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1 **(p)ppGpp modifies RNAP function to confer β -lactam resistance in a peptidoglycan-**
2 **independent manner**

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9

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13

14 **Little is known on the adaptation of the cell wall peptidoglycan metabolism to nutrient**
15 **deprivation. We hypothesized that (p)ppGpp might be involved in this process since**
16 **elevated levels of this alarmone confer resistance to the narrow-spectrum β -lactam**
17 **mecillinam. In addition, (p)ppGpp is essential for broad-spectrum β -lactam resistance**
18 **mediated by redirecting the flux of peptidoglycan precursors toward the β -lactam-**
19 **insensitive transpeptidase YcbB (LdtD). High-resolution mass spectrometry analyses of**
20 **peptidoglycan structure unexpectedly revealed that these (p)ppGpp-dependent resistance**
21 **mechanisms do not rely on any modification of peptidoglycan metabolism. Amino acid**
22 **substitutions in the β or β' RNA polymerase (RNAP) subunits, alone or in combination with**
23 **the CRISPR interference-mediated downregulation of three of seven ribosomal RNA**
24 **operons, was sufficient for resistance, although β -lactams have no known impact on the**
25 **RNAP or ribosomes. This implies that modifications of RNAP and ribosome functions are**
26 **critical to prevent downstream effects of the inactivation of peptidoglycan transpeptidases**
27 **by β -lactams.**

28

29 The ubiquitous stringent response is triggered in response to diverse stresses, such as amino
30 acid or phosphate starvation^{1,2}. The stringent response relies on the production of the
31 alarmones ppGpp (guanosine 5'-diphosphate 3'-diphosphate) and pppGpp (guanosine 5'-
32 triphosphate 3'-diphosphate) [collectively referred to as (p)ppGpp]. In *Escherichia coli*,
33 (p)ppGpp production involves two homologues of (p)ppGpp synthases, named RelA and SpoT.
34 RelA is a monofunctional enzyme that possesses only (p)ppGpp synthase activity while SpoT
35 is a bifunctional enzyme with both (p)ppGpp synthetic and hydrolytic activities. The (p)ppGpp
36 alarmone binds to and directly activates or inhibits several enzymes involved in multiple
37 metabolic pathways (Fig. 1)³. In *E. coli*, the alarmone also modulates the expression of *ca.* 1200
38 genes by binding to the RNA polymerase (RNAP) and altering its promoter selectivity and the
39 stability of the initiation complex^{2,4}.

40 Peptidoglycan is an essential macromolecule that surrounds the bacterial cell providing
41 resistance to the turgor pressure of the cytoplasm⁵. The disaccharide-pentapeptide subunit of
42 peptidoglycan is polymerized to form glycan strands cross-linked by short peptides. D,D-
43 transpeptidases belonging to the penicillin-binding protein (PBP) family form 4→3 cross-links
44 connecting the fourth residue (D-Ala⁴) of an acyl donor stem to the third residue (DAP³) of an
45 acyl acceptor stem (Extended Data Fig. 1a). The unrelated L,D-transpeptidases (LDTs) catalyze
46 the formation of 3→3 cross-links connecting two DAP³ residues (Extended Data Fig. 1b). PBPs
47 and LDTs show distinct inhibition profiles since PBPs are potentially inhibited by all classes of
48 β-lactams (including penams, cephems, and carbapenems), whereas LDTs are effectively
49 inhibited only by carbapenems⁶. Bypass of PBPs by LDTs, which leads to high-level resistance
50 to β-lactams of the penam (such as ampicillin) and cephem (ceftriaxone) classes, was
51 previously reported in recombinant *E. coli* strains that overproduce the YcbB L,D-
52 transpeptidase (also known as LdtD) and the (p)ppGpp alarmone⁷. (p)ppGpp production that
53 activates YcbB-mediated β-lactam resistance was triggered either by (i) mutations that impair
54 the activity of aminoacyl-tRNA synthetases and mimic amino acid starvation, leading to the
55 activation of the (p)ppGpp synthase activity of RelA or by (ii) expression of a truncated allele
56 of *relA* that encodes the RelA' synthase mediating constitutive production of (p)ppGpp. In the
57 absence of the YcbB L,D-transpeptidase, elevated (p)ppGpp on its own leads to high-level
58 resistance to the narrow spectrum β-lactam mecillinam, which specifically inactivates PBP2, a
59 peptidoglycan D,D-transpeptidase involved in cell elongation⁸.

60 The role of (p)ppGpp in resistance to mecillinam and to broad-spectrum β -lactams
61 mediated by YcbB implies a functional link between peptidoglycan synthesis and the stringent
62 response. The nature of this link remains fully unknown. This is surprising since (p)ppGpp-
63 mediated mecillinam-resistance was reported 30 years ago. Furthermore, mecillinam has
64 been extensively used as a chemical probe for exploring both peptidoglycan polymerization
65 and the mode of action of β -lactams. The pleiotropic effects of (p)ppGpp may account for this
66 knowledge gap. Here, we investigate every aspect of (p)ppGpp-mediated regulation and
67 narrow its role in β -lactam resistance down to specific alterations of RNAP and ribosomal
68 functions. The structure of peptidoglycan was not modified in response to these alterations
69 indicating that their contribution to β -lactam resistance did not rely on regulatory effects on
70 genes involved in peptidoglycan metabolism. We discuss the impact of these controls in the
71 light of recent models accounting for the cascade of events triggered by inactivation of
72 peptidoglycan polymerases by β -lactams.

73

74 RESULTS

75 **High levels of (p)ppGpp do not alter peptidoglycan structure.** Our first objective was to
76 determine whether (p)ppGpp directly affects peptidoglycan composition. In the absence of
77 induction of the plasmid copy of the *ycbB* gene by IPTG, production of the (p)ppGpp synthase
78 following induction of gene *relA'* did not lead to any modification of peptidoglycan structure
79 (Fig. 2a, 2c, and 2d). In this condition, the proportion of peptidoglycan dimers containing 3 \rightarrow 3
80 cross-links was low. In contrast, induction of *ycbB* alone resulted in an increase in the
81 proportion of 3 \rightarrow 3 cross-linked dimers and overall peptidoglycan cross-linking (Fig. 2b, 2c, and
82 2d). Additional induction of *relA'* did not result in further modifications of the peptidoglycan
83 structure. These observations indicate that the alarmone does not modulate the activity of
84 enzymes specifically involved in peptidoglycan polymerization. The formal possibility that
85 production of (p)ppGpp might alter the expression of the chromosomal copy of *ycbB* was ruled
86 out by repeating the peptidoglycan analysis in strains lacking the chromosomal copy of this
87 gene (Extended Data Fig. 2).

88

89 **Bypass of (p)ppGpp by mutational alteration of RNA polymerase.** Our second objective was
90 to determine whether the essential role of the (p)ppGpp alarmone in YcbB-mediated β -lactam

91 resistance depends upon modulation of RNA polymerase activity or upon direct binding to
92 other enzymes. To address this question, we anticipated that mutations enabling expression
93 of β -lactam resistance in a $\Delta relA \Delta spoT$ background [(p)ppGpp⁰] would map in the gene(s)
94 encoding the alarmone target(s) responsible for resistance and thereby lead to their
95 identification. YcbB-expressing mutants resistant to all β -lactams except carbapenems, the
96 hallmark of bypass of PBPs by YcbB, were obtained at a frequency of *ca.* 2×10^{-8} in media
97 containing the broad-spectrum β -lactam ampicillin. Genome sequencing of 5 mutants
98 revealed 4 distinct single mutations leading to 4 distinct amino acid substitutions in the β or
99 β' RNAP subunits (Fig. 3a). This observation indicates that the role of (p)ppGpp in YcbB-
100 mediated β -lactam resistance depends upon modulation of RNAP activity and not upon direct
101 binding of (p)ppGpp to other enzymes.

102 Our next objective was to extend our analysis to mutants selected on media containing
103 mecillinam in the absence of induction of *ycbB*. Mutants selected on this medium (frequency
104 of *ca.* 3×10^{-8}) were all (12 out of 12) additionally resistant to all other β -lactams except
105 carbapenems upon induction of *ycbB* by IPTG and harbored 10 distinct mutations leading to
106 10 distinct substitutions in the β or β' RNAP subunits (Fig. 3a). Thus, dissociated resistance to
107 mecillinam or to ampicillin (upon *ycbB* induction) was not observed indicating that (p)ppGpp
108 has similar roles in mecillinam resistance and in YcbB-mediated broad-spectrum β -lactam
109 resistance. For the sake of simplicity, we will hereafter refer to β -lactam resistance to
110 designate mecillinam resistance in the absence of *ycbB* induction combined to resistance to
111 cepheems and penams upon induction of this gene.

112 RNAP is the target of rifampicin and substitutions leading to resistance to this drug are
113 mostly clustered in narrow regions of the RNAP β subunit⁹ that was shown here to also contain
114 substitutions selected on media containing β -lactams (above, Fig. 3a and Extended Data Fig.
115 3a). Strikingly, pioneering work on (p)ppGpp-mediated mecillinam resistance revealed that
116 rifampicin at sub-inhibitory concentrations abolishes resistance to mecillinam⁸. The synergy
117 between the two drugs prompted us to determine whether selection for resistance to β -
118 lactams or rifampicin results in dissociated or co-resistance to these drugs. The collection of
119 17 mutants selected on β -lactams was screened for expression of rifampicin resistance
120 revealing co-resistance in 8 of 17 mutants (Fig. 3a). Conversely, mutants selected on rifampicin
121 (frequency of *ca.* 9×10^{-7}) harbored mutations in the RNAP β subunit (10 out of 10; 5 distinct
122 mutations; 5 distinct amino acid substitutions) and were resistant to rifampicin only (4 out of

123 10 mutants, 4 distinct amino acid substitutions) or co-resistant to β -lactams (6 out of 10; 1
124 distinct amino acid substitution). These results indicate that substitutions in the β or β' RNAP
125 subunits result both in dissociate and co-resistance to β -lactams and rifampicin. Substitutions
126 conferring co-resistance to β -lactams and rifampicin both mimic binding of (p)ppGpp to RNAP,
127 thereby leading to β -lactam resistance, and prevent binding of rifampicin to RNAP. Although
128 the modes of action of β -lactams and rifampicin differ, these results demonstrate the possible
129 emergence of cross-resistance in clinical isolates, a so-far neglected aspect of these two widely
130 used antibiotics. Of note, cross-resistance to rifampicin and the β -lactam cefuroxime in a
131 mutant selected in laboratory conditions was reported to result from the Ser⁴⁸⁷Leu
132 substitution in *Bacillus subtilis* RpoB, which is distinct from those reported in Fig. 3a for *E.*
133 *coli*¹⁰.

134
135 **RNAP (p)ppGpp binding site 2 is critical for β -lactam resistance.** Since RNAP harbors two
136 (p)ppGpp binding sites, our next objective was to determine whether β -lactam resistance is
137 specifically controlled by binding of the alarmone to one of these sites. These sites are located
138 at the interfaces defined by the β' subunit and either the ω subunit (site 1) or transcription
139 factor DksA (site 2)^{11,12}. Since the alarmone does not bind to site 1 or 2 in the absence of the
140 ω subunit or DksA, respectively, we independently deleted the corresponding genes to disrupt
141 either (p)ppGpp binding site. Deletion of *dksA* but not that of *rpoZ* abolished expression of β -
142 lactam resistance, indicating that resistance requires binding of the alarmone to site 2 but not
143 to site 1 (Extended Data Fig. 4). In agreement, the RNAP substitutions enabling β -lactam
144 resistance in the (p)ppGpp⁰ background were closer to site 2 than to site 1 (Fig. 3b) and were
145 clustered at positions in or very close to the path of DNA in the transcription complex, possibly
146 causing a reduction in the stability of the RNAP-promoter open complex (Extended Data Fig.
147 3b)⁹. The overexpression of *dksA*, known to modulate transcription on its own^{13,14}, bypassed
148 the requirement of (p)ppGpp for β -lactam resistance (see below). Together, these results
149 suggest that β -lactam resistance requires a modulation of RNAP activity mediated by binding
150 of the alarmone to site 2. This effect can be mimicked by modification of residues in the β and
151 β' RNAP subunits or by overproduction of DksA.

152
153 **A negative control of the growth rate is not sufficient for β -lactam resistance.** Since the
154 intracellular concentration of (p)ppGpp is one of the factors that affect growth rate¹⁵, we

155 considered the possibility that β -lactam resistance might merely depend upon this global
156 effect. To investigate this possibility, we determined the growth rate of 12 mutants
157 representative of dissociate and co-resistance to β -lactams and rifampicin (Fig. 3a). Strikingly,
158 all mutants, irrespective of their resistance phenotype, had an increased generation time
159 compared to the parental strain. For instance, one mutant (harboring the Gly⁴⁴⁹Val
160 substitution) was resistant to β -lactams but another (harboring the Gln¹⁴⁸Leu substitution)
161 was susceptible to β -lactams in spite of a similarly increased generation time (27 ± 1 min vs.
162 28 ± 2 min, respectively, instead of 24 ± 1 min for the parental strain) (see Extended Data Fig.
163 5 for the resistance phenotypes conveyed by the Gly⁴⁴⁹Val and Gln¹⁴⁸Leu substitutions). In
164 addition, supplementation of the growth medium with sub-inhibitory concentrations of
165 chloramphenicol resulted in a dose-dependent increase in the generation time without
166 enabling ceftriaxone resistance (Extended Data Fig. 6a and 6b). Furthermore, growth at 28 °C
167 instead of 37 °C also resulted in an increase in the generation time and the absence of
168 expression of β -lactam resistance (Extended Data Fig. 6c). These analyses revealed that a
169 reduced growth rate was not, in itself, sufficient for β -lactam resistance. The role of (p)ppGpp
170 in β -lactam resistance does not only depend upon a negative control of the growth rate, that
171 might have, for example, compensated for ineffective peptidoglycan polymerization in the
172 presence of the drugs.

173

174 **Single-gene overexpression identified *dksA* as the only gene bypassing (p)ppGpp for β -**
175 **lactam resistance.** To identify (p)ppGpp-regulated genes specifically involved in β -lactam
176 resistance, we first investigated unique genes by using the ASKA plasmid library, which enables
177 IPTG-inducible overexpression of each individual gene of the *E. coli* genome¹⁶. Selection for a
178 bypass of the (p)ppGpp requirement for β -lactam resistance revealed the same DksA-
179 encoding plasmid in 40 out of 40 transformants. These results suggest that the essential role
180 of (p)ppGpp in β -lactam resistance cannot be accounted for by the upregulation of a single
181 gene. However, the impact of the upregulation of a combination of several genes cannot be
182 assessed by this experimental approach.

183

184 **(p)ppGpp does not mediate β -lactam resistance by modulating the activity of alternative**
185 **sigma factors.** Since the various effects of (p)ppGpp on gene transcription include the
186 recruitment of alternative sigma factors by RNAP (Fig. 1)^{17,18}, we investigated the possibility

187 that the role of (p)ppGpp in β -lactam resistance could involve increased transcription of a set
188 of genes controlled by a specific sigma factor^{19–21}. Four of the six alternative sigma factors
189 were directly evaluated by gene deletion as they are dispensable for growth in *E. coli*. Deletion
190 of genes *rpoN*, *rpoS*, *rpoF*, or *fecI*, encoding the unessential sigma factors σ^N (σ^{54}), σ^S (σ^{38}), σ^F
191 (σ^{28}), and σ^{FecI} (σ^{19}), respectively, did not abolish resistance to β -lactams (Extended Data Table
192 1). As a complementary approach, we designed an experiment to test the impact of
193 overproduction of each one of the six alternative sigma factors on the expression of β -lactam
194 resistance. The rationale for this experiment arises from the possibility that overproduction of
195 these alternative factors could lead to effective competition with the vegetative sigma factor
196 σ^D for binding to the RNAP core enzyme, thereby mimicking the (p)ppGpp-dependent
197 increased affinity of RNAP for the alternative sigma factors. Overproduction of the essential
198 (σ^H and σ^E) and unessential (σ^N , σ^S , σ^F , and σ^{FecI}) sigma factors was obtained by cloning the
199 corresponding genes in the expression vector pHV7. Overproduction of the six alternative
200 sigma factors did not bypass the requirement of (p)ppGpp for β -lactam resistance in any of
201 the genetic backgrounds tested (Extended Data Table 1). Together, these results indicate that
202 modulation of the transcription of specific genes by alternative sigma factors does not account
203 for the essential role (p)ppGpp in β -lactam resistance.

204

205 **Downregulation of ribosomal RNA operons mediates β -lactam resistance in combination**
206 **with a specific alteration of the β RNAP subunit.** Since binding of (p)ppGpp to the RNAP
207 results in a decrease in the number of ribosomes per cell mediated by the downregulation of
208 the operons encoding ribosomal RNA genes^{13,22,23} (Fig. 1) our last objective was to determine
209 whether a decrease in the transcription of ribosomal RNAs could contribute to β -lactam
210 resistance. The CRISPR interference (CRISPRi) approach²⁴ was therefore used to downregulate
211 the transcription of multiple *rrn* operons at once (Fig. 4a and Extended Data Fig. 7a for the
212 rationale underlying the design of the RNA guides). To guide dCas9 on the *rrn* operons, we
213 independently cloned in the pFR56 plasmid four different single-guide RNAs (sgRNAs)
214 targeting all seven 23S rRNA genes (*rrlA*, *B*, *C*, *D*, *E*, *G*, and *H*; 7 *rrl* sgRNA) or only *rrlB*, *E*, and *G*
215 (3 *rrl* sgRNA). In addition, we tested as negative controls two sgRNAs (*ctrl1* and *ctrl2*) without
216 complementarity to the *E. coli* genome²⁵. Combined downregulation of the seven *rrn* operons
217 was not compatible with growth (Extended Data Fig. 7b). In the absence of (p)ppGpp,
218 downregulation of the *rrnB*, *E*, and *G* operons resulted in ceftriaxone resistance (in conditions

219 of induction of *ycbB*; Fig. 4b and 4c) and to mecillinam resistance in the absence of *ycbB*
220 induction (Extended Data Fig. 7c). The control sgRNAs had no phenotypic effect. A quantitative
221 mass spectrometric analysis of ribosomal proteins was designed to determine whether
222 CRISPRi-mediated downregulation of the distal portion of the *rrnB*, *E*, and *G* operons by the 3
223 *rrl* sgRNA was resulting in a modification of the number of ribosomes per cell (Extended Data
224 Fig. 8). The analysis of 188 unique tryptic fragments belonging to 49 ribosomal proteins
225 indicated that 3 *rrl*-mediated CRISPR interference resulted in a minor (15%) but significant
226 reduction in the number of ribosomes per cells (p -value < 0.0001). As a control, we sequenced
227 the genome of the strain producing the 3 *rrl* guide. This analysis revealed the presence of an
228 unexpected mutation causing the P³⁷⁶R substitution in the β subunit of the RNAP. To evaluate
229 whether the P³⁷⁶R substitution contributed to β -lactam resistance, we independently
230 introduced plasmids pKT2(*ycbB*) and pHV136(*dcas9*; 3 *rrl*) in six sub-cultures of the BW25113
231 Δ *relA* Δ *spoT* strain whose genome was re-sequenced to eliminate the possible presence of any
232 undesired mutation. The number of cultures required for the introduction of plasmids
233 pKT2(*ycbB*) and pHV136(*dcas9*; 3 *rrl*) in this (p)ppGpp⁰ strain and for the drug susceptibility
234 assay was minimized. Under such conditions, induction of the genes encoding dCas9 and YcbB,
235 in combination with constitutive transcription of the 3 *rrl* sgRNA, did not confer resistance to
236 any of the six independently-constructed BW25113 Δ *relA* Δ *spoT* pKT2(*ycbB*) pHV136(*dcas9*; 3
237 *rrl*) strains (Extended Data Fig. 9). Thus β -lactam resistance depended upon both CRISPRi-
238 mediated downregulation of *rrn* operons and the P³⁷⁶R substitution in the β subunit of the
239 RNAP.

240

241 **DISCUSSION**

242 We used a trial-and-error strategy for elucidating the mechanism underlying the contribution
243 of (p)ppGpp to β -lactam resistance, which has remained enigmatic for three decades. We first
244 showed that the alarmone has no direct impact on peptidoglycan structure, including the
245 proportion of 3 \rightarrow 3 and 4 \rightarrow 3 cross-links (Fig. 2 and Extended Data Fig. 2). This observation
246 indicates that the contribution of (p)ppGpp to β -lactam resistance does not depend upon a
247 modulation of the activity of peptidoglycan synthesis enzymes. Then, we established that a
248 (p)ppGpp-mediated increase in the generation time is unlikely to account for the role of the
249 alarmone in resistance. This conclusion was reached by showing that derivatives of a
250 (p)ppGpp⁰ strain remain susceptible to β -lactams in spite of increases in the generation time

251 resulting from (i) the production of RNAP with the Gln¹⁴⁸Leu, Gln¹⁴⁸Arg, Leu¹⁴⁹Arg, or Ile⁵⁷²Leu
252 substitutions in the β -subunit (Fig. 3), (ii) the exposure to sub-inhibitory concentrations of
253 chloramphenicol, or (iii) a reduction of the growth temperature from 37 °C to 28 °C (Extended
254 Data Fig. 6). The possibility that (p)ppGpp could bind to enzymes and directly modulate their
255 catalytic activity was dismissed based on selection of RNAP mutants and overexpression of
256 DksA, which both bypassed the requirement of the alarmone for resistance (Fig. 3, Extended
257 Data Fig. 3). This result prompted us to investigate the mechanisms by which a modulation of
258 the activity of the RNAP by (p)ppGpp could contribute to β -lactam resistance. First, we showed
259 that the deletion or the overexpression of genes encoding alternative sigma factors did not
260 affect the expression of β -lactam resistance (Extended Data Table 1). This observation
261 indicates that the role of (p)ppGpp in resistance is unlikely to involve a modulation of specific
262 genes that are expressed under the control of the alternative sigma factors. We then
263 evaluated the contribution of a negative control of rRNA gene transcription to β -lactam
264 resistance and showed that downregulation of three of the seven rRNA operons by CRISPR
265 interference contributed to β -lactam resistance in the absence of (p)ppGpp (Fig. 4 and
266 Extended Data Fig. 7). The *rrnB*, *E*, and *G* operons targeted by the 3 *rrl* sgRNA do not contain
267 any distal tRNA genes downstream of the 5S rRNA gene. Thus, CRISPR interference mediated
268 by the 3 *rrl* sgRNA could only result in decreased transcription of the 23S genes (directly
269 targeted by the 3 *rrl* sgRNA) and the distal 5S rRNA genes in the same operons. The fact that
270 the number of ribosomes per cell was only reduced by 15% (Extended Data Fig. 8), whereas
271 the 3 *rrl* sgRNA targeted three of the seven operons (43%), implies that the negative control
272 of the transcription of the *rrnB*, *E*, and *G* operons may be compensated by an increase in the
273 transcription of the remaining operons (*rrnA*, *C*, *D*, and *H*). Such a compensatory mechanism
274 has also been reported for deletions of one to four of the seven *rrn* operons of *E. coli*^{26,27}.
275 Strikingly, the reduction of the number of ribosomes resulting from the downregulation of
276 three *rrn* operons in our study (15%) is similar to the reduction in the rRNA cell content (12%)
277 previously reported for the deletion of three of the seven *rrn* operons²⁷. Since the
278 downregulation of the *rrnB*, *E*, and *G* operons was largely compensated by an increase in the
279 transcription of the remaining operons, the contribution of downregulation of these operons
280 to β -lactam resistance might not be quantitative in nature. A qualitative contribution instead
281 might involve a depletion of the content of ribosomes in the specific 23S and 5S ribosomal
282 RNAs encoded by the distal portion of the *rrnB*, *E*, and *G* operons. This hypothesis provokingly

283 implies that *E. coli* produces ribosomes that are functionally specialized depending upon their
284 content in specific rRNAs. Alternatively, the moderate decrease in the number of ribosomes
285 could account for resistance in combination with a pleiotropic effect of the P³⁷⁶R substitution,
286 which disorganizes the gate-loop of the RNAP β subunit and could result in the destabilization
287 of the open complex²⁸.

288 Strikingly, overproduction of (p)ppGpp had no impact on peptidoglycan structure (Fig.
289 2) indicating that the alarmone does not act by preventing binding of β -lactams to their PBP
290 targets or by compensating for impaired D,D-transpeptidase activity of the PBPs. Instead,
291 (p)ppGpp is likely to act by mitigating the consequences of PBP inactivation by β -lactams. The
292 most recent model for the mechanism of bacterial killing by β -lactams^{29–36} proposes that
293 inhibition of D,D-transpeptidases by these drugs uncouples the transglycosylation and cross-
294 linking reactions resulting in an energy-depleting futile cycle of peptidoglycan synthesis and
295 degradation. This is associated with a dysregulation of the energy metabolism and increased
296 protein synthesis, which ultimately leads to bacterial death due to the production of reactive
297 oxygen species^{32,35}. In agreement with this model, we observed in no instance a dissociation
298 between the narrow-spectrum mecillinam resistance phenotype and the broad-spectrum β -
299 lactam resistance phenotype conveyed by YcbB that involves distinct PBP targets.
300 Furthermore, this model provides a rational explanation for the double requirement of YcbB
301 and (p)ppGpp for β -lactam resistance as YcbB compensates for the loss of the transpeptidase
302 activity of PBPs whereas (p)ppGpp protects the bacterium from the downstream effects of
303 PBP inactivation by β -lactams. This model also accounts for the fact that production of
304 (p)ppGpp is, in itself, sufficient for mecillinam resistance since the alarmone mitigates the
305 deadly consequences of inactivation of the D,D-transpeptidase activity of PBP2, a β -lactam
306 target specifically inactivated by mecillinam.

307

308 MATERIALS AND METHODS

309 **Strains, plasmids, and growth conditions.** The characteristics and origin of plasmids and strains
310 used in the study are listed in Supplementary Table S1. Bacteria were grown at 37 °C in brain
311 heart infusion (BHI; Difco) agar or broth with aeration (180 rpm). Kanamycin at 50 μ g/ml was
312 used for selection of transductants carrying the Km^R cassette obtained from the Keio collection³⁷.
313 The growth media were systemically supplemented with drugs to counter-select plasmid loss: 10

314 $\mu\text{g/ml}$ tetracycline for vector pHV6 and derivatives, 20 $\mu\text{g/ml}$ chloramphenicol for pHV7, pFR56,
315 and derivatives, 25 $\mu\text{g/ml}$ zeocin for pHV63zeo. Induction of the P_{trc} , P_{araBAD} , and P_{hlf} promoters
316 was performed with isopropyl β -D-1-thiogalactopyranoside (IPTG), L-arabinose, and 2,4-
317 diacetylphloroglucinol (DAPG), respectively. Plasmids constructed in this study were obtained by
318 using NEBuilder HiFi DNA assembly (New England Biolabs) method, unless otherwise specified.
319 Deletions of specific genes were obtained by P1 transduction of the Km^R cassette of selected
320 mutants from the Keio collection^{37,38}. For multiple gene deletions, the Km^R cassette was removed
321 by the FLT recombinase encoded by plasmid pCP20.

322 **Preparation of sacculi.** Strains BW25113 $\Delta relA$ or BW25113 $\Delta relA \Delta ycbB$ harboring plasmids
323 pHV6 and pHV7 (vectors), pKT2(*ycbB*) and pHV7, pHV6 and pKT8(*relA'*), or pKT2(*ycbB*) and
324 pKT8(*relA'*) were grown in BHI broth in the presence of 40 μM IPTG for induction of the P_{trc}
325 promoter of pHV6 and pKT2(*ycbB*) and of 1% L-arabinose for induction of the P_{araBAD} promoter of
326 pHV7 and pKT8(*relA'*). pHV6 and pHV7 are the vectors used for construction of pKT2(*ycbB*) and
327 pKT8(*relA'*), respectively. The growth media contained tetracycline (to counterselect loss of pHV6
328 or pKT2) and chloramphenicol (to counterselect loss of pHV7 or pKT8). Bacteria were grown to
329 late growth phase, *i.e.* to an $\text{OD}_{600\text{nm}}$ greater than 1.0 (*ca.* 6 h at 37 °C under agitation). Ten μl of
330 the resulting bacterial suspensions were plated on BHI agar supplemented with the same
331 inducers (40 μM IPTG and 1% L-arabinose) and drugs (10 $\mu\text{g/ml}$ tetracycline and 20 $\mu\text{g/ml}$
332 chloramphenicol). For each replicate, 5 BHI agar plates were used in order to obtain a sufficient
333 amount of bacteria. Plates were incubated for 16 h at 37 °C. Bacteria were harvested in two steps
334 by scrapping each plate with 1 ml of phosphate-buffered saline (PBS) pH 7.2 followed by washing
335 the plate with an additional 1 ml of PBS. Bacteria were boiled in 0.5 x PBS supplemented with 4%
336 sodium dodecyl sulfate (SDS) in a final volume of 20 ml for 1 h. Sacculi were harvested by
337 centrifugation (20,000 x *g* for 20 min at 20 °C), washed five times with 20 ml of water,
338 resuspended in 1 ml of 20 mM Tris-HCl pH 7.5, and incubated with 100 $\mu\text{g/ml}$ pronase at 37 °C
339 for 16 h. Sacculi were washed five times with 1 ml of water, resuspended in 1 ml of 20 mM sodium
340 phosphate pH 8.0 and incubated with 100 $\mu\text{g/ml}$ trypsin at 37 °C for 16 h. Sacculi were washed
341 five times with 1 ml of water, boiled for 5 min, collected by centrifugation, resuspended in 300
342 μl of water, and stored at -20 °C.

343 **Peptidoglycan analysis.** Ten μl of purified sacculi were digested with 120 μM lysozyme in 40 mM
344 Tris-HCl pH 8.0 at 37 °C for 16 h. Insoluble material was removed by centrifugation at 12,000 rpm

345 in a microcentrifuge for 10 min and the soluble fraction containing muropeptides was reduced
346 with sodium borohydride in 125 mM borate buffer pH 9.0 for 1 h at room temperature.
347 Phosphoric acid was used to adjust the pH to 4.0. Muropeptides were separated by *rp*HPLC in a
348 C18 column (Hypersil GOLD aQ; 250 x 4.6 mm; 3 μ m, Thermo Scientific) at a flow rate of 1 ml/min
349 with a linear gradient (0 to 20%) applied between 10 and 60 min (buffer A, TFA 0.1%; buffer B,
350 acetonitrile 99.9%, TFA 0.1%, v/v). Absorbance was monitored at 205 nm and fractions were
351 collected, lyophilized, resuspended in water, and analyzed by mass spectrometry. Mass spectra
352 were obtained on a Bruker Daltonics maXis high-resolution mass spectrometer (Bremen,
353 Germany) operating in the positive mode (Analytical Platform of the Muséum National d'Histoire
354 Naturelle, Paris, France). Mass spectral data were explored using mineXpert³⁹.

355 **Mutant selection and phenotype analysis.** *E. coli* BW25113 Δ *relA* Δ *spoT* pJEH12(*ycbB*) was
356 streaked for isolated colonies on agar plates containing 10 μ g/ml tetracycline to counter-
357 select loss of plasmid pJEH12(*ycbB*). The selection procedure was independently carried out
358 starting with independent colonies. Briefly, a fresh colony of *E. coli* BW25113 Δ *relA* Δ *spoT*
359 pJEH12(*ycbB*) was inoculated in 5 ml of BHI broth supplemented with 10 μ g/ml tetracycline.
360 Bacteria were grown overnight at 37 °C with shaking (180 rpm). One hundred μ l of overnight
361 cultures were plated on BHI agar supplemented with 32 μ g/ml ampicillin and 50 μ M IPTG, 50
362 μ g/ml mecillinam, or 16 μ g/ml rifampicin. Plates were incubated overnight at 37 °C. The
363 frequency of mutants was determined by dividing the number of CFUs obtained on the
364 selective media by the total number of CFUs plated on the selective media. Each mutant
365 presented in this work originates from independent experiments. Activation of YcbB-mediated
366 β -lactam resistance in the mutants was confirmed using the disk diffusion assay in BHI agar
367 supplemented or not with 50 μ M IPTG to confirm that induction of *ycbB* was required for
368 resistance⁷. Resistance to tetracycline and imipenem was also tested to confirm the presence
369 of plasmid pJEH12(*ycbB*) and the dependence upon active YcbB, respectively. Disks were
370 loaded with 10 μ g of mecillinam, 10 μ g of ampicillin, 30 μ g of ceftriaxone, 10 μ g of imipenem,
371 30 μ g of tetracycline or 30 μ g of rifampicin.

372 **Whole-genome sequencing.** To identify the mutations, 5 ml of BHI broth containing 10 μ g/ml
373 tetracycline were inoculated with a single colony of each selected mutants and genomic DNA
374 was extracted (Wizard DNA extraction kit, Promega). Genomic DNA was either sequenced by
375 paired-end joining Illumina (Novogene) or used for PCR amplification of the *rpoB* gene

376 followed by Sanger sequencing. For Illumina sequencing, paired-end reads of 150 bp were
377 generated using a NovaSeq6000 (> 50x coverage). Read processing and identification of the
378 mutations were performed with the *breseq* pipeline (v0.35.5)⁴⁰. The positions of the mutations
379 identified in the RNAP subunits are shown on the structures deposited on the Protein Data
380 Bank [entry 5VSW (RNAP, DksA and ppGpp complex), 5UAC (RNAP and rifampicin complex),
381 and 7KHV (RNAP and *rrnB* P1 promoter open complex)] using the PyMOL software (v2.3.4).
382 Subunits of the structures were colored as indicated in the legends of the corresponding
383 figures.

384 **Determination of generation time.** For each mutant tested, a fresh colony was inoculated in
385 1 ml BHI broth. Fifty μ l of the suspension were inoculated in 150 μ l BHI broth in 5 wells of 96-
386 well plates. Incubation was performed at 37 °C overnight with agitation. Optical density at 600
387 nm was continuously determined with a plate-reader (Tecan Infinite 200 pro). For technical
388 and biological replicates as detailed in legends, the generation time (G) was determined using
389 the formula $G = \ln(\mu) / 2$, where μ corresponds to the slope of the linear part of the curved
390 obtained by plotting the $\ln(\text{OD}_{600\text{nm}})$ as a function of time.

391 **Plating efficiency assay.** Bacteria were grown to late growth phase, *i.e.* to an $\text{OD}_{600\text{nm}}$ greater
392 than 1.0 (*ca.* 6 h at 37 °C under agitation). The $\text{OD}_{600\text{nm}}$ was adjusted to 1.0 and 10-fold
393 dilutions (10^{-1} to 10^{-5}) were prepared in BHI broth. Five μ l of the resulting bacterial suspensions
394 were spotted on BHI agar plates supplemented with inducers and drugs as indicated in the
395 legend to figures. Plates were imaged after 16 h (or 24 h for plates containing ceftriaxone) of
396 incubation at 37 °C. Data shown in the figures are representatives of at least two biological
397 repeats.

398 **Identification of genes that bypass the requirement of (p)ppGpp.** To identify genes whose
399 overexpression might bypass the requirement of (p)ppGpp in YcbB-mediated β -lactam
400 resistance or mecillinam resistance, the pool of plasmids from the ASKA library^{16,41} was
401 transformed into BW25113 Δ *relA* Δ *spoT* pHV63zeo(*ycbB*). The resulting bacterial suspension
402 was plated on BHI agar supplemented with 8 μ g/ml ceftriaxone plus 1% L-arabinose to induce
403 expression of *ycbB*, or 50 μ g/ml mecillinam, and in the absence or presence of 20, 60, and 180
404 μ M IPTG to induce expression of the ASKA plasmid-encoded genes. The media contained
405 chloramphenicol to counter-select loss of the ASKA plasmids. Plates were incubated overnight
406 at 37 °C. Twenty transformants selected with ceftriaxone plus L-arabinose and 20

407 transformants selected with mecillinam were isolated and their resistance phenotype
408 confirmed. ASKA plasmids that produced resistant clones under the selective pressure of β -
409 lactams were extracted and Sanger sequencing revealed in all 40 transformants the presence
410 of the ASKA plasmid carrying the *dksA* gene.

411 **Downregulation of *rrn* operons by CRISPRi.** The options for choosing the RNA guide targeting
412 a subset of the *rrn* operons were limited by: (i) the presence of a polymorphism common to a
413 subset of the *rrn* operons; (ii) the presence of the polymorphism in the protospacer adjacent
414 motif (PAM) to avoid off-target effects; (iii) the targeting of the 23S rRNA genes to avoid any
415 effect on the tRNA genes located between the 16S and 23S rRNA genes; and (iv) the targeting
416 of *rrn* operons that do not contain any tRNA genes at the 3' end of the operons (i.e.
417 downstream of the distal 5S RNA genes). According to these four criteria, targeting of the *rrnB*,
418 *E*, and *G* was the unique solution. The sgRNAs were under the control of the constitutive
419 promoter of plasmid pFR56, which also mediates DAPG-inducible production of dCas9.

420 **Ribosome preparation and analysis.** *E. coli* BW25113 Δ *relA* Δ *spoT* harboring plasmids
421 pKT2(*ycbB*) and derivatives of pFR56 encoding dCas9 and either the *ctrl1* or 3 *rrl* single-guide
422 RNAs (sgRNAs) were grown overnight at 37 °C in 10 mL of BHI broth containing 10 μ g/mL
423 tetracycline and 20 μ g/mL chloramphenicol under vigorous shaking. Two mL of this preculture
424 were used to inoculate four Erlen-Meyer flasks containing 200 mL of BHI broth supplemented
425 with 10 μ g/mL tetracycline, 20 μ g/mL chloramphenicol, 40 μ M IPTG for induction of *ycbB*, and
426 50 μ M DAPG for induction of *dcas9*. Cells were grown at 37 °C under vigorous shaking to an
427 optical density at 600 nm (OD_{600}) of 0.6 (average of 0.607 ± 0.123 versus 0.619 ± 0.073 for the
428 *ctrl1* and 3 *rrl* cultures, respectively, $n = 4$ for each strain). Thirty mL of each culture were
429 centrifuged ($5,000 \times g$ at 4 °C for 8 min), harvested bacteria were washed twice with 30 mL of
430 cold PBS, and the pellet was frozen in liquid nitrogen. To obtain fully labeled cells, *E. coli*
431 BW25113 was grown in 500 mL of M9 minimal medium containing 0.2% ^{13}C D-glucose and ^{15}N
432 ammonium chloride (Eurisotop) at 37 °C under vigorous shaking to an OD_{600} of 1.0. Cells were
433 harvested by centrifugation ($5,000 \times g$ at 4 °C for 8 min), washed twice with 30 mL of cold PBS,
434 and resuspended in 25 mL of ribosome lysis buffer (RLB; 50 mM Tris-HCl pH 7.5, 50 mM
435 magnesium acetate, 100 mM ammonium chloride, 1 mM DTT, and 0.5 mM EDTA), and frozen
436 in liquid nitrogen. An aliquot of 1 mL (equivalent of 20 mL of culture) of labeled cells was used
437 to resuspend the bacterial pellets corresponding to 30 mL of each one of the four unlabeled

438 cultures. The resulting bacterial suspensions were sonicated on ice (3 x 30 s). Lysates were
439 clarified by centrifugation (20,000 x *g* at 4 °C for 20 min). The clarified lysates were deposited
440 on top of 30 mL of a 1.1 M saccharose solution in ribosome lysis buffer. Ribosomes were
441 pelleted (100,000 x *g* overnight at 4 °C) in a fixed angle rotor and resuspended in 300 µL of
442 100 mM ammonium acetate buffer pH 7.5, distributed in four aliquots of 75 µL, and frozen in
443 liquid nitrogen.

444 Identification of the purified proteins and quantification of the tryptic fragments was
445 performed by liquid chromatography-mass spectrometry (LC-MS/MS). The ribosomal
446 preparations (30 µL) were processed by subjecting them to a short-time SDS-polyacrylamide
447 gel electrophoresis to let the proteins enter the stacking gel but not to get resolved. The gel
448 band was excised and a third of it was sliced to perform the reduction-alkylation step prior to
449 the trypsin-based hydrolysis of the proteins. For each sample the peptidic content was
450 estimated by measuring the absorbance at 280 nm. The sample volume was adjusted so that
451 a volume of 4 µL of sample would carry 400 ng of peptides for injection into a nanoLC-Ultra
452 system (Eksigent, Dublin, CA, USA). The chromatographic column was a Biosphere C18 (length:
453 31 cm, inner diameter: 75 µm, particle size: 3 µm, pore size: 120 Å). Chromatography was
454 performed at a flow rate of 300 nL/min (buffer A: 99.9% water, 0.1% formic acid; buffer B:
455 99.9% acetonitrile, 0.1% formic acid). The chromatographic separation was run over 80 min
456 with two gradient development steps (i) from 5 % to 30 % buffer B in 75 min and (ii) to 95 %
457 buffer B in 5 min. The analytes eluting from the chromatographic column were injected in a Q
458 ExactivePlus mass spectrometer (Thermo Scientific, Courtaboeuf, France) using a nano-
459 electrospray interface and ionized using a liquid junction and an uncoated capillary probe (10
460 µm inner diameter; New Objective, Woburn, MA, USA).

461 Full scan MS acquisition settings were as follows: resolution, 70,000; AGC target, 3×10^6 ;
462 maximum ion time, 60 ms; scan range, 350-1400 *m/z*; acquisition type, profile. Data
463 dependent MS/MS acquisition settings were as follows: resolution, 17,500; AGC target, 10^5 ;
464 maximum ion time, 120 ms; topN, 8; isolation window, 1.5 *m/z*; normalized collision energy,
465 27%; acquisition type, profile. The mass spectrometer was directed (i) to select ions of charge
466 state either 2+ or 3+ showing an expected isotopic pattern for unlabeled peptide, (ii) to
467 exclude secondary isotopic peaks, and (iii) to exclude already fragmented ions for 50 s.

468 A standard bottom-up proteomics approach yielded ribosomal protein identifications
469 based on the unlabeled peptides contained in each sample. The identification and
470 quantification processes were carried out using an updated version of the i2MassChroQ (the
471 preferred software version for this work is 1.0.0)^{42,43}. By using the elemental composition of
472 each identified unlabeled peptide ion (along with its charge restricted to $z = 2$), the m/z value
473 of the fully labeled peptide ion counterpart was computed. The (rt, m/z , z) triplets of the
474 labeled and unlabeled ions eluting at the same retention time (rt) were used to perform ion
475 current extractions (an area-under-the-curve measurement). Further data processing was
476 restricted to peptides from ribosomal proteins having identified light and heavy isotopologues
477 in all eight samples corresponding to the four biological repeats for both the *ctrl1* and 3 *rrl*
478 sgRNAs. For each peptide found in all eight samples, the ratio of the unlabeled to labeled ion
479 peak areas (light-to-heavy-area ratio) was computed. The mean value (\bar{x}) of the light-to-heavy-
480 area ratio measurements was computed separately in each group (*ctrl1* and 3 *rrl* samples).
481 The identified peptides in the *ctrl1* and 3 *rrl* groups that have any one of the four light-to-
482 heavy-area ratios outside of the $[0.5 < \bar{x} < 2]$ interval were filtered out of the data set.

483 **Statistics and reproducibility.** For the plating efficiency assays, at least two biological repeats
484 were performed. For peptidoglycan analysis by *rp*HPLC and mass spectrometry, three
485 independent biological repeats were performed. Antibiotic susceptibility testing data are
486 medians from three independent biological repeats. Generation time was determined from at
487 least three biological repeats. For proteomic analysis of ribosome content, four independent
488 biological repeats were performed. The light-to-heavy mean ratios of 188 unique tryptic
489 fragments belonging to 49 ribosomal proteins were compared between the two conditions,
490 and the p -value (< 0.0001) was obtained from unpaired two-tailed t -test using Welch's
491 correction for heteroscedasticity.

492

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500

501 **AUTHOR CONTRIBUTIONS**

502 HV: conception and design; acquisition, analysis, and interpretation of data; drafting and
503 revising the article. CAP: conception and design; acquisition, analysis, and interpretation of
504 data; revising the article. FR and OL: mass spectrometric analysis of ribosome preparations
505 and quantitative mass data processing. JEH and MA: conception and design; analysis and
506 interpretation of data; drafting and revising the article.

507

508 **CONFLICT OF INTEREST**

509 The authors declare that there is no conflict of interest.

510

511 **DATA AVAILABILITY**

512 Source data are provided with this paper. Whole genome sequencing raw data of RNAP
513 mutants are available at the Sequence Read Archive database (SRA) under accession code
514 PRJNA1044113.

515

516 **CODE AVAILABILITY**

517 The i2MassChroQ software used to identify and quantify the tryptic peptides is freely available
518 at <https://forgemia.inra.fr/pappso/i2masschroq/-/releases> (the preferred software version
519 for this work is 1.0.0).

520

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637 **Figure 1. Pleiotropic effects of (p)ppGpp in *E. coli*.** The (p)ppGpp alarmone binds to enzymes involved in multiple
638 metabolic pathways, such as the GuaB inosine monophosphate dehydrogenase, the DnaG DNA primase, and the
639 PPX polyphosphate kinase³. The alarmone also modulates the expression of approximately 1200 genes by
640 binding to two sites on RNA polymerase (RNAP)⁴. Modulation of gene expression involves (1) activation of specific
641 promoters involved in amino acid synthesis, (2) recruitment of alternative sigma factors, and (3) downregulation
642 of ribosomal RNA operons^{2,17}.

643
644 **Figure 2. Impact of (p)ppGpp and YcbB production on peptidoglycan structure.** (a) Representative *rp*HPLC
645 profiles of muropeptides from strains BW25113 Δ *relA* harboring vectors pHV6 and pHV7 (grey) or pHV6 and
646 plasmid pKT8(*relA'*) (dark red). (b) Representative *rp*HPLC profiles of muropeptides from strains BW25113 Δ *relA*
647 harboring plasmids pKT2(*ycbB*) and pHV7 (grey) or pKT2(*ycbB*) and pKT8(*relA'*) (dark red). (c) Structure of the
648 muropeptides eluted in *rp*HPLC peaks 1 to 10 deduced from mass spectrometry analyses. Tetra, tetrapeptide L-
649 Ala-D-iGlu-DAP-D-Ala; Tri, tripeptide L-Ala-D-iGlu-DAP; Tri-KR, tripeptide L-Ala-D-iGlu-DAP substituted by the
650 dipeptide Lys-Arg originating from the Braun lipoprotein; Tri-Tri and Tri-Tetra, dimers containing a tripeptide
651 donor stem linked to a tripeptide or tetrapeptide acceptor stem, respectively. These dimers contained a 3→3
652 cross-link formed by the YcbB L,D-transpeptidase. Tetra-Tri and Tetra-Tetra, dimers containing a tetrapeptide
653 donor linked to a tripeptide or tetrapeptide acceptor, respectively. These dimers contained a 4→3 cross-link
654 formed by D,D-transpeptidases belonging to the PBP family. Tetra(Gly), Tri-Tetra(Gly), and Tetra-Tetra(Gly),
655 muropeptides containing a Gly residue instead of D-Ala at the 4th position of the free stem. These muropeptides
656 originated from the exchange of D-Ala by Gly catalyzed by L,D-transpeptidases. Data are representative
657 chromatograms from three biological repeats. (d) Relative peak area of muropeptides extracted from strains
658 BW25113 Δ *relA* harboring plasmids pHV6 and pHV7 (grey), pHV6 and pKT8(*relA'*) (dark red), pKT2(*ycbB*) and
659 pHV7 (dashed grey), or pKT2(*ycbB*) and pKT8(*relA'*) (dashed dark red). Induction of *relA* had no impact on
660 peptidoglycan structure in these strains (panel d) as well as in derivatives of the same strains lacking the
661 chromosomal copy of *ycbB* (Extended Data Fig. 2). Data are means and standard deviations from three biological
662 repeats (n = 3). Tri and Tetra, disaccharide-tripeptide and disaccharide-tetrapeptide monomers, respectively.
663 3→3 and 4→3, dimers containing 3→3 and 4→3 cross-links, respectively.

664
665 **Figure 3. Localization of amino acid substitutions mediating β -lactam and rifampicin resistance in the RNAP
666 holoenzyme.** (a) Table of RNAP substitutions and the corresponding generation times (GT) of (p)ppGpp⁰ mutants
667 selected on media containing ampicillin plus IPTG (Amp), mecillinam (Mec), or rifampicin (Rif). ND, not
668 determined. Note that the table includes 16 distinct amino acid substitutions including Arg⁴⁵¹Cys [due to the
669 same mutation (CGT→TGT)] that was independently obtained on media containing ampicillin or mecillinam and
670 Thr⁵⁶³Pro (due to ACC→CCC) that was obtained on all three selectors. GT was determined from at least three
671 biological repeats (n ≥ 3). (b) Cartoon and surface representations of the PDB entry 5VSW are shown with
672 subunits highlighted in various colors. ppGpp molecules at the two binding sites of RNAP are indicated by red
673 arrows and circles. The inset shows the localization of amino acid substitutions conferring resistance to β -lactams
674 only (green), to rifampicin only (red), and to both β -lactams and rifampicin (yellow). Substitutions are localized
675 in the β subunit (not indicated) or β' subunit (indicated in parentheses). The figure was generated with PyMOL
676 (v2.3.4). See Potrykus, K. and Cashel, M. (2013)⁴⁴ for an exhaustive review of amino acid substitutions detected
677 in RNAP subunits.

678
679 **Figure 4. Impact of downregulation of rRNA transcription on β -lactam resistance.** (a) Schematic representation
680 of the CRISPR interference approach used to decrease expression of several *rrn* operons at once. The *rrnA* operon
681 is displayed as an example. The catalytically inactive Cas9 (dCas9) associates to the single-guide RNA (sgRNA) and
682 binds to the chromosomal target (a sequence within the 23S rRNA gene *rrlA*). The elongating RNAP that
683 encounters dCas9 dissociates from the DNA matrix, leading to downregulation of the targeted gene. The rational
684 design of the 3 *rrl* guide is described in the Extended Data Fig. 7a. (b) Plating efficiency of (i) the RpoB G⁴⁴⁹V
685 mutant harboring pKT2(*ycbB*) (positive control), (ii) the parental strain BW25113 Δ *relA* Δ *spoT* pKT2(*ycbB*)
686 (negative control), and (iii) derivatives of the latter strain harboring plasmids encoding dCas9 under the control
687 of the DAPG-inducible P_{hlf} promoter and one of the four sgRNAs expressed under the control of a constitutive
688 promoter. The sgRNAs targeted seven (7 *rrl*) or three (3 *rrl*) of the seven 23S rRNA genes. The remaining sgRNAs,
689 *ctrl1* and *ctrl2*, do not target any sequence in the *E. coli* chromosome²⁵. P³⁷⁶R*, genome re-sequencing identified
690 a substitution (P³⁷⁶R) in the β subunit of the RNAP in the strain expressing the 3 *rrl* sgRNA. Growth was tested in
691 the presence of ceftriaxone at 8 μ g/ml (+ ceftriaxone) or in the absence of the drug (- ceftriaxone). Induction of
692 the *dcas9* gene was performed with 50 μ M DAPG. BHI agar plates contained 40 μ M IPTG for induction of *ycbB*.
693 (c) Colony forming units (CFUs) were enumerated for the same strains and the same growth conditions by plating

694 100 μ L of 10-fold dilutions on agar plates. The detection limit was 10^2 cfu/mL. Horizontal bars indicate the means
695 from three biological repeats ($n = 3$).
696

697 **Extended Data Figure 1. Reactions catalyzed by PBPs and L,D-transpeptidases.** (a) Peptidoglycan cross-linking
698 by PBPs is a two-step reaction initiated by the activation of the catalytic Ser residue for nucleophilic attack of the
699 carbonyl of D-Ala⁴ in a pentapeptide donor stem. This first step results in the release of D-Ala⁵ and formation of
700 an acyl-enzyme. In the second step, the carbonyl of the resulting ester bond undergoes nucleophilic attack by
701 the side-chain amino group of the diaminopimelyl (DAP) residue of an acceptor. This results in the release of the
702 PBP and in the formation a peptidoglycan dimer (Tetra \rightarrow Tri or Tetra \rightarrow Tetra) containing a 4 \rightarrow 3 cross-link, which
703 connects D-Ala at the 4th position of the donor to DAP at the 3rd position of the acceptor. Tetra \rightarrow Tri and
704 Tetra \rightarrow Tetra dimers differ by the presence of a tripeptide or a tetrapeptide in the acceptor position, respectively.
705 D,D-transpeptidase catalytic domains are found in Class A PBPs PBP1a, PBP1b, and PBP1c, which also contain a
706 glycosyltransferase domain for glycan chain elongation. D,D-transpeptidase catalytic domains are also found in
707 monofunctional Class B PBP2 and PBP3. (b) The L,D-transpeptidases YcbB (LdtD) and YnhG (LdtE) also catalyze
708 peptidoglycan in a two-step reaction similar to that catalyzed by PBPs. Main differences involve the nature of (i)
709 the nucleophile: a cysteine instead of a serine, (ii) the donor stem: a tetrapeptide instead of a pentapeptide, and
710 (iii) the cross-link: 3 \rightarrow 3 instead of 4 \rightarrow 3.
711

712 **Extended Data Figure 2. Impact of (p)ppGpp and YcbB production on peptidoglycan structure in strains lacking**
713 **the chromosomal copy of *ycbB*.** Relative peak area of muropeptides extracted from strains BW25113 Δ *relA*
714 Δ *ycbB* harboring plasmids pHV6 and pHV7 (blue), pHV6 and pKT8(*relA'*) (orange), pKT2(*ycbB*) and pHV7 (dashed
715 blue), or pKT2(*ycbB*) and pKT8(*relA'*) (dashed orange). Data are means and standard deviations from three
716 biological repeats ($n = 3$). Tri and Tetra, disaccharide-tripeptide and disaccharide-tetrapeptide monomers,
717 respectively. 3 \rightarrow 3 and 4 \rightarrow 3, dimers containing 3 \rightarrow 3 and 4 \rightarrow 3 cross-links, respectively.
718

719 **Extended Data Figure 3. Position of the amino acid substitutions in the RNAP β subunit at the proximity of the**
720 **rifampicin binding site (a) and DNA in the open promoter complex (b).** Cartoon and surface representations of
721 the PDB entry 5UAC (a) and 7KHB (b) are shown with subunits highlighted in various colors. (p)ppGpp binding
722 sites of RNAP are indicated by red arrows and dashed circles. Substitutions led to resistance to β -lactams only
723 (green), to rifampicin only (red), and to both β -lactams and rifampicin (yellow). The inset in panel (a) shows
724 rifampicin (pink) bound to RNAP (for the sake of simplicity, only the β subunit is shown). The inset in panel (b)
725 shows DNA (grey) of the *rrnB* P1 open promoter complex. All substitutions are localized in the β subunit except
726 substitutions in positions 333, 1144, 1147, and 1308 of β' subunit (indicated in parentheses). The figure was
727 generated with PyMOL (v2.3.4).
728

729 **Extended Data Figure 4. Role of (p)ppGpp binding to the two RNAP binding sites.** Plating efficiency of BW25113
730 Δ *relA* pKT2(*ycbB*) pKT8(*relA'*) and its derivatives obtained by deletion of the *rpoZ* or *dfsA* genes. Growth was
731 tested in the presence of ceftriaxone at 8 μ g/ml (+ ceftriaxone) or in the absence of the drug (- ceftriaxone) on
732 BHI agar plates supplemented with 40 μ M IPTG and 1% L-arabinose for induction of *ycbB* and *relA'*, respectively
733 ($n = 3$).
734

735 **Extended Data Figure 5. Role of the Q¹⁴⁸L and G⁴⁴⁹V RpoB substitutions in β -lactam and rifampicin resistance.**
736 Plating efficiency was performed for BW25113 Δ *relA* Δ *spoT* pKT2(*ycbB*) and its derivatives harboring mutations
737 leading to the G⁴⁴⁹V or Q¹⁴⁸L substitution in the β -subunit of the RNAP (encoded by *rpoB*). Growth was tested in
738 BHI agar supplemented by the indicated antibiotics and 40 μ M IPTG for induction of *ycbB*. DNA was extracted
739 from the cultures that were used for the phenotypic analysis and whole genome sequencing did not reveal any
740 additional mutation in the genes encoding the RNAP subunits. This control indicates that our analysis is not
741 biased by the accumulation of suppressors of the Δ *relA* and Δ *spoT* mutations. Data are a representative of three
742 independent experiments ($n = 3$).
743

744 **Extended Data Figure 6. Impact of sub-inhibitory concentrations of chloramphenicol on the generation time**
745 **and expression of ceftriaxone resistance.** (a) Generation time (GT) as a function of supplementation of BHI broth
746 with various concentrations of chloramphenicol. Each point is the median from five biological repeat. (b) Plating
747 efficiency of the RpoB G⁴⁴⁹V mutant harboring pKT2(*ycbB*) (positive control) and of the parental BW25113 Δ *relA*
748 Δ *spoT* pKT2(*ycbB*) strain in the absence (- Cm) or presence (+ Cm) of chloramphenicol at various concentrations.
749 All plates contained 40 μ M IPTG for induction of *ycbB*. Plates contained 8 μ g/ml ceftriaxone (+ ceftriaxone) or no

750 drug (- ceftriaxone). Data are a representative of five biological repeats. (c) Plating efficiency of the RpoB G^{449V}
751 mutant harboring pKT2(*ycbB*) (positive control) and of the parental BW25113 Δ *relA* Δ *spoT* pKT2(*ycbB*) strain
752 grown at 28 °C. Plates contained 40 μ M IPTG for induction of *ycbB*, 8 μ g/ml ceftriaxone (+ ceftriaxone) or no drug
753 (- ceftriaxone). Data are a representative of five biological repeats. The generation time of the parental strain
754 was 38 \pm 4 min at 28 °C versus 24 \pm 1 min at 37 °C (determined from five biological repeats).

755

756 **Extended Data Figure 7. Lowering rRNA transcription bypasses the requirement of (p)ppGpp for mecillinam**
757 **resistance.** (a) Sequence alignments of portions of the *rrl* genes targeted by the sgRNAs. Variable positions
758 exploited to downregulate *rrlB*, *E*, and *G* without affecting *rrlA*, *C*, *D*, and *H* expression are marked by asterisks.
759 sgRNA sequences shown are the reverse complements (RC) of the sgRNA sequences used to downregulate *rrl*
760 transcription. See Material and Methods for the design of sgRNAs. (b) Plating efficiency of derivatives of
761 BW25113 Δ *relA* Δ *spoT* harboring plasmids encoding dCas9 under the control of the DAPG-inducible P_{hif} promoter
762 and four sgRNAs under the control of a constitutive promoter. The sgRNAs targeted seven (7 *rrl*) or three (3 *rrl*)
763 of the seven 23S rRNA genes. The remaining sgRNAs, *ctrl1* and *ctrl2*, do not target any sequence in the *E. coli*
764 chromosome²⁵. Induction of the *dcas9* gene was performed with 25, 50, or 200 μ M DAPG. Induction of *dcas9* in
765 the presence of the 7 *rrl* sgRNA prevented growth of BW25113 Δ *relA* Δ *spoT* at DAPG concentrations of 50 and
766 200 μ M. Effective downregulation of all 7 *rrl* copies is likely to account for the absence of growth. (c) Plating
767 efficiency of the RpoB G^{449V} mutant (positive control), the parental strain BW25113 Δ *relA* Δ *spoT* pKT2(*ycbB*)
768 (negative control) and its derivatives harboring plasmids encoding dCas9 under the control of the DAPG-inducible
769 P_{hif} promoter and the four sgRNAs under the control of a constitutive promoter. Growth was tested in the
770 presence of mecillinam at 16 μ g/ml (+ mecillinam) or in the absence of the drug (- mecillinam). Induction of the
771 *dcas9* gene was performed with 50 μ M DAPG. Mecillinam resistance was obtained in the absence of *ycbB*
772 induction. Induction of the gene encoding dCas9 was associated with a 1.4-fold increase in the generation time
773 (from 28 \pm 2 min versus 37 \pm 1 min; n = 3).

774

775 **Extended Data Figure 8. Impact of the downregulation of *rrn* operons on the relative number of ribosomes per**
776 **cell.** (a) Unlabeled ribosomes were purified from derivatives of BW25113 Δ *relA* Δ *spoT* pKT2(*ycbB*) harboring
777 pHV137(*dcas9*; *ctrl1*) or pHV136(*dcas9*; 3 *rrl*) (n = 4). Tet, 10 μ g/mL tetracycline; Cm, 20 μ g/mL chloramphenicol;
778 RLB, Ribosome lysis buffer. 40 μ M IPTG and 50 μ M DAPG were used to induce expression of *ycbB* and *dcas9*,
779 respectively. Labeled ribosomes were purified from BW25113. (b) Sample preparation and data acquisition.
780 Ribosomes digested with trypsin were analyzed by LC-MS/MS. The representative full scan spectrum obtained
781 for the VANLGLSLGDQVNVK peptide of protein L9 shows the isotopic distributions for the unlabeled and labeled
782 isotopologues. (c) Distribution of light-to-heavy mean ratios. The boxes extend from the 25th to 75th percentiles.
783 The line in the middle of the box is plotted at the median. The whiskers extend from the 5th to 95th percentiles.
784 Outliers (< 5% or > 95%) are represented outside of the box plot by circles and squares. ****, *p*-value < 0.0001
785 for comparison of the means; unpaired two-tailed *t*-test with Welch's correction for heteroscedasticity.

786

787 **Extended Data Figure 9. Expression of ceftriaxone resistance mediated by downregulation of the transcription**
788 **of *rrl* genes in the absence of the P³⁷⁶R substitution in the β subunit of the RNAP.** Plating efficiency of the
789 BW25113 Δ *relA* Δ *spoT* pKT2(*ycbB*) pHV136(*dcas9*; 3 *rrl*). The parental strain BW25113 Δ *relA* Δ *spoT* was re-
790 sequenced to ensure the absence of mutations in the genes encoding the RNAP subunits. Plasmids pKT2(*ycbB*)
791 and pHV136(*dcas9*; 3 *rrl*) plasmids were independently introduced in six independent cultures of this strain
792 (clones 1 to 6). The strain appearing in the top row and in Fig. 4 additionally harbored the unexpected P³⁷⁶R
793 substitution in the β subunit of the RNAP. Growth was tested in the presence of 8 μ g/mL ceftriaxone
794 (+ ceftriaxone), 16 μ g/mL mecillinam (+ mecillinam), or in the absence of the drugs (- β -lactam). Induction of
795 *dcas9* was performed with 50 μ M DAPG (+ DAPG). BHI agar plates contained 40 μ M IPTG for induction of *ycbB* (n
796 = 6).

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Extended Data Table 1. YcbB- and (p)ppGpp-mediated β -lactam resistance does not rely on the recruitment of a specific alternative sigma factor

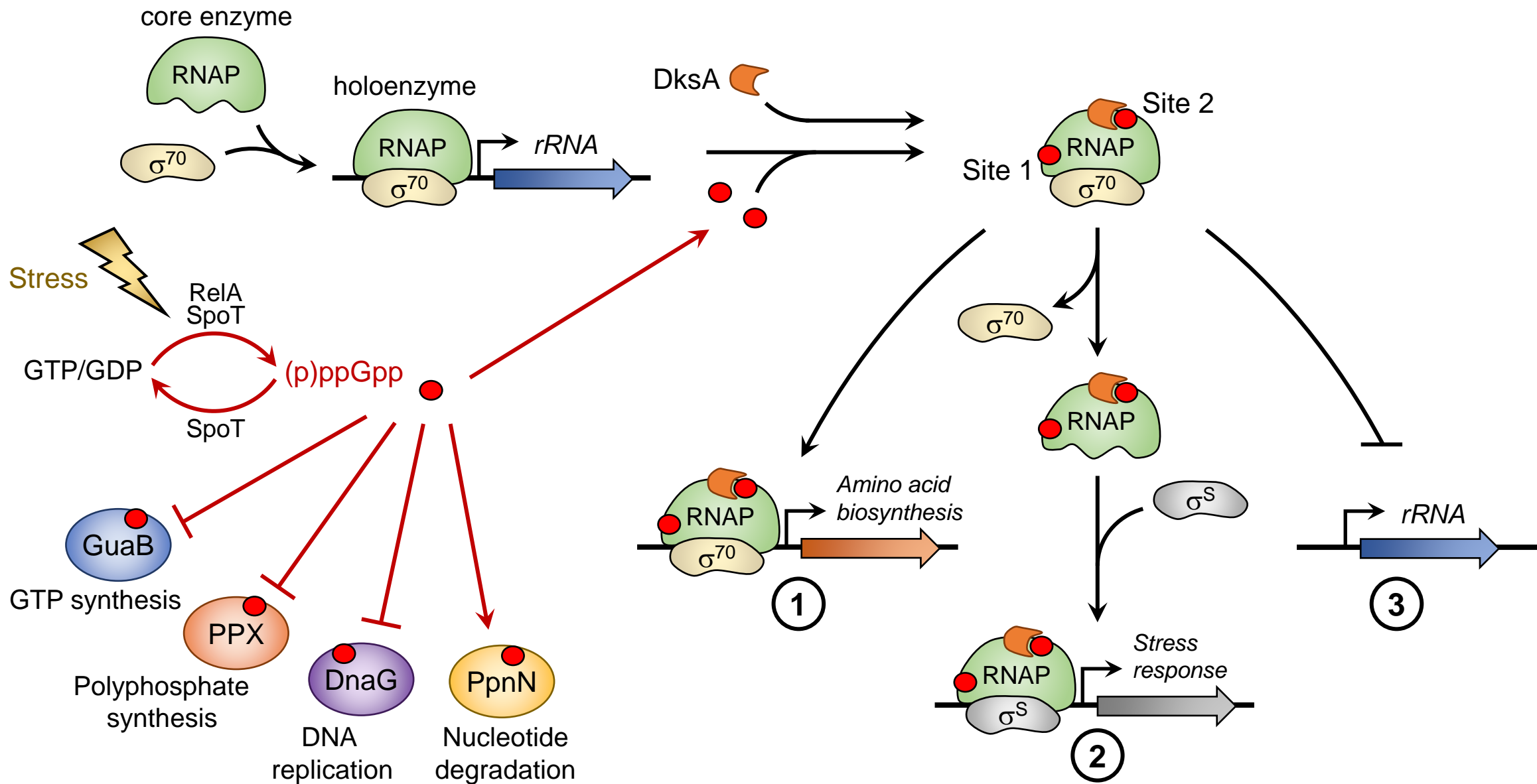
Host	Plasmid	Inducers ^b	Inhibition zones (mm) ^a		
			Mel	Amp	Cro
BW25113 $\Delta relA$		None	20	18	34
	pKT2(<i>ycbB</i>) pKT8(<i>relA'</i>)	IPTG + Ara	< 6	< 6	15
Deletion of genes encoding unessential alternative σ factors					
BW25113 $\Delta relA \Delta rpoN$					
	pKT2(<i>ycbB</i>) pKT8(<i>relA'</i>)	IPTG + Ara	< 6	< 6	16
BW25113 $\Delta relA \Delta rpoS$					
	pKT2(<i>ycbB</i>) pKT8(<i>relA'</i>)	IPTG + Ara	< 6	< 6	15
BW25113 $\Delta relA \Delta rpoF$					
	pKT2(<i>ycbB</i>) pKT8(<i>relA'</i>)	IPTG + Ara	< 6	< 6	15
BW25113 $\Delta relA \Delta fecI$					
	pKT2(<i>ycbB</i>) pKT8(<i>relA'</i>)	IPTG + Ara	< 6	< 6	14
Overexpression of genes encoding essential alternative σ factors in BW25113 $\Delta relA \Delta spoT$					
	pKT2(<i>ycbB</i>) pHV7 ^c	IPTG + Ara	31	24	40
	pKT2(<i>ycbB</i>) pHV17(<i>rpoH</i>)	IPTG + Ara	32	26	43
	pKT2(<i>ycbB</i>) pHV18(<i>rpoE</i>)	IPTG + Ara	31	25	41
Overexpression of genes encoding alternative σ factors in BW25113					
	pKT2(<i>ycbB</i>) pHV17(<i>rpoH</i>)	IPTG + Ara	31	18	37
	pKT2(<i>ycbB</i>) pHV18(<i>rpoE</i>)	IPTG + Ara	28	17	33
	pKT2(<i>ycbB</i>) pHV66(<i>rpoN</i>)	IPTG + Ara	30	17	36
	pKT2(<i>ycbB</i>) pHV67(<i>rpoS</i>)	IPTG + Ara	32	19	37
	pKT2(<i>ycbB</i>) pHV68(<i>rpoF</i>)	IPTG + Ara	35	18	40
	pKT2(<i>ycbB</i>) pHV70(<i>fecI</i>)	IPTG + Ara	30	17	37
Overexpression of genes encoding alternative σ factors in BW25113 $\Delta ycbB$					
	pKT2(<i>ycbB</i>) pHV17(<i>rpoH</i>)	IPTG + Ara	30	17	32
	pKT2(<i>ycbB</i>) pHV18(<i>rpoE</i>)	IPTG + Ara	29	17	32
	pKT2(<i>ycbB</i>) pHV66(<i>rpoN</i>)	IPTG + Ara	31	17	35
	pKT2(<i>ycbB</i>) pHV67(<i>rpoS</i>)	IPTG + Ara	30	17	36
	pKT2(<i>ycbB</i>) pHV68(<i>rpoF</i>)	IPTG + Ara	33	18	40
	pKT2(<i>ycbB</i>) pHV70(<i>fecI</i>)	IPTG + Ara	29	16	37

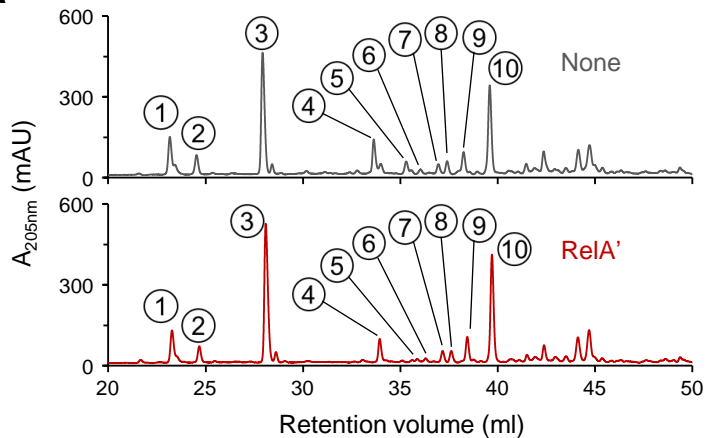
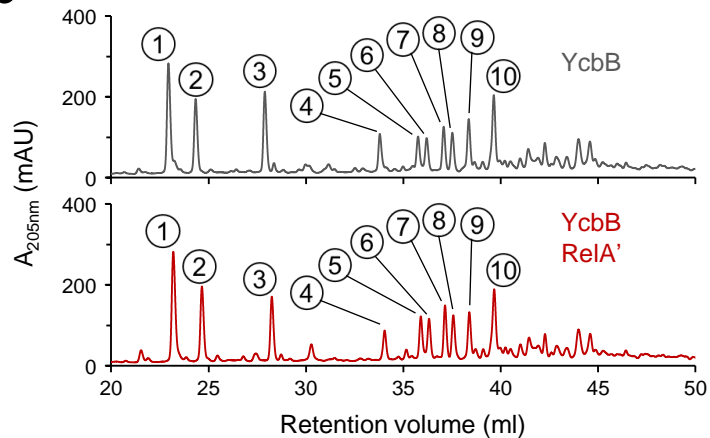
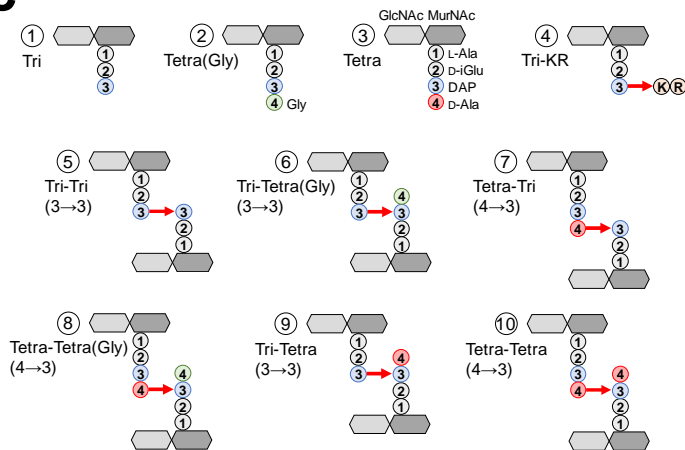
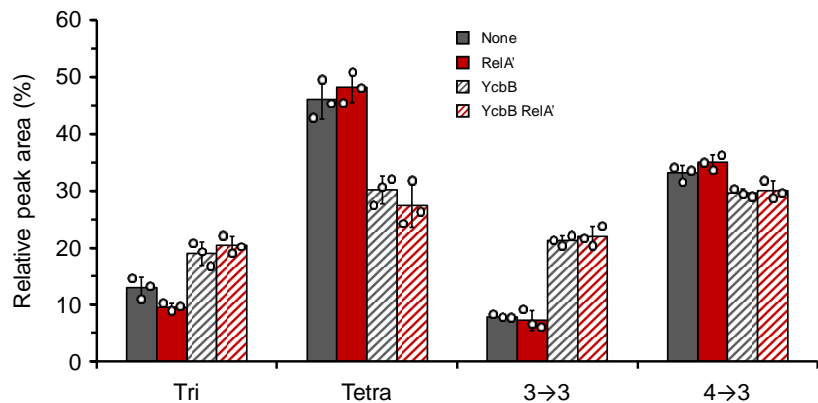
802 ^a The diameter of inhibition zones was determined by the disk diffusion assay using
803 disks containing 10 μ g mecillinam (Mel), 10 μ g ampicillin (Amp), or 30 μ g ceftriaxone
804 (Cro). Data are the medians from three experiments.

805 ^b The *ycbB* gene carried by plasmid pKT2 was induced with 40 μ M IPTG. The *relA'* gene
806 was induced with 1% L-arabinose (Ara). The genes encoding alternative sigma factor
807 were induced with 0.2% L-arabinose.

808 ^c pHV7 is the vector used for construction of pKT8(*relA'*), pHV17(*rpoH*), pHV18(*rpoE*),
809 pHV66(*rpoN*), pHV67(*rpoS*), pHV68(*rpoF*), and pHV70(*fecI*).

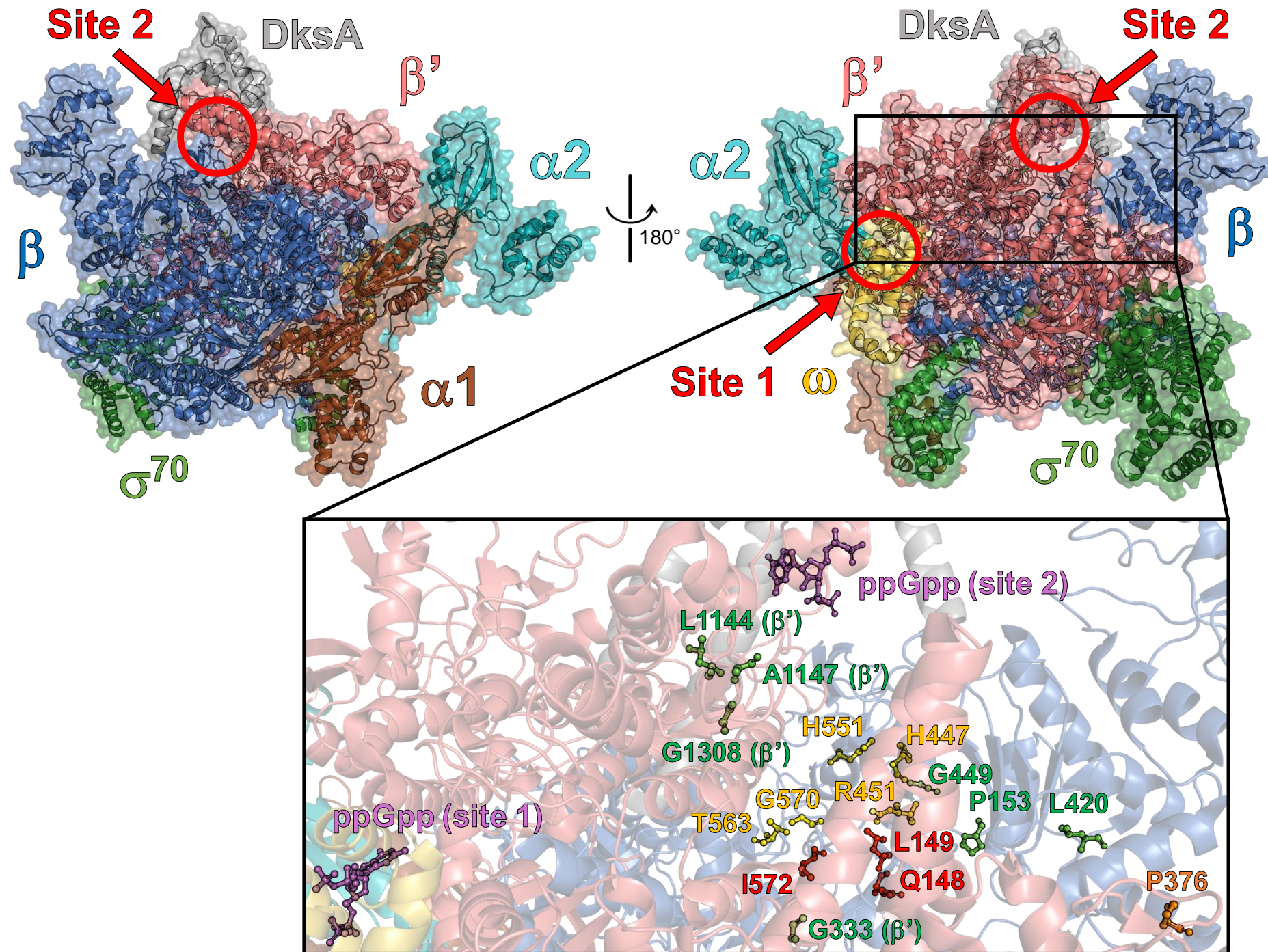
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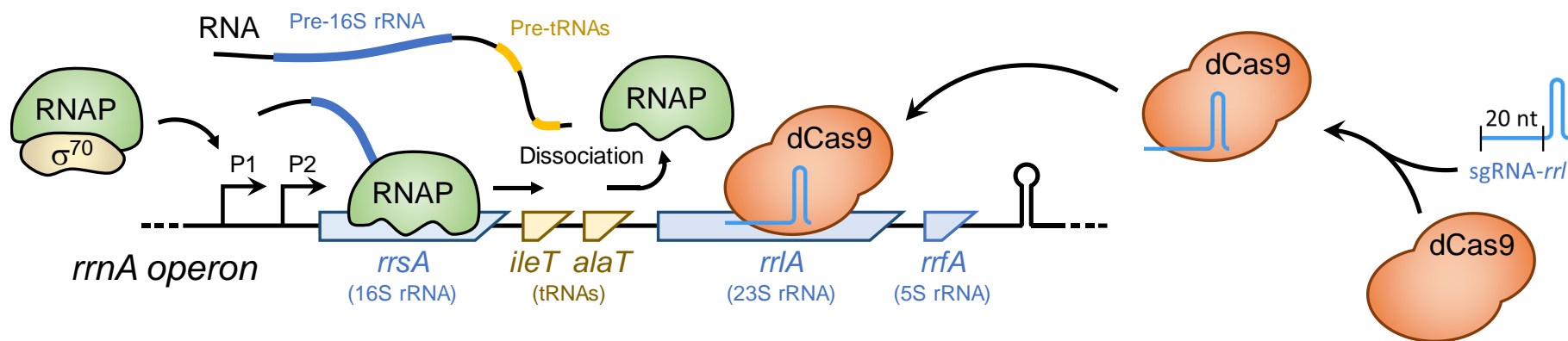
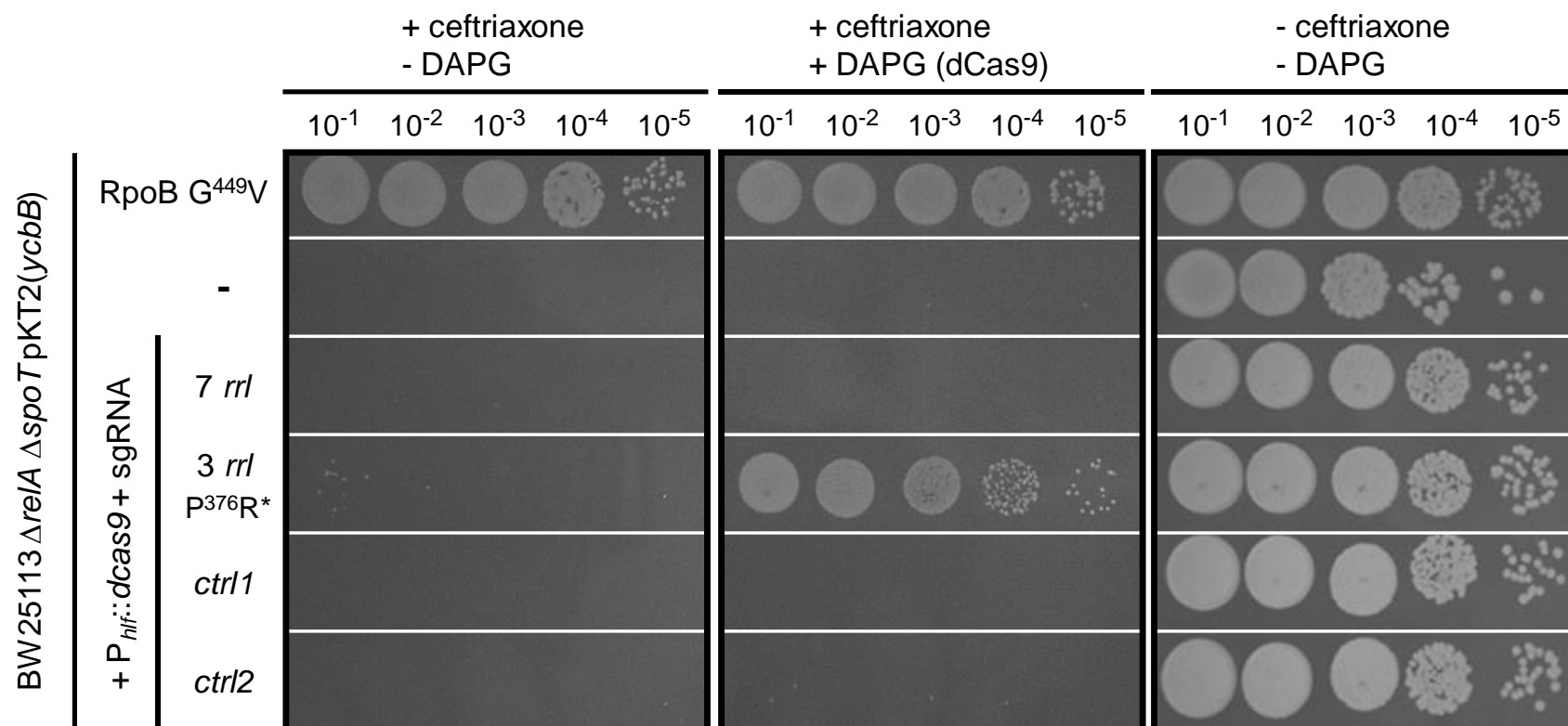
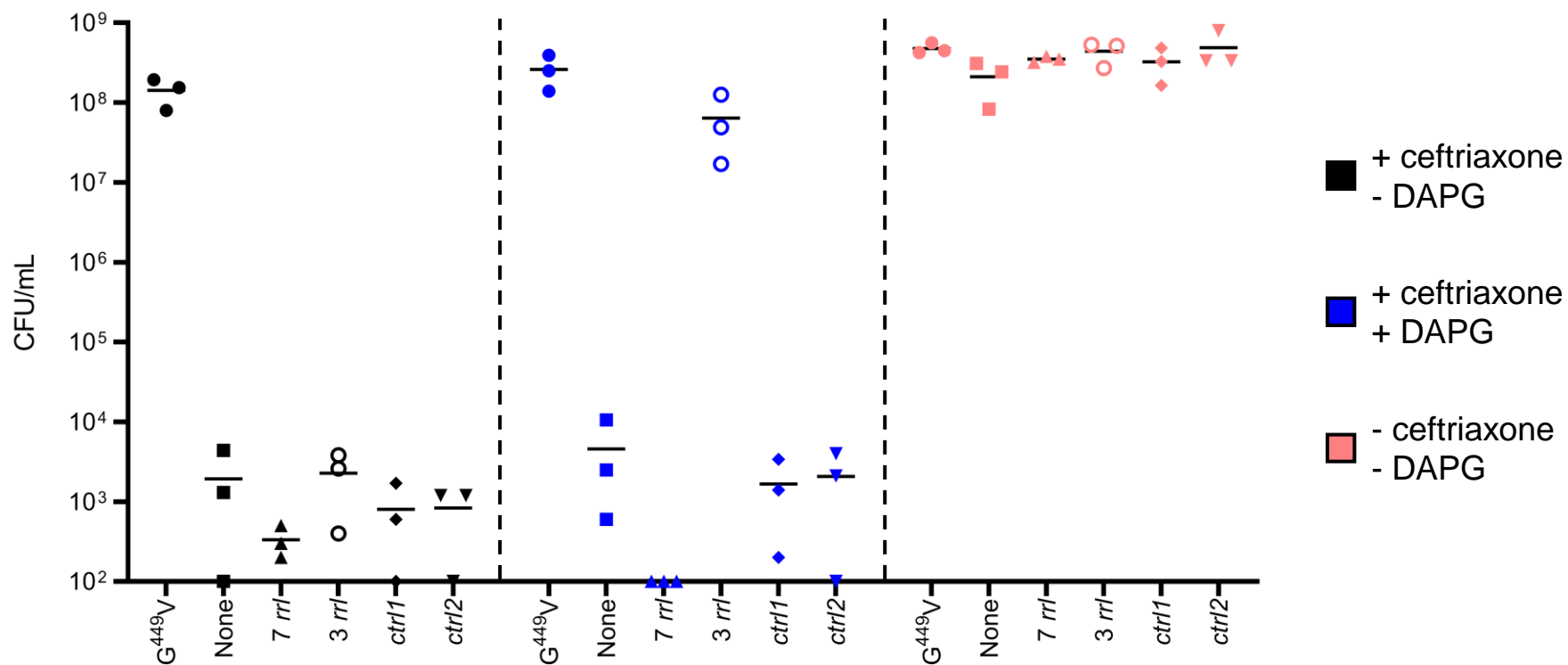


a**b****c****d**

a**RNAP substitutions obtained by selection with β -lactams or rifampicin**

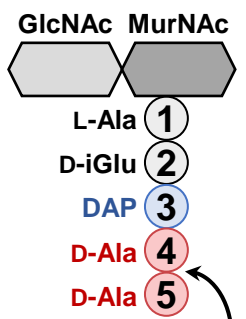
Substitution (subunit)	Mutation(Selector)	GT (min)
Wild-type	None	24 \pm 1
β-lactam resistance		
Pro¹⁵³Leu (β)	CCG \rightarrow CTG ^(Mel)	ND
Leu⁴²⁰Arg (β)	CTG \rightarrow CGG ^(Mel)	37 \pm 1
Gly⁴⁴⁹Val (β)	GGC \rightarrow GTC ^(Mel)	27 \pm 1
Gly³³³Asp (β')	GGT \rightarrow GAT ^(Mel)	ND
Leu¹¹⁴⁴Pro (β')	CTC \rightarrow CCG ^(Mel)	ND
Ala¹¹⁴⁷Pro (β')	GCA \rightarrow CCA ^(Mel)	ND
Gly¹³⁰⁸Asp (β')	GGT \rightarrow GAT ^(Amp)	40 \pm 2
Rifampicin resistance		
Gln¹⁴⁸Leu (β)	CAG \rightarrow CTG ^(Rif)	28 \pm 2
Gln¹⁴⁸Arg (β)	CAG \rightarrow CGG ^(Rif)	28 \pm 2
Leu¹⁴⁹Arg (β)	CTG \rightarrow CGG ^(Rif)	28 \pm 3
Ile⁵⁷²Leu (β)	ATC \rightarrow CTC ^(Rif)	28 \pm 2
Co-resistance		
His⁴⁴⁷Leu (β)	CAC \rightarrow CTC ^(Amp)	48 \pm 2
Arg⁴⁵¹Cys (β)	CGT \rightarrow TGT ^(Amp; Mel)	34 \pm 2
His⁵⁵¹Pro (β)	CAC \rightarrow CCC ^(Mel)	34 \pm 1
Thr⁵⁶³Pro (β)	ACC \rightarrow CCC ^(Amp; Mel; Rif)	50 \pm 3
Gly⁵⁷⁰Ser (β)	GGT \rightarrow AGT ^(Mel)	36 \pm 3

b

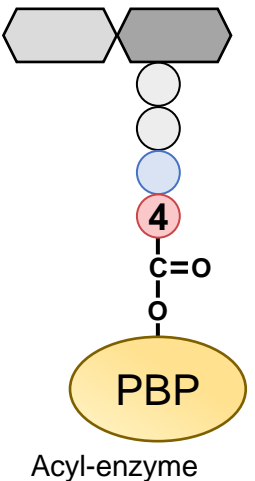
a**b****c**

a

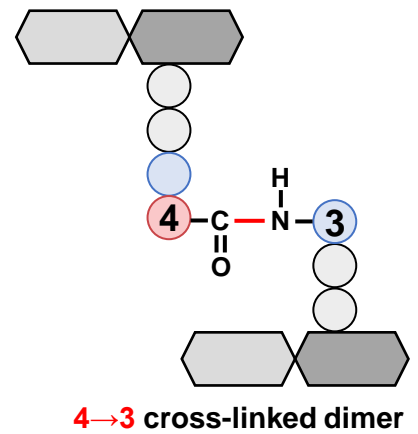
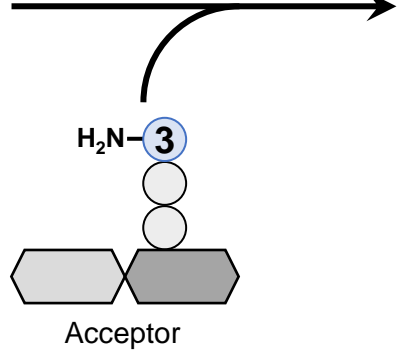
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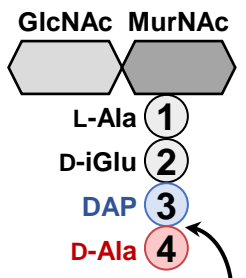
Penams
Cephems
Carbapenems



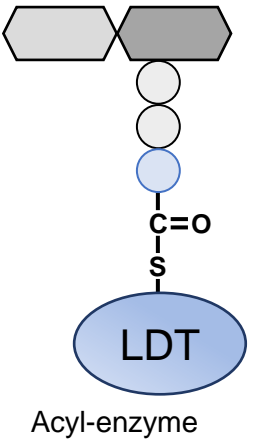
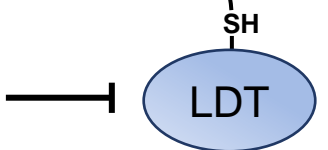
D,D-transpeptidation

**b**

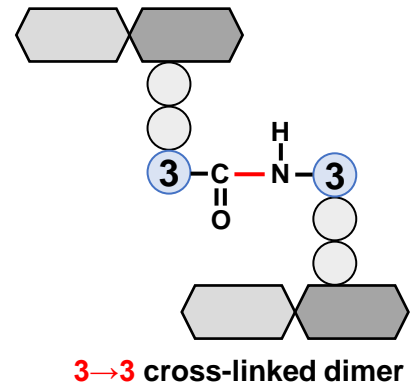
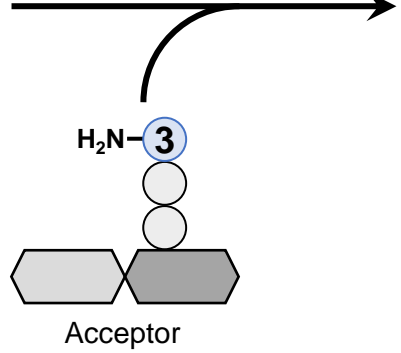
Tetrapeptide donor

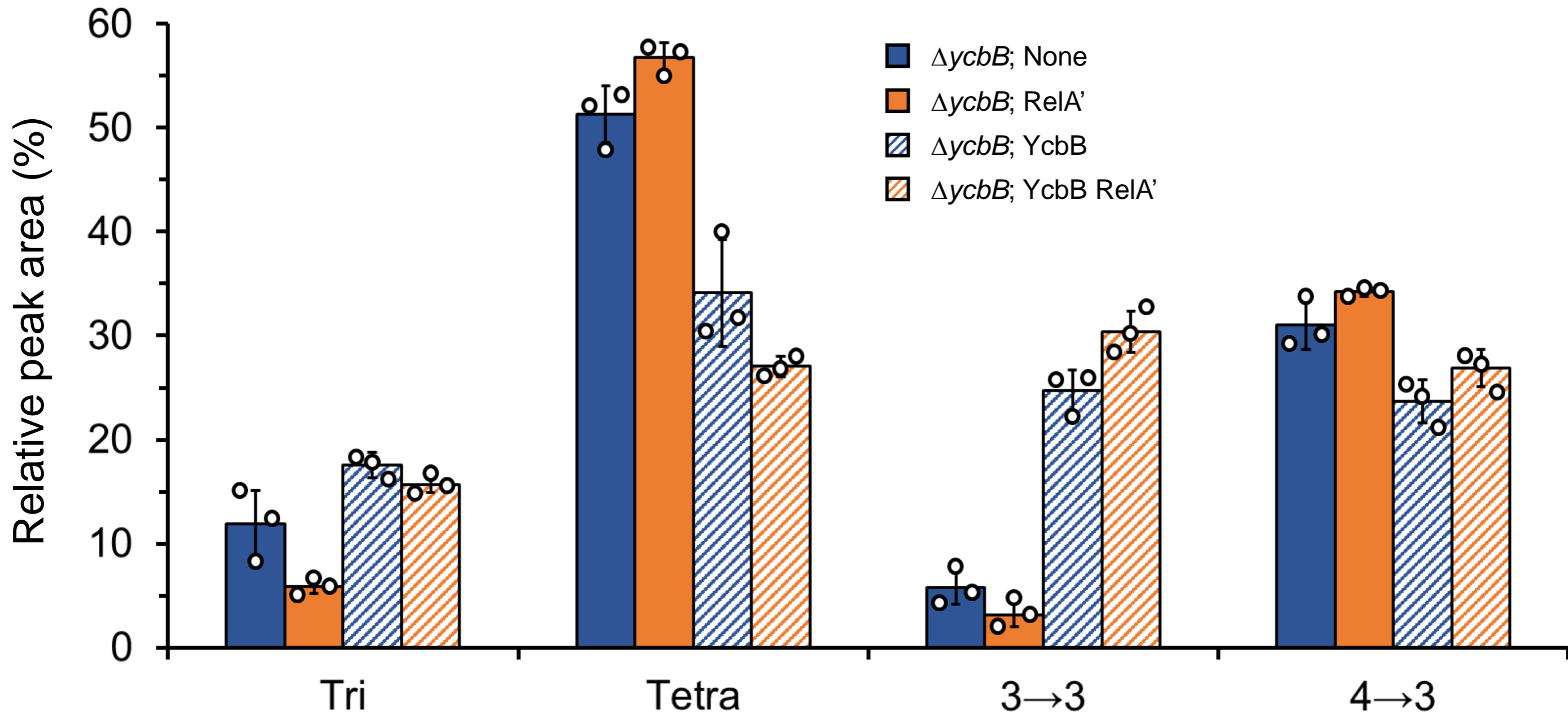


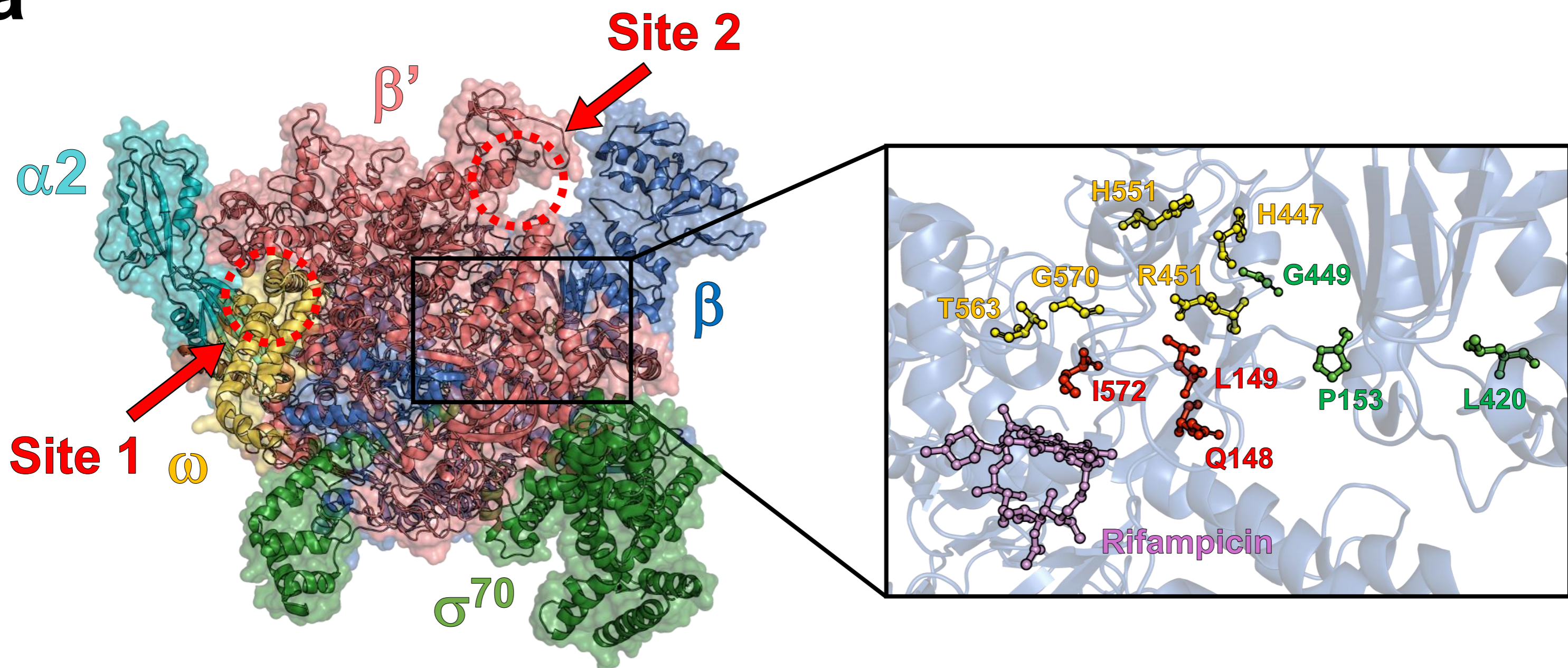
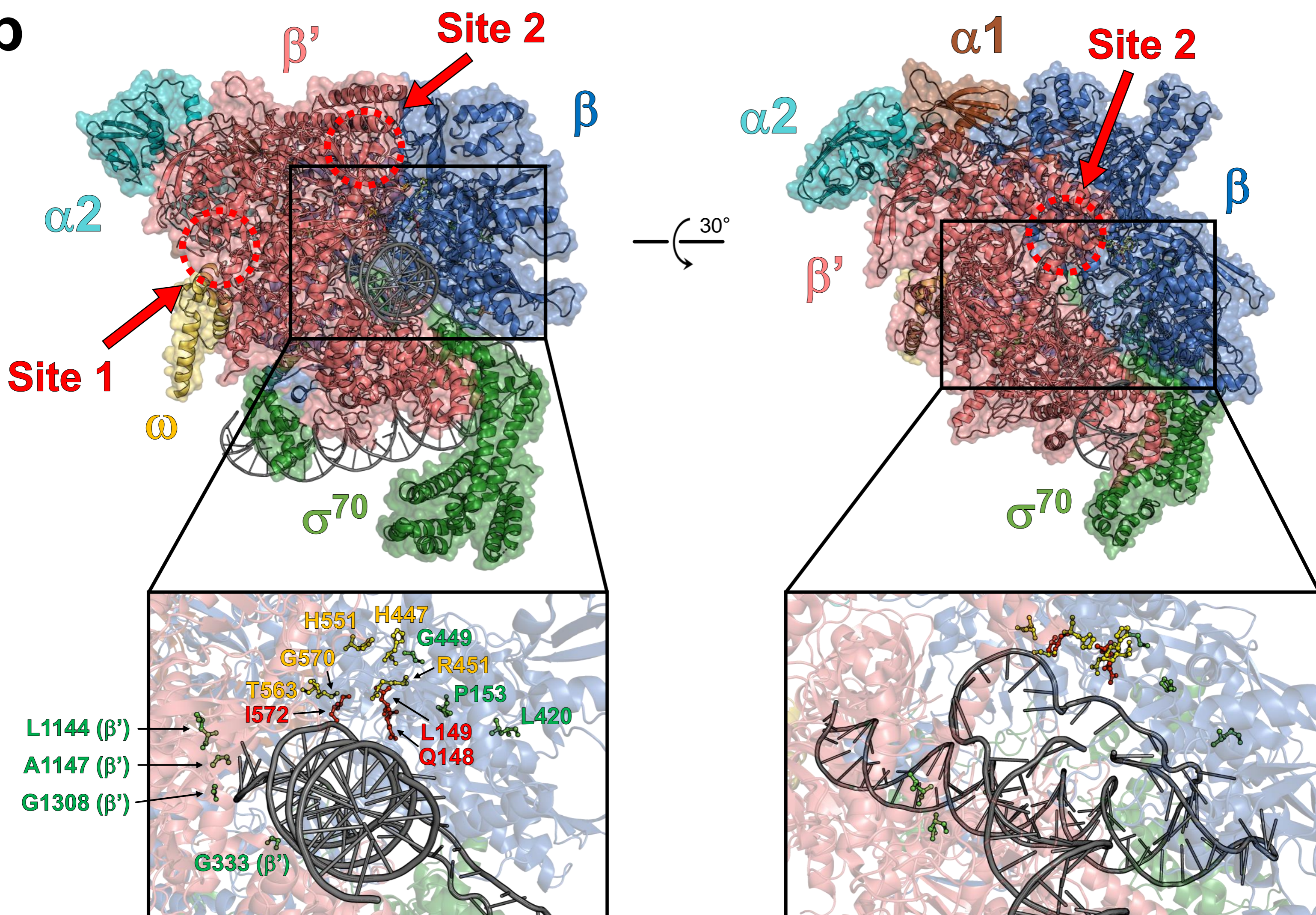
Carbapenems



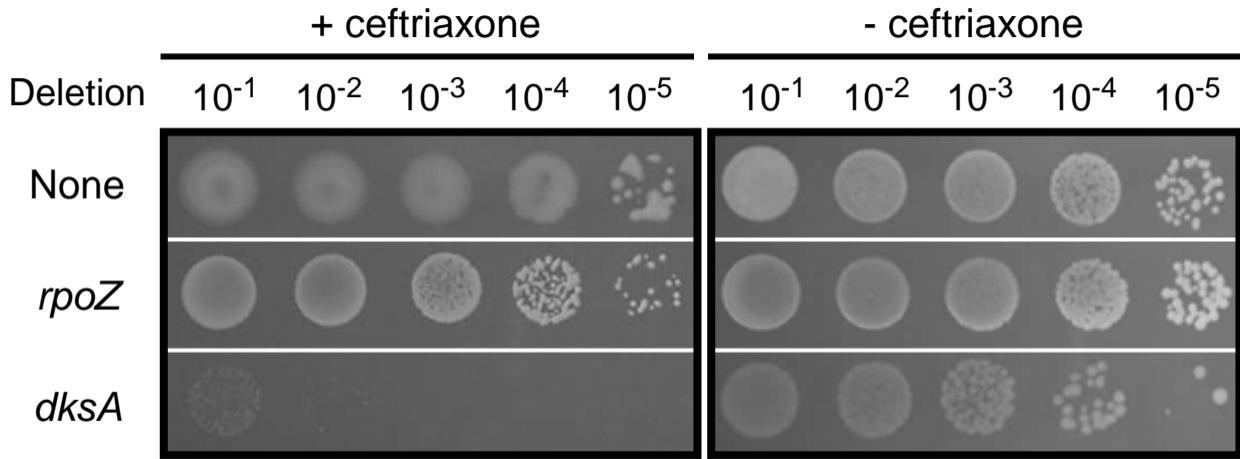
L,D-transpeptidation



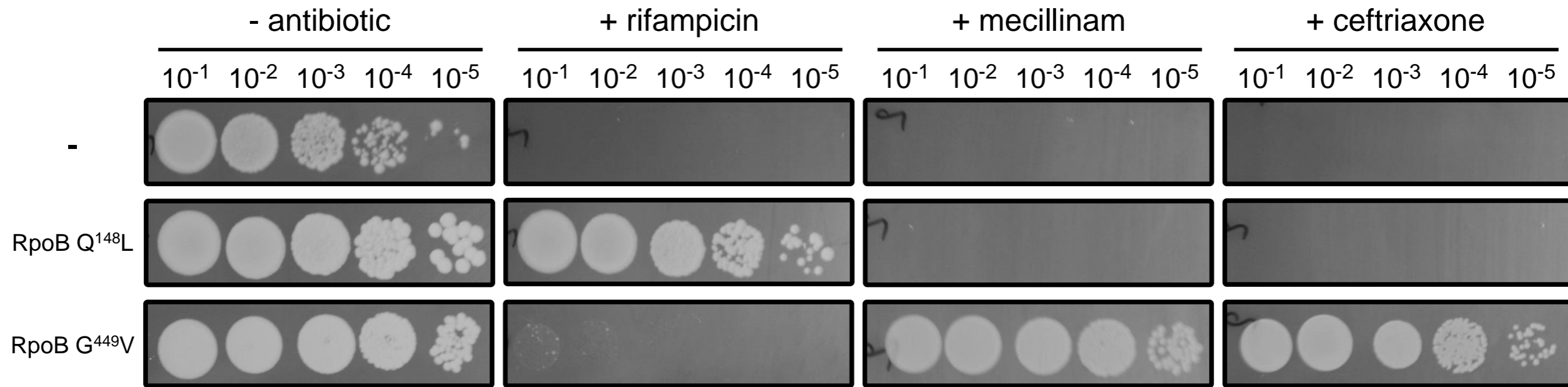


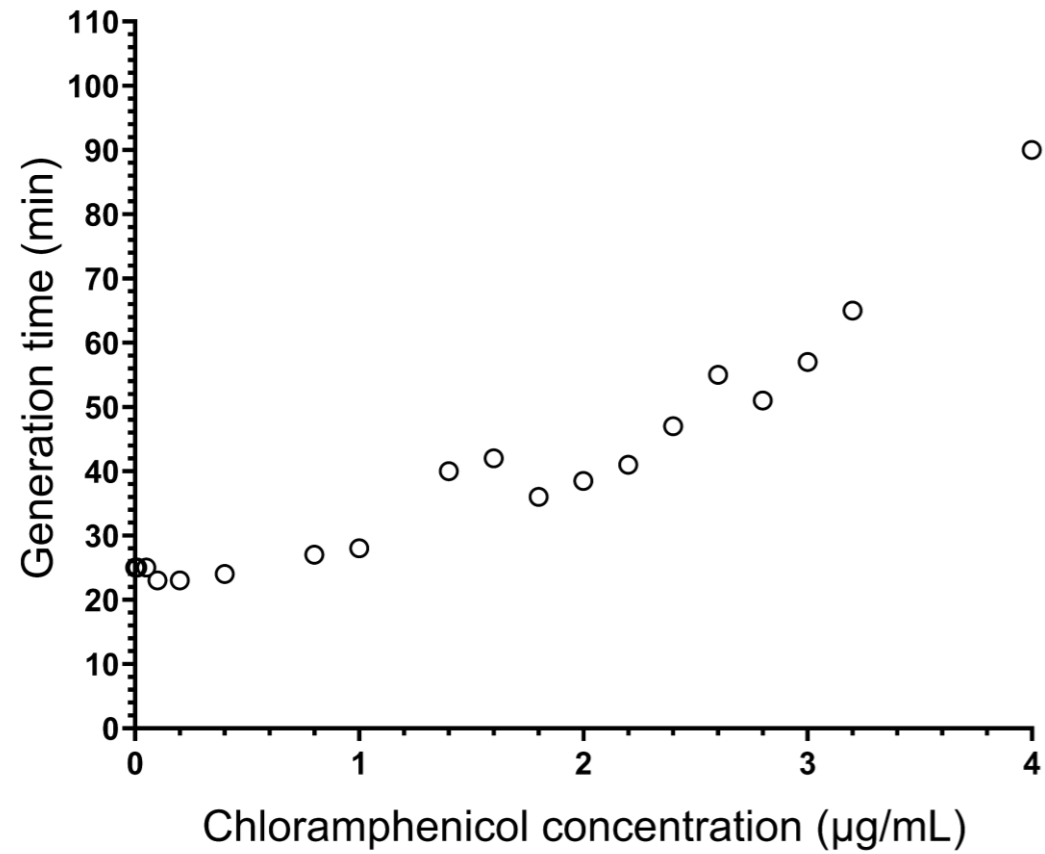
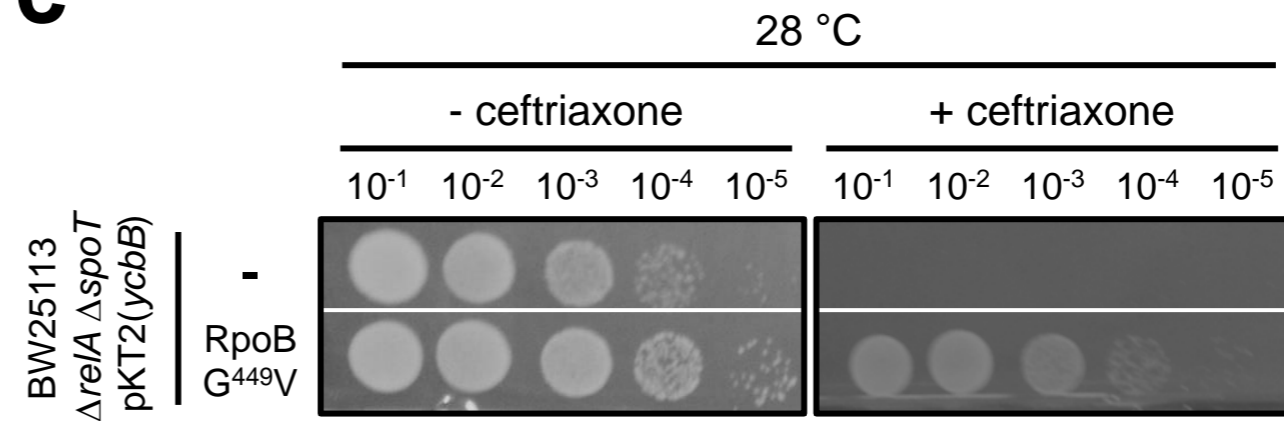
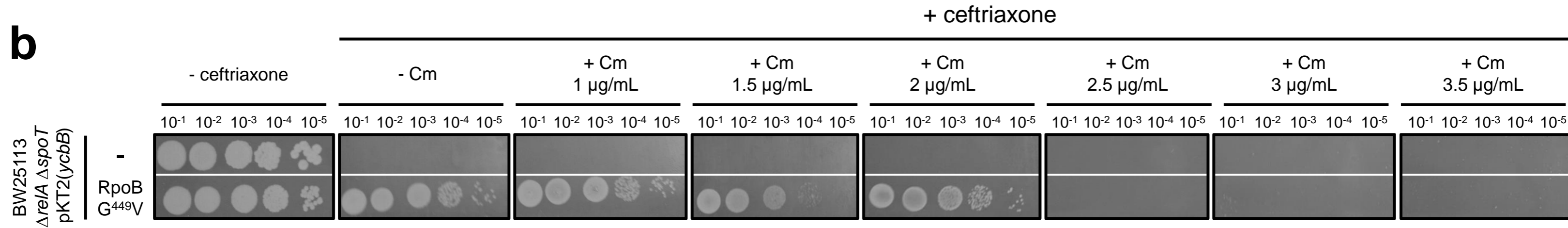
a**b**

BW25113(*ycbB*, *relA*)



BW25113 Δ relA Δ spoT pKT2(*ycbB*)



a**c****b**

a

Position 1350 1360 1370 1380 1390 1400

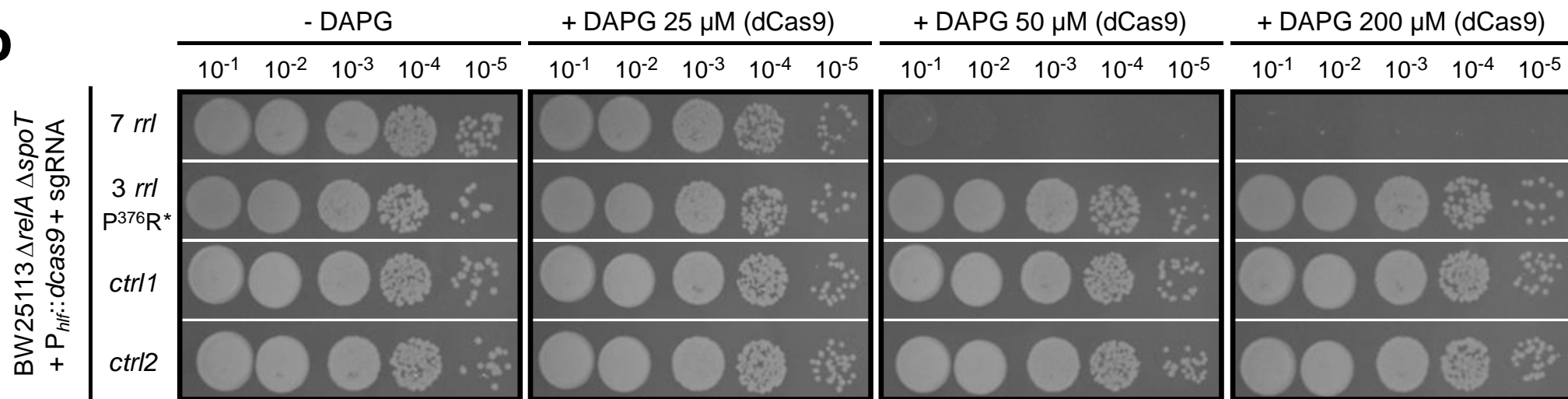
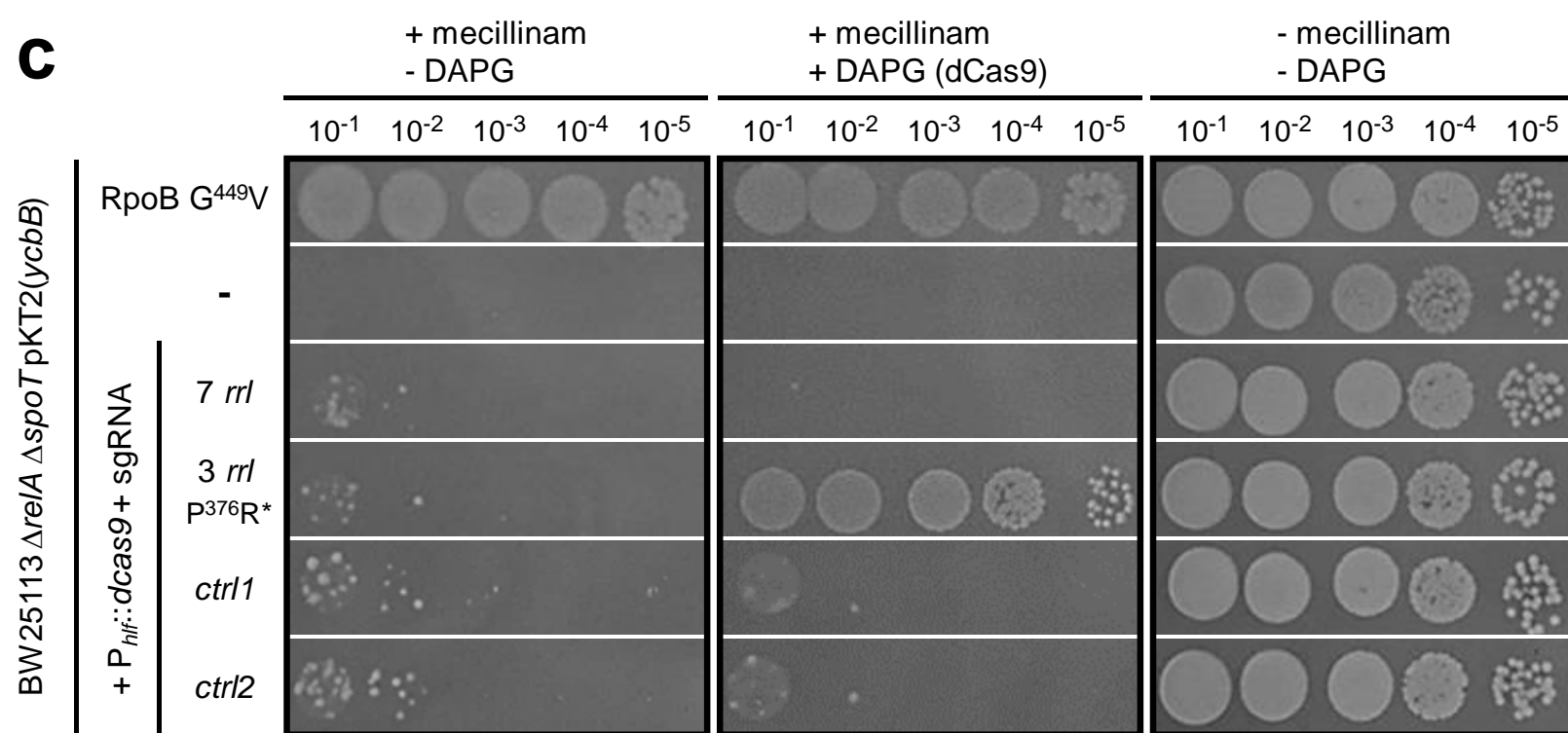
|...|...|...|...|...|...|...|...|...|...|

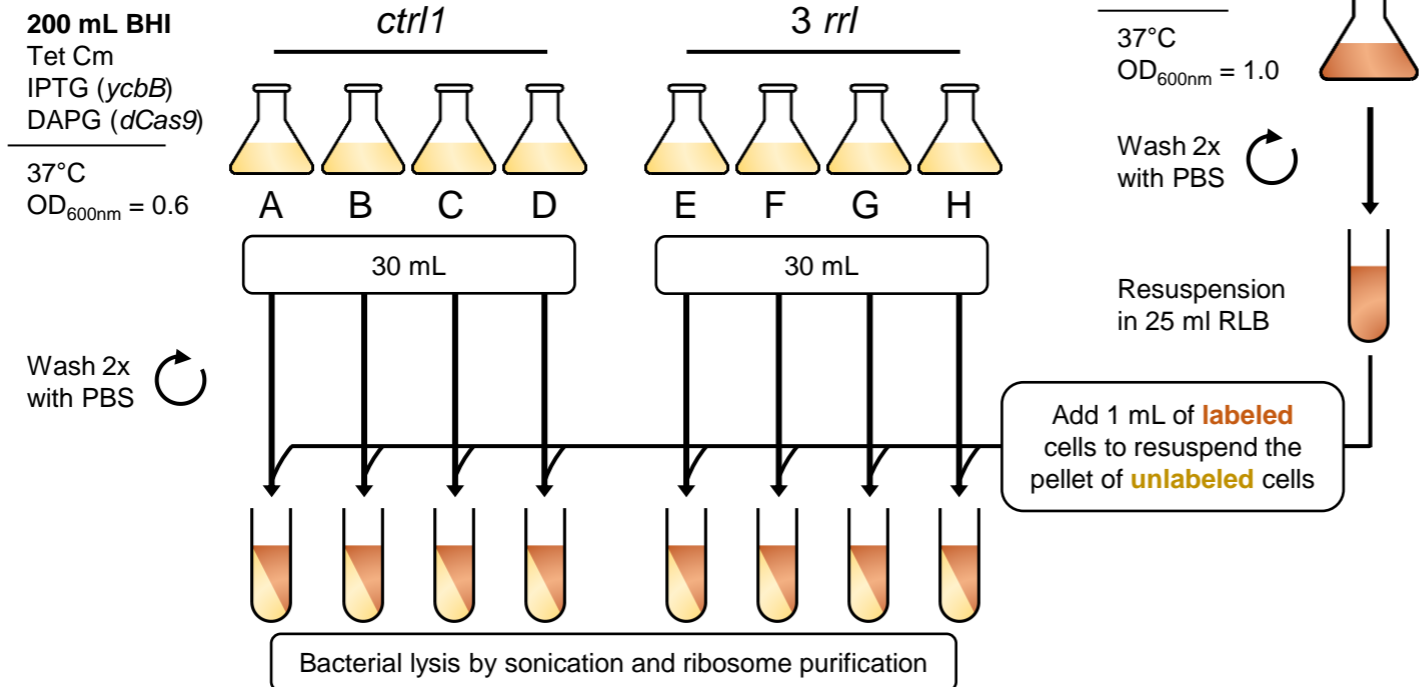
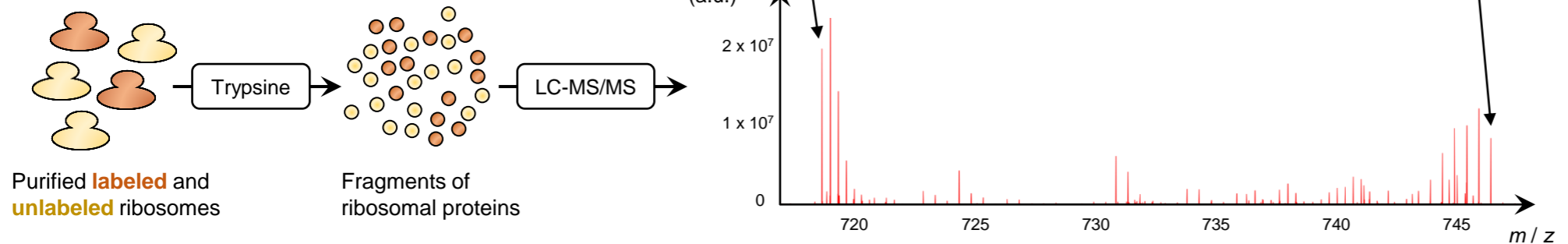
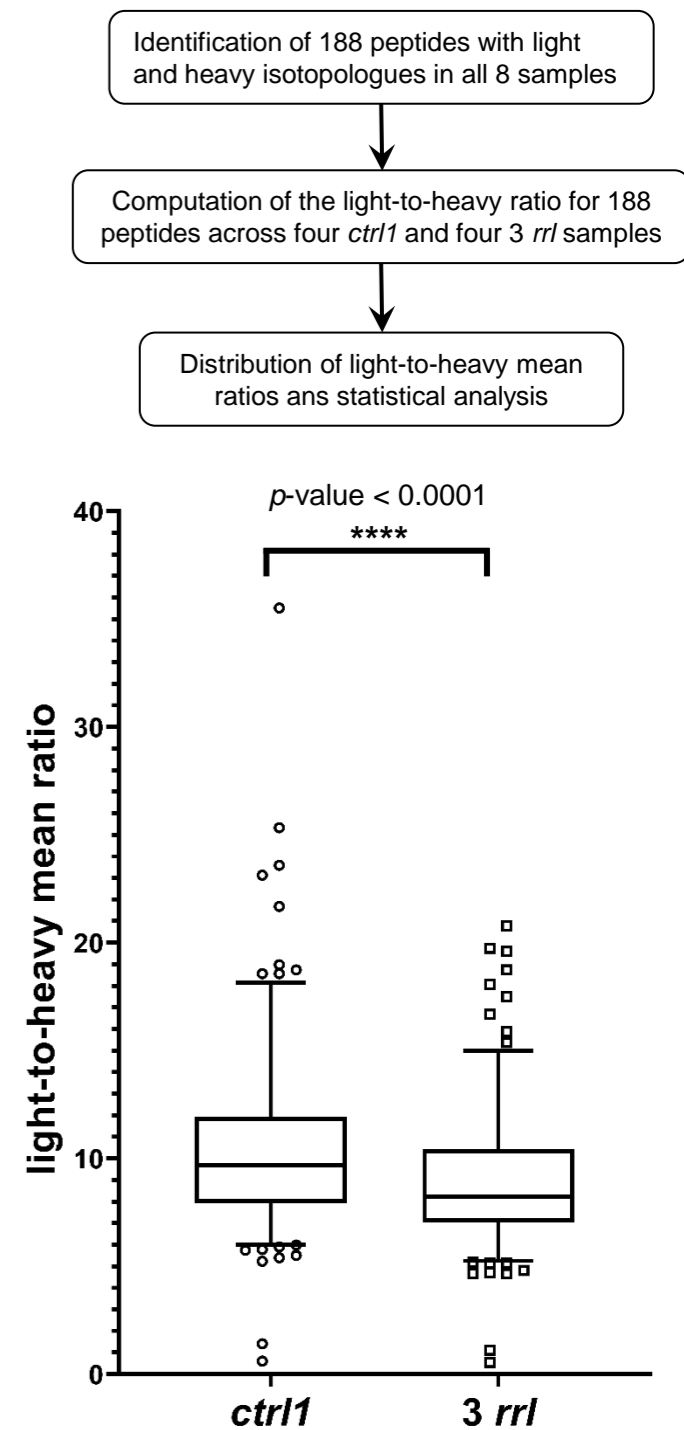
rrLA ...CCTAAGGCGAGGCCGAAAGGCGTAGTCGATGGGAAACAGGTTAATATTCCT...
rrLB ...CCTAAGGCGAGGCCGAAAGGCGTAGTCGATGGGAAACAGGTTAATATTCCT...
rrLC ...CCTAAGGCGAGGCCGAAAGGCGTAGTCGATGGGAAACAGGTTAATATTCCT...
rrLD ...CCTAAGGCGAGGCCGAAAGGCGTAGTCGATGGGAAACAGGTTAATATTCCT...
rrLE ...CCTAAGGCGAGGCCGAAAGGCGTAGTCGATGGGAAACAGGTTAATATTCCT...
rrLG ...CCTAAGGCGAGGCCGAAAGGCGTAGTCGATGGGAAACAGGTTAATATTCCT...
rrLH ...CCTAAGGCGAGGCCGAAAGGCGTAGTCGATGGGAAACAGGTTAATATTCCT...
RC sgRNA 7 *rrL*(ABCDEFGH) AAAGGCGTAGTCGATGGGAA

Position 1710 1720 1730 1740 1750 1760

|...|...|...|...|...|...|...|...|...|...|

rrLA ...GATATGTAGGTGAAGCGACTTGCTCGTGGAGCTGAAATCAGTCGAAGATAC...
rrLB ...GATATGTAGGTGAGGTCCCTCGCGGATGGAGCTGAAATCAGTCGAAGATAC...
rrLC ...GATATGTAGGTGAAGCGACTTGCTCGTGGAGCTGAAATCAGTCGAAGATAC...
rrLD ...GATATGTAGGTGAAGCGACTTGCTCGTGGAGCTGAAATCAGTCGAAGATAC...
rrLE ...GATATGTAGGTGAGGTCCCTCGCGGATGGAGCTGAAATCAGTCGAAGATAC...
rrLG ...GATATGTAGGTGAGGTCCCTCGCGGATGGAGCTGAAATCAGTCGAAGATAC...
rrLH ...GATATGTAGGTGAAGCGACTTGCTCGTGGAGCTGAAATCAGTCGAAGATAC...
RC sgRNA 3 *rrL*(BEG) * *** TCGCGGATGGAGCTGAAATC
* ***

b**c**

a**b****c**

BW25113 Δ relA Δ spoT pKT2(*ycbB*)
+ $P_{hff}::$ dcas9 + sgRNA 3 *rrl*

RpoB P^{376R}

without RpoB P^{376R}

clone 1

clone 2

clone 3

clone 4

clone 5

clone 6

+ ceftriaxone
- DAPG

10⁻¹ 10⁻² 10⁻³ 10⁻⁴ 10⁻⁵

+ ceftriaxone
+ DAPG (dCas9)

10⁻¹ 10⁻² 10⁻³ 10⁻⁴ 10⁻⁵

+ mecillinam
- DAPG

10⁻¹ 10⁻² 10⁻³ 10⁻⁴ 10⁻⁵

+ mecillinam
+ DAPG (dCas9)

10⁻¹ 10⁻² 10⁻³ 10⁻⁴ 10⁻⁵

- β -lactam
- DAPG

10⁻¹ 10⁻² 10⁻³ 10⁻⁴ 10⁻⁵

