SI APPENDIX for:

The LpoA activator is required to stimulate the peptidoglycan polymerase activity of its cognate cell wall synthase PBP1a

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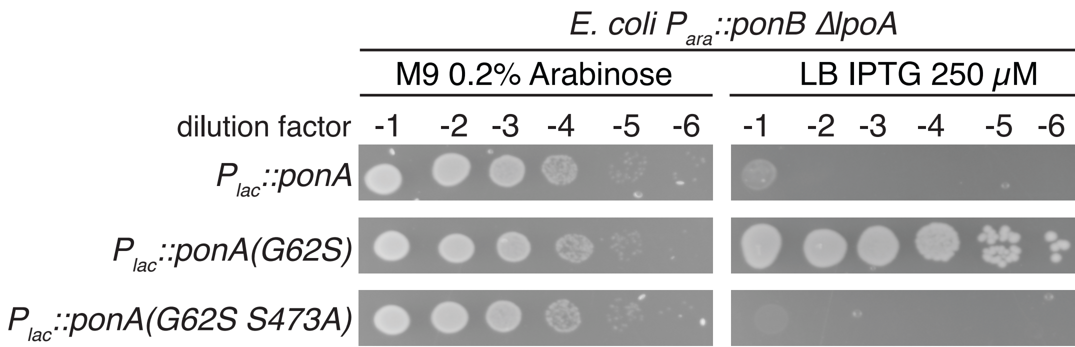

Figure S1. TPase activity is required for the LpoA bypass function of PBP1a(G62S). Overnight cultures of MM20 [Δ*lpoA* P_{ara}*::ponB*] containing the plasmids pJLB16 [P_{lac}::*ponA*], pJLB20 [P_{lac}::*ponA(G62A)*], or pJLB77 [P_{lac}::*ponA(G62A, S473A)*] were grown, diluted, and spotted on the indicated media as described in Figure 2A.

Table S1: Strains used in this study.

E. coli **strains**	**[a]Genotype**	**[b]Source/Reference**
DH5αpir	*endA1 hsdR17 glnV44 (= supE44) thi-1 recA1 gyrA96 relA1 φ80dlacΔ(lacZ)M15 Δ(lacZYA-argF)U169 zdg-232::Tn10 uidA::pir+*	Gibco BRL
Rosetta2(DE3)	F^- *ompT* $hsdS_B(r_B^-\ m_B^-)$ *gal dcm* (DE3) pRARE2 (Cam^R)	Novagen
MG1655	*rph ilvG rfb*-50	(1)
TB28	*rph ilvG rfb*-50 Δ*lacIZYA*::frt	(2)
MM13	TB28 *ΔlpoA::*Kan^R *(frt araC Para)::ponB*	(3)
MM20	TB28 *ΔlpoA::frt (frt araC* P_{ara}*)::ponB*	(3)
JW3340**v**	*Δ(araD-araB)567 ΔlacZ4787(::rrnB-3) rph-1 Δ(rhaD-rhaB)568 hsdR514 yhfT::*Kan^R	(4)
JLB123	MG1655 *yhft::* Kan^R	P1 JW3340 x MG1655
JLB124	JLB123 *ponA(T38P)*	This Study
JLB127	JLB123 *ponA(T38P)*	This Study
JLB128	MM20 *ponA(G62S) yhft::*Kan^R	P1 (JLB124) x MM20
JLB129	MM20 *ponA(T38P) yhft::*Kan^R	P1 (JLB127) x MM20
CB173	*rph1 ilvG rfb-50 ΔlacIZYA::frt ycbC::frt ΔlpoA::kan*	(5)
HC529	MG1655 *ΔlysA::frt ΔpbpC::frt ΔmtgA::frt ΔampD::frt ponB(S247C)*	(6)
HC529(*att*HKHC859)	HC529 (P_{tac}*::sulA*)	P1(HC533(*att*HKHC859) x HC529
MFS8(*att*HKHC859)	HC529 *ΔlpoA::*Kan^R (P_{tac}*::sulA*)	P1(CB173) x HC529(*att*HKHC859)
HC533(*att*HKHC859)	MG1655 *ΔlysA::frt ΔponA::frt ΔpbpC::frt ΔmtgA::frt ΔampD::frt ponB(S247C)* (P_{tac}*::sulA*)	(6)
HC533(*att*HKHC859) (attλTB309)	HC533(*att*HKHC859) (P_{ara}*::gfp-ponA*)	P1[TU121(attλTB309)] x HC533(*att*HKHC859)
MFS7(*att*HKHC859) (attλTB309)	HC533(*att*HKHC859) (attλTB309) *ΔlpoA::*Kan^R	P1(CB173) x HC533(*att*HKHC859) (attλTB309)
HC533(*att*HKHC859) (attλMFS28)	HC533(*att*HKHC859) (P_{ara}*::gfp-ponA(G62S)*)	P1[MG1655(attλMFS12)] x HC533(*att*HKHC859)
MFS7(*att*HKHC859) (attλMFS28)	HC533(*att*HKHC859) (attλMFS12) *ΔlpoA::*Kan^R	P1(CB173) x HC533(*att*HKHC859) (attλMFS12)
P. aeruginosa **strain**	**Genotype**	**Source/Reference**
PA686	Δ*ponB* Δ*lpoA*	(7)

[a] The kanamycin resistance cassette (Kan^R) is flanked by *frt* sequences for removal by FLP recombinase. Removal of the cassette leaves an *frt* scar sequence behind.

[b] Strains generated by P1 transduction are described as follows: P1(donor strain) X recipient strain.

Table S2. Plasmids used in this study.

Plasmid	[a]Genotype	ori	Source/reference
pCP20	*bla cat cI875 repA(Ts) PR::flp*	pSC101	(8)
pCB21	*bla* $lacI^q$ P_{T7}*::h-sumo-ponA*	pBR/colE1	(3)
pJLB16	*cat* $lacI^q$ P_{lac}*::ponA*	pBR/colE1	This study
pJLB20	*cat* $lacI^q$ P_{lac}*::ponA(G62A)*	pBR/colE1	This study
pJLB21	*cat* $lacI^q$ P_{lac}*::ponA(G62S)*	pBR/colE1	This study
pJLB22	*cat* $lacI^q$ P_{lac}*::ponA(L146I)*	pBR/colE1	This study
pJLB25	*cat* $lacI^q$ P_{lac}*::ponA(W494G)*	pBR/colE1	This study
pJLB29	*cat* $lacI^q$ P_{lac}*::ponA(T38P)*	pBR/colE1	This study
pJLB77	*cat* $lacI^q$ P_{lac}*::ponA(G62S, S473A)*	pBR/colE1	This study
pJLB78	*sacB cat ponA(G62S)*	R6K	This study
pJLB83	*sacB cat ponA(T38P)*	R6K	This study
pPSV38	*aacC1* $lacI^q$ P_{lacUV5}::empty	pBR322/ pRO1614	(9)
pNG91	*aacC1* $lacI^q$ P_{lacUV5}::^{Pa}*ponA*	pBR322/ pRO1614	This study
pNG94	*aacC1* $lacI^q$ P_{lacUV5}:: ^{Pa}*ponA(E158K)*	pBR322/ pRO1614	This study
pNG95	*aacC1* $lacI^q$ P_{lacUV5}:: ^{Pa}*ponA(G62S)*	pBR322/ pRO1614	This study
pNG96	*aacC1* $lacI^q$ P_{lacUV5}:: ^{Pa}*ponA(G284D)*	pBR322/ pRO1614	This study
pMFS1	*bla* $lacI^q$ P_{T7}*::h-sumo-ponA(G62S)*	pBR/colE1	This study
pMFS4	*bla* $lacI^q$ P_{T7}*::h-sumo-ponA(L146I)*	pBR/colE1	This study
pMFS5	*bla* $lacI^q$ P_{T7}*::h-sumo-ponA(E278K)*	pBR/colE1	This study
pMFS6	*bla* $lacI^q$ P_{T7}*::h-sumo-ponA(W494G)*	pBR/colE1	This study
pMFS19	*bla* $lacI^q$ P_{T7}*::h-sumo-ponA(T38P)*	pBR/colE1	This study
pMFS28	*cat att*λ P_{ara}*::gfp-ponA(G62S)*	R6K	This study
pMM18	*bla* $lacI^q$ P_{T7}*::h-sumo-lpoA*	pBR/colE1	(3)
pMM36	*cat att*λ P_{ara}*::gfp-ponA*		(3)

[a] P_{lac}, P_{ara}, and P_{T7} refer to the lactose, arabinose, and phage T7 promoters, respectively. The His_6 tag is abbreviated as h. The superscript *Pa* designates the allele is from *P. aeruginosa*. All other alleles are from *E. coli*.

46 **SUPPLEMENTAL METHODS AND MATERIALS**
47
48 *PBP1a purification*

49 His_6-SUMO-tagged derivatives of *E. coli* PBP1a or PBP1a* variants were expressed in *E.*
50 *coli* Rosetta(λDE3)/pLysSRARE. Protein expression was carried out according to a
51 modified protocol based on that described previously (3). Briefly, *E. coli*
52 Rosetta(λDE3)/pLysSRARE transformed with the appropriate pMFS plasmid were grown
53 overnight at 37°C in Terrific Broth (TB, 2.4% w/v yeast extract, 2% w/v tryptone, 0.4% v/v
54 glycerol, 0.017 M KH_2PO_4, 0.072 M K_2HPO_4) supplemented with Kan and Cam. The next
55 day, 1L of similarly supplemented TB was inoculated with 5mL of the overnight culture and
56 cells were allowed to reach an OD_{600}=0.6. Subsequently, the cultures were transferred to
57 an incubator set at 20°C for 1h. Protein expression was then induced with 1.5mM IPTG
58 addition and growth was continued overnight. Cells were harvested the next morning with
59 centrifugation at 4,000 x g for 30min at 4°C. Cell paste was resuspended in a volume (ml)
60 of 20mM Tris-HCl pH=7.4, 150 mM NaCl, 10% v/v glycerol that was 10 times the wet pellet
61 weight in grams. After the cell suspension was completely homogeneous, the solution was
62 supplemented with 5000U of DNase I, 5mM $MgCl_2$ and 5mM $CaCl_2$ and 1 tablet of
63 Complete, EDTA-free Protease Inhibitor Tablet (Roche). Cells were briefly sonicated and
64 lysed in a Constant Systems Cell Disruptor CF at 25 KPSI. After centrifugation at 40,000 x
65 g for 60min at 4°C the supernatant was removed and the membrane pellet was collected
66 and resuspended using a glass homogenizer in 1% w/v CHAPS, 20mM Tris-HCl pH=7.4,
67 150 mM NaCl, 10% v/v glycerol. The material that did not solubilize was removed with
68 centrifugation at 40,000 x g for 60min at 4°C. The supernatant was collected and filtered
69 through a 0.22μm PES filter. Proteins were isolated using 1mL prepacked Ni-NTA fast flow

70 Sepharose (GE) in an AKTA pure 25. The column was equilibrated with 5 column volumes
71 (5 mL) and washed with 10 column volumes (10 mL) of 0.1% v/v reduced Triton X-100, 20
72 mM Tris-HCl pH=7.4, 500 mM NaCl, 10% v/v glycerol. Captured protein was eluted in a
73 linear gradient of 40 column volumes (40 mL) up to 100% of 500mM imidazole, 20mM
74 Tris-HCl pH=7.4, 500 mM NaCl, 10% glycerol, 0.1% v/v reduced Triton X-100. Elution
75 fractions of 0.5mL were collected and analyzed to identify those containing the
76 PBP1a/PBP1a* proteins. The flow rate throughout the protocol was 1 ml/min and the
77 isolation was performed in a refrigerated cabinet. The fractions that contained
78 PBP1a/PBP1a* proteins were pooled and 5mM EDTA was added in the solution to remove
79 any Ni^{2+} ions that leached out during the purification. Imidazole was removed with dialysis
80 in 20 mM Tris-HCl pH=7.4, 500 mM NaCl, 10% glycerol, 0.1% v/v reduced Triton X-100.
81 When protein concentration was required, Amicon Centrifugal Filter Unites (MWCO
82 10kDA) were used. The His_6-SUMO- tag was removed by adding His_6-Ulp1 protease to
83 the sample before the overnight dialysis at 4°C with stirring. The next day this solution was
84 loaded again on a 1 mL prepacked Ni-NTA fast flow Sepharose (GE) in an AKTA pure 25
85 and the procedure of chromatography was performed as described above. In this case, the
86 flow through contained the PBP1a/PBP1a* proteins whereas the His_6-SUMO- tag and
87 His_6-Ulp1 protease were captured on the Ni-NTA beads.
88
89 ***LpoA purification***
90 His_6-SUMO-LpoA(28-678) was expressed from plasmid pCB40 in Rosetta™ 2(DE3) cells
91 and purified very similarly to the protocol described above for PBP1a purification.
92 Following overnight induction, cells were harvested and resuspended in 20mM Tris-HCl

93 pH=7.4, 150mM NaCl, 10% v/v glycerol as described above. After the cell suspension was
94 completely homogeneous, the solution was supplemented with 5000U of DNase I, 5mM
95 $MgCl_2$ and 5mM $CaCl_2$ and 1 tablet of complete, EDTA-free Protease Inhibitor Tablet
96 (Roche). Cells were briefly sonicated and lysed in a Constant Systems Cell Disruptor CF
97 at 25KPSI. After centrifugation at 40,000 x g for 60min at 4°C the supernatant was
98 collected and filtered through a 0.22μm PES filter. His_6-SUMO-LpoA(28-678) was isolated
99 using 1mL prepacked Ni-NTA fast flow Sepharose (GE) in an AKTA pure 25. The column
100 was equilibrated with 5 column volumes (5 mL) and washed with 10 column volumes (10
101 mL) of 20mM Tris-HCl pH=7.4, 500mM NaCl, 10% v/v glycerol. Captured protein was
102 eluted in a linear gradient of 40 column volumes (40 mL) up to 100% of 500 mM imidazole,
103 20 mM Tris-HCl pH=7.4, 500 mM NaCl, 10% glycerol. Elution fractions of 0.5 mL were
104 collected and analyzed to determine which ones contained the His_6-SUMO-LpoA(28-678).
105 The flow rate throughout the protocol was 1 ml/min and the isolation was performed in a
106 refrigerated-cabinet. The fractions that contained His_6-SUMO-LpoA(28-678) were pooled
107 and 5 mM EDTA was added in the solution to remove any Ni^{2+} ions that leached out during
108 the purification. Imidazole was removed with dialysis in 20mM Tris-HCl pH=7.4, 500 mM
109 NaCl, 10% glycerol. The His_6-SUMO tag was then cleaved and removed as described
110 above.
111

112 ***Isolation of lipid II from E. coli and E. faecalis***

113 Isolation of *E. faecalis* lipid II was performed as described previously (10). *E. coli* lipid II
114 was isolated from strain NR2186 using a similar method. The strain encodes
115 MurJ(A29C), which can be inactivated with MTSES to block lipid II flipping and promote

116 the accumulation of lipid II. NR2186 was inoculated into 100 ml LB and grown overnight
117 at 37°C. The next morning, four flasks with 1L LB each was inoculated with 5mL of the
118 overnight cell culture. When the OD_{600} reached 0.5-0.6, MTSES (100 μM final) was
119 added to each 1L flask and growth was continued for 10min. Cells were then harvested
120 by centrifugation at 4,000 x g for 10min at 4°C. The supernatant was discarded, and the
121 cell pellet was resuspended in Phosphate Buffered Saline (PBS) pH=7.5. $CHCl_3$ and
122 CH_3OH was added in the PBS solubilized pellet to achieve a ratio of 1:2 :0.8
123 ($CHCl_3$:CH_3OH :PBS). This mixture was stirred vigorously with a magnetic stir bar for 1
124 hr at high speed (approximately 800rpm) at room temperature. The mixture was
125 transferred into Teflon tubes and centrifuged at 4,000 x g for 15 min at 4°C. The
126 supernatant was collected, and the pellet discarded. The supernatant was mixed with
127 $CHCl_3$, CH_3OH, and PBS so that the final ratio of all components was 2: 2:1.8
128 ($CHCl_3$:CH_3OH :PBS). At this ratio a biphasic system will form. This mixture was stirred
129 vigorously with a magnet bar for 1hr at high speed (approximately 800rpm) at room
130 temperature. After the mixture was homogenized completely it was transferred into
131 Teflon tubes and centrifuged at 4,000 x g for 15 min at 4°C. After centrifugation, the
132 tubes were transferred into a refrigerator and were kept at 4°C overnight. The next day
133 three layers were visible in each tube; the upper one contains the aqueous phase; a thin
134 middle interface contains the lipid II, and the lower phase contains the organic phase.
135 The next day the top aqueous layer was removed carefully with a glass pipette. The
136 interface was collected carefully in a round bottom glass flask. The lipid II-containing
137 mixture was then rotary evaporated under vacuum at 37°C. The dried material was
138 resuspended in 7.5 mL of solution of 33.3% v/v 6 M pyridinium acetate (51.5% v/v

139 glacial acetic acid, 48.5% v/v pyridine) and 66.6% v/v n-butanol (organic phase). The
140 resuspended lipid II-containing solution was transferred to a small separatory funnel and
141 was washed with 15 mL n-butanol saturated water (aqueous phase) to remove the
142 nucleotide precursors from the lipid II-containing organic phase. This mixture forms a
143 biphasic system. The nucleotides are found in the lower aqueous phase whereas lipid II
144 remains in the upper organic phase. The upper organic phase was collected. The
145 resulting lower aqueous phase was also collected and washed with 7.5 mL of fresh
146 organic phase to maximize extraction of lipid II. The organic phases were combined to
147 give a total 15 mL and washed with 15 mL of aqueous phase three times. The washed
148 organic phase was evaporated in a rotary evaporator under vacuum. The evaporated
149 material that contains lipid II was resuspended in 1 mL of methanol, transferred to a
150 glass vial and evaporated under a stream of air. The final film of material was
151 resuspended in 100% DMSO.
152
153 **Quantification of lipid II from *E. coli* and *E. faecalis*.**
154 The concentration of purified lipid II was quantified with fluorescamine. Fluorescamine is
155 not fluorescent by itself but when it interacts with primary amines on lipid II, it forms
156 products that are fluorescent. A stock 200 mM fluorescamine in methanol (for 200μL
157 solution we used 11 mg of fluorescamine) solution was prepared and used for the
158 quantifications. Solutions of 400 μM L-lysine-L-lysine or L-Ala in 400 mM borate pH=9.5
159 were used as standards. Serial dilutions of these solutions were prepared down to a
160 concentration of 3μM, and 100μL of each dilution was added to wells of a black 96-well
161 plate. Similarly, the lipid II preparations were diluted 1/50 and one 1/100 DMSO and
162 100μL of each sample added to the black 96-well plate. All samples were then mixed

163 with 10μL of 200 mM fluorescamine in methanol. The samples were incubated for 10
164 min at room temperature and then were then analyzed for fluorescence with 365nm
165 excitation and 525nm emission wavelengths. All samples were processed in triplicate. A
166 standard curve was generated from the amino acid standards and used to determine
167 the concentration of lipid II samples from their fluorescence measurements.
168
169 **PG polymerase assays**
170 The PGTase activity of PBP1a/PBP1a* proteins was monitored as described previously
171 (10–12). The reaction buffer was 20 mM HEPES pH=7.5, 20 mM $MnCl_2$, 0.2% w/v
172 reduced Triton X-100. Each reaction contained 5 μM PBP1a or PBP1a*. The reactions
173 were started with addition of 10 μM lipid II from *E. faecalis*. The concentration of lipid II
174 stock was adjusted so that the final concentration of DMSO in the reaction did not
175 exceeded 30% v/v. The final volume of each reaction was 10μL. Reactions were
176 incubated at 25°C for the indicated time points. Reactions were terminated by heat
177 denaturation at 95°C for 5 min. After heat denaturation, the samples were cooled on ice
178 and then mixed with 5μM *S. aureus* PBP4 and 10 μM Biotinylated D-Lysine (BDL).
179 Samples were then incubated at 25°C for 1h. Each reaction was quenched with 13 μL of
180 6 x Laemmli SDS-sample buffer and loaded on a 4-20% gradient SDS polyacrylamide
181 gel and run at 30 mA for 45min. The analyzed products were transferred to a PVDF
182 membrane. The membrane was fixed with a solution of 0.4% parafolmaldehyde in
183 phosphate-saline-buffered saline (PBS) for 30 min. The membrane was washed with
184 PBS and then it was blocked with SuperBlock blocking buffer. The blocked membranes
185 were incubated with a solution of IRDye 800CW Streptavidin (1:5,000 dilution in PBS).

186 Finally, the membranes were washed three times with TBST (50mM Tris-HCl, pH=7.5,
187 150mM NaCl, 1% v/v Tween 20) and then once with PBS (0.1M NaCl, 3mM KCl, 10mM
188 Na_2HPO_4, 2mM KH_2PO_4). The products of the reaction were visualized and imaged
189 using an Odyssey CLx imaging system (LI-COR Biosciences).
190

191 **PG crosslinking assays**

192 TPase assays of PBP1a/PBP1a* proteins were performed in 20 mM HEPES pH=7.5,
193 20mM $MnCl_2$, 0.2% w/v reduced Triton X-100. The protocol is similar to that described
194 previously (12). Each reaction contained 5 μM PBP1a/PBP1a*. The reactions were
195 started with addition of 200 μM lipid II from *E. coli*. The concentration of the lipid II stock
196 was adjusted so that the final concentration of DMSO did not exceeded 30% v/v. The final
197 volume of each reaction was 30 μL. Moenomycin or penicillin G were added in negative
198 control samples. Reactions were incubated at 25°C for 5 minutes. Reactions were
199 terminated by heat denaturation at 95°C for 5 min. The volume of the reaction was
200 increased with HPLC-grade water to 130μL to lower the concentration of
201 DMSO. Mutanolysin (from *Streptomyces globisporus*; 4,000 U/ml) was added (5μL) to
202 each reaction to digest the polymerized PG for 3h at 37°C. Sodium borohydrite (50μL of
203 10mg/mL) was added per reaction to reduce the sugar ring in the muropeptides generated
204 by the mutanolysin digestion. The pH was adjusted to about 4 with 20% phosphoric acid
205 and the samples were lyophilized under vacuum overnight. The samples were
206 resuspended in 20 μL HPLC-grade water and analyzed by LC-MS on an Agilent 6210
207 TOF mass spectrometer with ESI-MS in positive mode. The muropeptides were
208 separated on a Waters Symmetry Shield RP18 column (5μM; 3.9mm x 150 mm). The

209 HPLC method used was as follows: flow rate 0.5 mL/min; isocratic wash with 100%
210 solvent A (0.1% v/v HPLC-grade Formic Acid in HPLC-grade water) for 5 min and then a
211 linear gradient of solvent B (0.1% v/v HPLC-grade Formic Acid in acetonitrile) up to 40%
212 over 25 min. Molecular ions for the muropeptides were extracted from the chromatograms
213 using ChemDraw 18.0. To quantify the amount of monomer and dimers we extracted
214 chromatograms using Agilent Mass Hunter Qualitative Analysis B.06.00. The extraction
215 was done by selecting chromatograms that contained the corresponding ions (m/z and
216 isotopic profiles; (M+H)+1=1013.4521 and (M+H)+2=507.2297 for the monomers and
217 (M+H)+2=968.9283, (M+H)+3=646.2879 and (M+H)+4=484.9678 for the dimer, in EIC
218 positive polarity mode with symmetric (m/z)$\pm$0.5.
219
220 ***In vivo* PG polymerase assay**
221 The assay was performed essentially as described previously (6, 13). Cells of
222 HC533(*att*HKHC859) [Δ*lysA* Δ*ampD* Δ*pbpC* Δ*mtgA* *ponB(S247C)* (P_{tac}::*sulA*)] or its
223 Δ*lpoA* derivative were grown overnight in M9 medium supplemented with 0.4% v/v
224 glycerol and 0.2% w/v casamino acids. The overnight cultures were diluted to OD_{600} =
225 0.04 in the same medium and were grown at 30°C until their OD_{600} reached 0.25-0.3.
226 Cell division was inhibited with induction of *sulA* with 1.5 mM IPTG and growth was
227 continued for 30 min. In strains harboring P_{ara}::*gfp-ponA* constructs, the overproduction
228 of the PBP was also induced with the addition of 0.4% v/v arabinose during this time.
229 The OD_{600} of each culture was then adjusted to 0.3, and cells were treated with A22 (10
230 μ/ml), MTSES (1 mM), and cefsulodin (100 μg/ml) as indicated. Growth was continued
231 for 5 min before 1μCi of [^{3}H]-mDAP was added. Labeling proceeded for another 10 min.

232 After labelling, cells were centrifuged in a tabletop centrifuge at maximum speed
233 (21,000 x g) at 4°C. The supernatant was removed, and the cell pellets were
234 resuspended in 0.7mL of cold (4°C) HPLC grade water and placed on a heat block at
235 90°C for 30min to extract hydrophilic molecules from the cells (hot water extract). After
236 the heat treatment, tubes were cooled down on ice, and each solution was centrifuged
237 at 200,000 x g for 20min at 4°C. The supernatant that contains the hydrophilic PG
238 turnover intermediates was removed and lyophilized under vacuum overnight. The
239 pellets were used to determine how much [^{3}H]-mDAP was incorporated into the PG.
240

241 To measure the incorporation of material into the PG layer, each pellet was washed with
242 1mL of 20mM Tris-HCl, pH=8.0, 25 mM NaCl and then resuspended in 1mL of the same
243 buffer containing 0.25 mg lysozyme. These suspensions were incubated at 37°C
244 overnight. After the overnight incubation the insoluble material was pelleted by
245 centrifugation at 21,000 x g for 30 min at 4°C. The supernatant that contains PG
246 fragments released after lysozyme digestion of the cell wall was mixed with 10 mL
247 EcoLite (MP biomedicals) scintillation fluid. Incorporated [^{3}H]-mDAP was then quantified
248 in a Microbeta Trilux 1450 liquid scintillation counter (Perkin Elmer).
249

250 Labeled PG turnover products in the lyophilized hot water extracts were separated on a
251 Shimadzu HPLC system with a Symmetry Shield RP18 Column (100Å pore size, 5
252 µm resin size, 3.9 mm X 150 mm, Waters). The run was performed with the column
253 at 50°C. The lyophilized supernatants were resuspended in 150 µL of 0.1% v/v HPLC-
254 grade Formic Acid in HPLC-grade water. Resuspended samples were centrifuged at

255 21,000 x g for 10min at 4°C for 10min. After injection of 100 μL, the hot water extract
256 was eluted isocratically for 5min with a flow rate of 0.5mL/min of 98% solvent A and
257 2% solvent B (solvent A: 0.1% v/v/ HPLC-grade Formic acid in HPLC-grade water;
258 solvent B: 0.1% v/v/ HPLC-grade Formic acid in HPLC-grade acetonitrile). This
259 elution was followed by a linear gradient up to 83% solvent A/17% solvent B for
260 40min, 10% solvent A/90% solvent B for 15min and finally a 98% solvent A/2%
261 solvent B for 15 min. Eluates were mixed at a ratio of 1:3 with Perkin Elmer Ultima-
262 Flo M scintillation liquid and passed through a Berthold LB513 FlowStar radio flow
263 detector. The resulting chromatograms were analyzed with Prism 7. The area under
264 the curve of each anhydromuropeptide was quantified and used to determine the
265 total level of PG turnover, which in the presence of cefsulodin is a reflection of the
266 PG polymerase activity of PBP1a.
267
268 ***Plasmid construction***
269
270 **pPR66**
271 pPR66 was constructed as follows: The NotI/HindIII fragment of pHC800 [*cat*
272 *Ptac::empty*] containing ColE1 was used to replace this fragment of the pMT77 [*cat*
273 *lacIq Plac::*Empty] vector.
274
275 **pJLB16**
276 The plasmid pJLB16 is a derivative of pPR66. pJLB16 [*cat* $lacI^q$ P_{lac}*::ponA*] was
277 constructed as follows: The XbaI/HindIII fragment of pMM21 [*attHK022 bla* $lacI^q$
278 P_{lac}*::ponA-sfgfp*] containing *ponA* was used to replace the corresponding fragment of
279 the pPR66 [*cat* $lacI^q$ P_{lac}::Empty] vector. MM21 was derived from pTB279 [*attHK022 bla*
280 $lacI^q$ P_{lac}*::sfgfp*] by digesting the plasmid with XbaI/HindIII and ligating with the *ponA*
281 containing XbaI/HindII fragment from pTB309 [*attλ cat* P_{ara}*::ponA*].

282

283 **pJLB20-22, and pJBL25**

284 pJLB20 [*cat lacI*^{q} P_{lac}*::ponA(G62A)*], pJLB21 [*cat lacI*^{q} P_{lac}*::ponA(G62S)*], pJLB22 [*cat*
285 *lacI*^{q} P_{lac}*::ponA(L146I)*], and pJLB25 [*cat lacI*^{q} P_{lac}*::ponA(W494G)*], were all derived from
286 pJLB16 via single primer quick-change mutagenesis with the indicated primers: pJLB20:
287 ponA-G70A-SDM-F (5'-
288 GCGAGCTGATTGCTCAATACGCTGAGAAACGTCGTATTCCG-3').
289 pJLB21: ponA-G70S-SDM-F (5'-
290 GCGAGCTGATTGCTCAATACAGTGAGAAACGTCGTATTCCG-3').
291 pJLB22: ponA-L154I-SDM-F (5'-
292 GTAAGATTAAGGAAGTCTTCATCGCGATTCGCATTGAACAG-3').
293 pJLB25: ponA-W502G-SDM-F (5'-
294 ACGATGTGCCAATTTCTCGCGGGGATGCAAGTGCCGGTTCT-3').

295

296 **pJLB29**

297 pJLB29 was derived from pJLB16 by digesting the pJLB16 vector with XhoI/XbaI and
298 ligating with the corresponding *ponA(T38P)* containing fragment from the suppressor
299 plasmid isolated during the *E. coli* PBP1a* selection.

300

301 **pJLB77**

302 The plasmid pJLB77 is a derivative of pPR66. The insert was generated by overlap
303 extension PCR using a pJLB21 template and the primers indicated. Fragments 1 and 2
304 were amplified using primers PBP1aS473A-OE-primerA (5'-
305 CTACTCTAGACCGCGCGTTTGTTTATAAACTGCCC-3') and PBP1aS473A-OE-
306 primerB (5'-GTTTGATGTTGGCACCCACCTGACGCAGTGCCTG-3') for fragment 1 and
307 PBP1aS473A-OE-primerC (5'-CAGGTGGGTGCCAACATCAAACCGTTCCTC-3') and
308 PBP1aS473A-OE-primerD
309 (5'-GTATCGGTGATAAGCTTTTGTCAGCAAACTGAAAAGGCG-3') for fragment 2.
310 Then, fragments 1 and 2 were combined into the final insert in the overlap PCR using
311 primers PBP1aS473A-OE-primerA and PBP1aS473A-OE-primerD. The insert PCR was
312 then purified, digested with XhoI/HindIII and ligated into similarly digested pPR66.

313

314 **pJLB78 and pJLB83**

315 The plasmids pJLB78 [*sacB cat ponA(G62S)*] and pJLB83 [*sacB cat ponA(T38P)*] are
316 derivatives of pDS132 [*sacB cat*] (14). The insert for each plasmid was generated by
317 overlap extension PCR using *E. coli* MG1655 genomic DNA as a template and the
318 indicated primers.
319 Fragments 1 and 2 for pJLB78 were amplified using primers PBP1aG62S-OE-primerA
320 (5'-GACTGAGCTCCAGTTCGCGACTCCACGGTAACAACG-3') and PBP1aG62S-OE-
321 primerB (5'-CGTTTCTCACTGTATTGAGCAATCAGCTCGC-3') for fragment 1 and
322 PBP1aG62S-OE-primerC (5'-GCTCAATACAGTGAGAAACGTCGTATTCCGG-3') and
323 PBP1aG62S-OE-primerD (5'-GATCTCTAGACCGCGACAGCACGACGTTACGC-3') for
324 fragment 2. Then, fragments 1 and 2 were combined into the final insert for pJLB78 in
325 the overlap PCR using primers PBP1aG62S-OE-primerA and PBP1aG62S-OE-
326 primerD.
327 Fragments 1 and 2 for pJLB83 were amplified using primers PBP1aG62S-OE-primerA
328 (5'-GACTGAGCTCCAGTTCGCGACTCCACGGTAACAACG-3') and PBP1aT38H-
329 primerB (5'-CATCTTTTAATGGCGCCACATCCGGCAGTTGTG-3') for fragment 1 and
330 PBP1aT38H-primerC (5'-GGATGTGGCGCCATTAAAAGATGTTCGCCTG-3') and
331 PBP1aG62S-OE-primerD (5'-GATCTCTAGACCGCGACAGCACGACGTTACGC-3') for
332 fragment 2. Then, fragments 1 and 2 were combined into the final insert for pJLB83 in
333 the overlap PCR using primers PBP1aG62S-OE-primerA and PBP1aG62S-OE-
334 primerD.
335 The insert PCRs for both plasmids were then purified, digested with XbaI/HindIII and
336 ligated into similarly digested pDS132.

337

338 **pNG91, pNG94, pNG95, and pNG96**

339 The plasmids pNG91, pNG94, pNG95, and pNG96 are all derivatives of pPSV38
340 [*aacC1 lacIq PlacUV5::*Empty] (9). pNG91 [*aacC1 lacIq PlacUV5::ponA*] was
341 constructed as follows: The *ponA* insert was PCR amplified from *P. aeruginosa* PAO1
342 genomic DNA
343 using the primers oNG340

344 (5'ATGCGAATTCGAGGAGGATACATATGCGCCTGCTGAAGTTCCTG-3')
345 and oNG341 (5'-AATTCCCGGGTCAGAACAGGTCGATCGGCG-3'). The PCR was
346 then purified, digested with EcoRI/XmaI, and ligated into the similarly digested pPSV38
347 vector. pNG94 [*aacC1 lacIq PlacUV5::ponA(E158K)*], pNG95 [*aacC1 lacIq*
348 *PlacUV5::ponA(G62S)*], and pNG96 [*aacC1 lacIq PlacUV5::ponA(G284D)*] were all
349 constructed by amplifying the *ponA* gene from the genomic DNA an individual *P.*
350 *aeruginosa* PBP1a* suppressor strain using the primers oNG340 and oNG341. The
351 PCR was then purified, digested with EcoRI/XmaI, and ligated into the similarly digested
352 pPSV38 vector.
353
354 **pMFS1, pMSF4, pMFS5, and pMSF19**
355 These plasmids were constructed by site-directed mutagenesis of pCB21 (3), which has
356 the gene for P_{T7}: MRGS-His_6-MASG-SUMO-PBP1a cloned on a pET28a backbone that
357 carries kanamycin resistance. The mutations altering the coding sequence of PBP1a
358 were Quick-change style PCR using Phusion DNA polymerase and the following
359 primers: For pMFS1, 5'-CTG ATT GCT CAA TAC TCT GAG AAA CGT CGT ATT CCG
360 GTT ACG TTG GAT CAA ATC CC-3' and 5'-CG TTT CTC AGA GTA TTG AGC AAT
361 CAG CTC GCC ATC GGC GCT GTA AAT CTG-3'. For pMFS4, 5'-G ATT AAG GAA
362 GTC TTC ATC GCG ATT CGC ATT GAA C-3' and 5'-G TTC AAT GCG AAT CGC GAT
363 GAA GAC TTC CTT AAT C-3'. For pMSF5, 5'-G TAT AAC CGT TAT GGC AAA AGT
364 GCC TAT GAA GAC GG-3 and 5'-CC GTC TTC ATA GGC ACT TTT GCC ATA ACG
365 GTT ATA C-3'. For pMFS19, 5'-CTG CCG GAT GTG GCG CCG TTA AAA GAT GTT
366 CG-3' and 5'-CG AAC ATC TTT TAA CGG CGC CAC ATC CGG CAG-3'.
367
368 **pMFS28**
369 G62S mutation was introduced on pMM36 (P_{ara}::gfp-*ponA)* with PCR using Phusion DNA
370 polymerase and the mutagenic primers X21 [5'-CTG ATT GCT CAA TAC TCT GAG AAA
371 CGT CGT ATT CCG GTT ACG TTG GAT CAA ATC CC-3'] and X22 [5'-CG TTT CTC
372 AGA GTA TTG AGC AAT CAG CTC GCC ATC GGC GCT GTA AAT CTG-3']. This
373 construct resulted in a CRIM plasmid carrying P_{ara}::gfp-*ponA (G62S)*.
374

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