intrinsic resistance to trimethoprim and
755 sulfamethoxazole in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **40**,
756 2288–2290 (1996).
- 757 54. Peters, J. M. *et al.* A comprehensive, CRISPR-based functional analysis of essential
758 genes in bacteria. *Cell* **165**, 1493–1506 (2016).
- 759 55. Dhar, S., Kumari, H., Balasubramanian, D. & Mathee, K. Cell-wall recycling and synthesis

- 760 in *Escherichia coli* and *Pseudomonas aeruginosa* – Their role in the development of
761 resistance. *J Med Microbiol* **67**, 1–21 (2018).
- 762 56. Juan, C., Moyá, B., Pérez, J. L. & Oliver, A. Stepwise upregulation of the *Pseudomonas*
763 *aeruginosa* chromosomal cephalosporinase conferring high-level β -lactam resistance
764 involves three AmpD homologues. *Antimicrob. Agents Chemother.* **50**, 1780–1787
765 (2006).
- 766 57. Borisova, M., Gisin, J. & Mayer, C. Blocking peptidoglycan recycling in *Pseudomonas*
767 *aeruginosa* attenuates intrinsic resistance to fosfomycin. *Microb. Drug Resist.* **20**, 231–
768 237 (2014).
- 769 58. Davies, D. T. *et al.* ANT2681: SAR Studies Leading to the Identification of a Metallo- β -
770 lactamase Inhibitor with Potential for Clinical Use in Combination with Meropenem for the
771 Treatment of Infections Caused by NDM-Producing *Enterobacteriaceae*. *ACS Infect. Dis.*
772 **6**, 2419–2430 (2020).
- 773 59. Jacobs, P. A. *et al.* Catalysis and Sulfa Drug Resistance in Dihydropteroate Synthase.
774 *Science.* **317**, 490–494 (2007).
- 775 60. Richards, R. M. E., Taylor, R. B. & Zhu, Z. Y. Mechanism for synergism between
776 sulphonamides and trimethoprim clarified. *J. Pharm. Pharmacol.* **48**, 981–984 (1996).
- 777 61. Minato, Y. *et al.* Mutual potentiation drives synergy between trimethoprim and
778 sulfamethoxazole. *Nat. Commun.* **9**, 1003 (2018).
- 779 62. Fumeaux, C. & Bernhardt, T. G. Identification of MupP as a New Peptidoglycan Recycling
780 Factor and Antibiotic Resistance Determinant in *Pseudomonas aeruginosa*. *MBio* **8**,
781 (2017).
- 782 63. Borisova, M., Gisin, J. & Mayer, C. The N-acetylmuramic acid 6-phosphate phosphatase
783 MupP completes the *Pseudomonas* peptidoglycan recycling pathway leading to intrinsic
784 Fosfomycin resistance. *MBio* **8**, (2017).
- 785 64. Jorgenson, M. A., Chen, Y., Yahashiri, A., Popham, D. L. & Weiss, D. S. The bacterial
786 septal ring protein RlpA is a lytic transglycosylase that contributes to rod shape and
787 daughter cell separation in *Pseudomonas aeruginosa*. *Mol. Microbiol.* **93**, 113–128
788 (2014).
- 789 65. Cheung, H. Y., Vitkovic, L. & Freese, E. Rates of peptidoglycan turnover and cell growth

- 790 of *Bacillus subtilis* are correlated. *J. Bacteriol.* **156**, 1099–1106 (1983).
- 791 66. Uratani, B., Lopez, J. M. & Freese, E. Effect of decoyinine on peptidoglycan synthesis
792 and turnover in *Bacillus subtilis*. *J. Bacteriol.* **154**, 261–268 (1983).
- 793 67. Corrigan, R. M., Abbott, J. C., Burhenne, H., Kaefer, V. & Gründling, A. C-di-AMP is a
794 new second messenger in *Staphylococcus aureus* with a role in controlling cell size and
795 envelope stress. *PLoS Pathog.* **7**, (2011).
- 796 68. Mamou, G. *et al.* Peptidoglycan maturation controls outer membrane protein assembly.
797 *Nature* **606**, 953–959 (2022).
- 798 69. Hummels, K. R. *et al.* Coordination of bacterial cell wall and outer membrane
799 biosynthesis. *Nature* **615**, 300–304 (2023).
- 800 70. Bernhardt, T. G. & De Boer, P. A. J. SImA, a nucleoid-associated, FtsZ binding protein
801 required for blocking septal ring assembly over chromosomes in *E. coli*. *Mol. Cell* **18**,
802 555–564 (2005).
- 803 71. Parks, D. H. *et al.* A standardized bacterial taxonomy based on genome phylogeny
804 substantially revises the tree of life. *Nat. Biotechnol.* **36**, 996 (2018).
- 805 72. Price, M. N., Dehal, P. S. & Arkin, A. P. FastTree 2 - Approximately maximum-likelihood
806 trees for large alignments. *PLoS One* **5**, e9490 (2010).
- 807 73. Letunic, I. & Bork, P. Interactive tree of life (iTOL) v5: An online tool for phylogenetic tree
808 display and annotation. *Nucleic Acids Res.* **49**, W293–W296 (2021).
- 809 74. Cavallari, J. F., Lamers, R. P., Scheurwater, E. M., Matos, A. L. & Burrows, L. L.
810 Changes to its peptidoglycan-remodeling enzyme repertoire modulate β -lactam
811 resistance in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **57**, 3078–3084
812 (2013).
- 813 75. French, S. *et al.* A robust platform for chemical genomics in bacterial systems. *Mol. Biol.*
814 *Cell* **27**, 1015–1025 (2016).
- 815 76. Weaver, A. *et al.* Lytic transglycosylases mitigate periplasmic crowding by degrading
816 soluble cell wall turnover products. *Elife* **11**, (2022).
- 817 77. Lamers, R. P., Nguyen, U. T., Nguyen, Y., Buensuceso, R. N. C. & Burrows, L. L. Loss of
818 membrane-bound lytic transglycosylases increases outer membrane permeability and β -

- 819 lactam sensitivity in *Pseudomonas aeruginosa*. *Microbiologyopen* **4**, 879–895 (2015).
- 820 78. Krueger, A. S. *et al.* Simulating Serial-Target Antibacterial Drug Synergies Using Flux
821 Balance Analysis. *PLoS One* **11**, e0147651 (2016).
- 822 79. Pettersen, E. F. *et al.* UCSF ChimeraX: Structure visualization for researchers,
823 educators, and developers. *Protein Sci.* **30**, 70–82 (2021).
- 824