Genome location dictates the transcriptional response to PolC-inhibition in *Clostridium difficile*

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Abstract

Clostridium difficile is a potentially lethal gut pathogen that causes nosocomial and community acquired infections. Limited treatment options and reports of reduced susceptibility to current treatment emphasize the necessity for novel antimicrobials. The DNA-polymerase of gram-positive organisms is an attractive target for the development of antimicrobials. ACX-362E (N^2-(3,4-Dichlorobenzyl)-7-(2-[1-morpholinyl]ethyl)guanine; MorE-DCBG) is a DNA polymerase inhibitor in pre-clinical development as a novel therapeutic against *C. difficile* infection. This synthetic purine shows preferential activity against *C. difficile* PolC over those of other organisms *in vitro* and is effective in an animal model of *C. difficile* infection. In this study we have determined its efficacy against a large collection of clinical isolates. At concentrations below the minimal inhibitory concentration, the presumed slowing (or stalling) of replication forks due to ACX-362E leads to a growth defect. We have determined the transcriptional response of *C. difficile* to replication inhibition and observed an overrepresentation of up-regulated genes near the origin of replication in the presence of PolC-inhibitors, but not when cells were subjected to sub-inhibitory concentrations of other antibiotics. This phenomenon can be explained by a gene dosage shift, as we observed a concomitant increase in the ratio between origin-proximal versus terminus-proximal gene copy number upon exposure to PolC-inhibitors. Moreover, we show that certain genes differentially regulated under PolC-inhibition are controlled by the origin-proximal general stress response regulator sigma factor B. Together, these data suggest that genome location both directly and indirectly determines the transcriptional response to replication inhibition in *C. difficile*.

46 **Background**

47 *Clostridium difficile* (*Clostridioides difficile* (1)) is a gram-positive, anaerobic bacterium that can
48 asymptomatically colonize the intestine of humans and other mammals (2-4). However, when the
49 normal flora is disturbed *C. difficile* can overgrow and cause fatal disease, as has been dramatically
50 demonstrated in the Stoke Mandeville Hospital outbreaks in 2004 and 2005 (5). The ability to form
51 highly resistant endospores coupled to its extensive antibiotic resistance have contributed to its success
52 as a nosocomial and community acquired pathogen (2-4). Recent years have seen an increase in the
53 incidence and severity of *C. difficile* infections (CDI), due to the emergence of certain PCR ribotypes (3,
54 6). Antibiotic use is a well-established risk factor for CDI (7), and the emergence of the epidemic PCR
55 ribotype 027 has been linked to fluoroquinolone resistance (8). At present two antibiotics,
56 metronidazole and vancomycin are commonly used to treat CDI and a third, fidaxomicin, is indicated for
57 the treatment of relapsing CDI (9, 10). Clearly, limited treatment options and reports of reduced
58 susceptibility to current treatment (11-13) emphasize the necessity for the development of novel
59 antimicrobials and a better understanding of tolerance and resistance towards existing therapeutics.

60 It is increasingly realized that off-target effects that occur when cells are exposed to
61 antimicrobials can contribute to their efficacy, but also facilitate the emergence of tolerance and/or
62 resistance (14). Antimicrobials may act as signaling molecules which modulate gene expression (14).
63 Additionally, in particular those targeting DNA replication (such as polymerase inhibitors) can cause
64 transcriptional effects as a result of differences in gene dosage (15).

65 The polymerase of gram-positive organisms is an attractive target for the development of novel
66 antimicrobials (16). First, these PolC-type polymerases are absent from gram-negative organisms and
67 humans (17, 18). HPUra, one of the first such compounds, is therefore highly active against a wide range
68 of gram-positive bacteria, but does not affect gram-negative bacteria (17, 18). Template-directed
69 elongation is blocked by the inhibitor through simultaneous binding to the cytosine of the DNA strand

70 and near the active site of PolC. Second, compounds can be derived that have an increased specificity
71 towards specific microorganisms. ACX-362E **(Figure 1**) is a compound in pre-clinical development as a
72 novel therapeutic against *C. difficile* as it is shows preferential activity against *C. difficile* PolC over those
73 of other organisms *in vitro* (19, 20), and will progress to clinical trials in the near future (Acurx
74 Pharmaceuticals, personal communication). PolC-inhibitors can cause a stress response and cell death
75 after prolonged exposure. In *Bacillus subtilis*, this stress is characterized by a combination of DNA
76 damage (SOS) response, and an SOS-independent pathway dependent on the DNA replication initiator
77 DnaA (21, 22). In *Streptococcus pneumoniae* cells, devoid of an SOS-response, competence for genetic
78 transformation is induced upon replication stress (23). The response of *C. difficile* to this particular class
79 of compounds is unknown.

80 In this study, we characterized aspects of the action of PolC-inhibitors towards *C. difficile*.
81 Minimal inhibitory concentrations for HPUra and ACX-362E were determined using agar dilution for a
82 large collection of clinical isolates. Next, we investigated the effects of sub-inhibitory levels of PolC-
83 inhibitors on growth of *C. difficile* in liquid medium and performed RNAseq analyses to determine the
84 transcriptional response to PolC-inhibitors in our laboratory strain 630Δ*erm.* Finally, marker frequency
85 analysis and transcriptional reporters were used to provide a mechanistic explanation for the observed
86 up-regulation of origin-proximal genes under conditions of replication inhibition.

87

88 **Results**

89 *ACX-362E is a potent inhibitor of diverse clinical isolates of C. difficile*

90 To date, reports on activity of PolC-inhibitors towards *C. difficile* are limited. For only 4 and 23 *C. difficile*
91 strains minimal inhibitory concentrations were published (19, 20), and no analysis was performed on
92 possible differences in efficacy between various phylogenetic groups (24, 25). Therefore, we assessed

93 the sensitivity of a diverse collection of *C. difficile* clinical isolates towards PolC-inhibitors and
94 determined if ACX-362E was indeed superior to the general PolC–inhibitor HPUra.

95 HPUra and ACX-362E were tested by the agar dilution method, according Clinical and Laboratory
96 Standards Institute (CLSI) guidelines for testing of antimicrobial susceptibility of anaerobes (26, 27),
97 against 364 *C. difficile* clinical isolates collected earlier in the framework of a pan-European study (6, 28).

98 We found that ACX-362E (MIC_{50}: 2 µg/ml; MIC_{90}: 4 µg/ml) demonstrates lower inhibitory
99 concentrations than the general gram-positive PolC-inhibitors HPUra (MIC_{50}: 16 µg/ml; MIC_{90}: 32 µg/ml)
100 (**Figure 2, Supplemental Table 1),** consistent with previous *in vitro* activities against purified PolC (19).
101 We observed no significant difference in ACX-362E susceptibility between clades **(Table 1)** and the
102 different PCR ribotypes demonstrated a similar distribution in MIC values (data not shown). No growth
103 at the highest concentration of compounds tested (resistance) for either one of the PolC-inhibitors was
104 observedamong the clinical isolates tested (N=363). Notably, we observed only a 2-fold difference in
105 MIC_{50} and $MIC_{90'}$ indicating that the compounds have similar activity against nearly all strains. In
106 contrast, the gram-negative obligate anaerobe *Bacteroides fragilis* was resistant to both polymerase
107 inhibitors under the conditions tested (MIC >265 µg/ml), as expected for an organism lacking PolC. The
108 gram-positive bacterium *Staphylococcus aureus*, which was included as a control for the activity of
109 HPUra against this group of bacteria (16, 29), was sensitive to both HPUra and ACX-362E, with MIC
110 values of 2 µg/mL and 1 µg/mL, respectively.

111 We conclude that ACX-362E is highly active against diverse clinical isolates of *C. difficile* and
112 resistance is not a concern in currently circulating strains.

113

114 *Treatment with ACX-362E leads to a pleiotropic transcriptional response*

115 In order to determine the transcriptional response of PolC-inhibitors, we established the optimal
116 concentration of both inhibitors which affected growth of *C. difficile* in liquid medium. The laboratory

117 strain *C. difficile* 630*Δerm* (PCR ribotype 012, MLST Clade 1)(30, 31) was grown in medium with varying
118 amounts of HPUra (10-40 µg/mL) or ACX-362E (0.25-8 µg/mL). We note that concentrations up to the
119 MIC_{90} (as determined by agar dilution) did not lead to a complete growth arrest in liquid medium in the
120 time course of the experiment (**Supplemental Figure 1**). A difference between MIC values from agar
121 dilution and (micro)broth has been observed before (32). The growth kinetics of *C. difficile* under the
122 influence of varying concentrations of HPUra was marginally affected when using concentrations from
123 10-40 µg/ml, at >80 percent of the non-treated culture. Growth kinetics of cultures containing PolC-
124 inhibitor ACX-362E 1-8 µg/ml were similar and resulted in -30-40 percent reduced growth compared to
125 the untreated culture. For subsequent experiments we used concentrations of PolC-inhibitors that were
126 at or close to the MIC_{90} (**Table 1**) and that result in a maximum reduction in growth of 30% compared to
127 a non-treated culture (**Supplemental Figure 1**).

128 Above, we established that growth of *C. difficile* is partially inhibited at certain concentrations of
129 PolC-inhibitors. Slowing down or stalling of replication forks might lead to a stressed state, as observed
130 for other organisms (22, 23). As nothing is known about the effect of replication inhibition on the
131 physiology of *C. difficile*, we determined the transcriptional response to replication inhibition by sub-MIC
132 levels of PolC-inhibitors through strand-specific RNA sequencing (RNA-Seq).

133 *C. difficile* 630*Δerm* was grown for 5h in medium with HPUra (35 µg/mL) or ACX-362E (4 µg/mL)
134 starting from an OD_{600} of 0.05 after which cells were harvested for RNA isolation. Purified RNA was
135 converted to cDNA and used for RNA-Seq as described in the Materials and Methods. For ACX-362E, 722
136 genes were differentially expressed, of which 438 genes were up-regulated and 284 genes were down-
137 regulated. The number of differentially expressed genes in HPUra treated samples was approximately 2-
138 fold lower: 360, of which 124 genes were up-regulated and 236 genes were down-regulated. The full list
139 of differentially regulated genes is available as **Supplementary Table 2** and the top 10 of up- and down-

140 regulated genes are shown in **Table 2** (HPura) and **Table 3** (ACX-362E). Here, we highlight three aspects
141 of the results.

142 First, we performed a Gene Set Enrichment Analysis (GSEA)(33) via the Genome2D web server
143 (34) using the locus tags of the differentially regulated genes (**Supplementary Table 1**) as input. Among
144 the genes up-regulated by ACX-362E, there is a strong overrepresentation of those involved in
145 translation, ribosome structure and ribosome biogenesis. Not unexpectedly, also replication,
146 recombination and repair processes are affected. This suggests that genes from these pathways show a
147 coordinated response in the presence of ACX-362E. Among the genes down-regulated in the presence of
148 ACX-362E, the levels of significance for specific processes are generally much lower, suggesting that
149 there is a more heterogeneous response among genes from the same pathway. Nevertheless, metabolic
150 pathways (especially carbon metabolism and coenzyme A transfer) and tellurite resistance were found
151 to be significantly affected. Strikingly, a GSEA analysis on lists of genes that are differentially expressed
152 in the presence of HPUra revealed similar processes to be affected.

153 The findings from the GSEA analysis prompted us to evaluate the overlap in the lists of
154 differentially regulated genes between the ACX-362E and HPUra datasets in more detail. If both
155 compounds act via a similar mechanism, we expect a conserved response. Indeed, we observe that
156 >90% of the genes that are up-regulated in the presence of HPUra compared to the non-treated
157 condition, are also identified as up-regulated in the presence of ACX-362E (**Figure 3A**). Though the
158 overlap is not as strong for the down-regulated genes, we find that >30% of the genes affected by HPUra
159 are also identified as affected by ACX-362E (**Figure 3B**). Notably, the directionality of the response is
160 conserved, as no genes were found to be up-regulated in one but down-regulated in the other
161 condition. Based on these observations, we believe that the differentially expressed genes identified in
162 this study are representative for a typical response to inhibition of PolC in *C. difficile*.

163 Finally, we related the changes in transcription to genome location. *C. difficile* has a single
164 circular chromosome and one origin of replication (*oriC*) from which the process of DNA replication
165 occurs bi-directionally towards the terminus (*terC*) (**Figure 4A**). Though neither *oriC* nor *terC* has been
166 definitively defined for *C. difficile*, it is assumed that *oriC* is located at or near *dnaA* (CD0001;
167 CD630DERM_RS00005). The terminus region is generally located at the inflection point of a GC skew ([G
168 - C]/[G + C]) plot. Such a plot places the *terC* region around 2.2Mb from CD0001, near the CD1931
169 (CD630DERM_RS10465) open reading frame (**Figure 4A**) (35). We noted that the differential expression
170 appeared to correlate with genome location (**Table 2, Table 3** and **Supplemental Table 2**): many of the
171 up-regulated genes have either low or high gene identifiers (CD numbers) indicative of an origin
172 proximal location and, conversely, many of the down-regulated genes appear to be located away from
173 *oriC*. Though this correlation is not absolute, we observed a clear trend when plotting the mean fold-
174 change against genome location for all genes (**Figure 4B**).

175 Overall, our data shows that inhibition of DNA replication by PolC-inhibitors causes a consistent
176 and pleiotropic transcriptional response that is at least in part is directly dictated by genome location.

177

178 *Gene dosage shift occurs at sub-inhibitory concentration of ACX-362E PolC-inhibitor*

179 A possible explanation for the relative up-regulation of *oriC*-proximal genes and down-regulation of
180 *terC*-proximal genes is a gene dosage shift (36-38), due to the fact that PolC-inhibition slows down
181 replication elongation but does not prevent re-initiation of DNA replication (23, 39). To determine if this
182 in fact occurs in *C. difficile* when replication elongation is inhibited, we performed a marker frequency
183 analysis (MFA) to determine the relative abundance of an origin proximal gene relative to terminus
184 proximal gene on chromosomal DNA isolated from treated and untreated cells.

185 We designed qPCR probes against the CD0001 and CD1931 regions, representing *oriC* and *terC*,
186 respectively (31, 35). Using these probes, we could show that *C. difficile* demonstrates multi-fork

187 replication in exponential growth phase and that the MFA assay detects the expected decrease in
188 *oriC*:*terC* ratio when cells enter stationary growth phase (data not shown). Next, we analyzed the effects
189 of PolC-inhibitors on the *oriC*:*terC* ratio. When cells were treated with HPUra (35 µg/mL), the MFA
190 showed a modest increase in *oriC:terC* ratio of 2,6-fold compared to untreated cells. However, when
191 cells were treated with ACX-362E (4 µg/ml), the MFA showed a >8-fold increase in the *oriC*:*terC* ratio
192 compared to untreated cells. In contrast, such an increase was not observed for cells treated with
193 metronidazole (0.25 ug/mL; a DNA damaging agent), fidaxomicin (0.00125 ug/mL; an RNA polymerase
194 inhibitor) or surotomycin (0.625 ug/mL; a cell-wall synthesis inhibitor) (**Figure 5**) or chloramphenicol (2
195 µg/mL; a protein synthesis inhibitor)(**Supplemental Figure 2**).

196 We conclude that inhibition of PolC-activity, but not the actions of any of the other tested
197 antimicrobials, leads to a gene dosage shift in *C. difficile*.

198

199 *Origin proximal* sigB *contributes to the transcriptional response*

200 Elegant work in *S. pneumoniae* has shown that the transcriptional response to replication inhibition can
201 also be affected by origin-proximal regulators that respond to the gene dosage effect (23). In *C. difficile*
202 the gene encoding the general stress response sigma factor σ^B (*sigB*, CD0011) is located close to the
203 origin of replication (40). We wondered whether this regulator contributes to the transcriptional effects
204 observed in our studies.

205 First, we compared the list of differentially regulated genes from our study (**Supplemental Table**
206 **2**) to those under the control of σ^B (41). In contrast to most anaerobic gram-positive organisms, *C.*
207 *difficile* encodes a homolog of the general stress response sigma factor, σ^B (40, 42). A transcriptome
208 analysis comparing *sigB* mutant versus wild type cells was recently published (41). Strikingly, we find
209 ~40% of the genes (21/58) identified as involved in stress response under the control of σ^B to be
210 differentially expressed in our ACX-362E dataset (**Supplemental Table 3**). Similarly, we observed that

211 7/20 (~35%) of the genes containing a transcriptional start site with a σ^B consensus sequence are
212 differentially expressed in our ACX-362E dataset (**Supplemental Table 3**). These data suggest that the
213 response to DNA replication inhibition is at least partially dependent on σ^B.

214 To demonstrate that exposure to ACX-362E causes a transient up-regulation of *sigB*, we
215 constructed a reporter fusion of the secreted luciferase reporter $sLuc^{opt}$ with the predicted promoter of
216 the *sigB* operon (P_{cd0007}). We monitored luciferase activity of a strain harboring a plasmid containing this
217 reporter fusion (WKS2003) after dilution of an overnight culture into fresh medium with or without ACX-
218 362E (**Figure 6**). In non-treated cells, expression from the *sigB* promoter is relatively stable over the
219 course of 5.5h. By contrast, luciferase activity strongly increases from 1.25h to 3h after inoculation into
220 medium with ACX-362E. These data show that exposure to ACX-362E transiently induces transcription of
221 the *sigB* operon.

222 Next, a *sigB* mutant was constructed using Allele-Coupled Exchange (43) as described in the
223 Materials and Methods. The chromosomal deletion of *sigB* and absence of σ^B protein was verified by
224 PCR and Western Blot, respectively (**Supplemental Figure 3**). To directly demonstrate a role for *sigB* in
225 the regulation of genes with altered transcription upon PolC-inhibition, we fused the predicted
226 promoter regions of selected genes to a secreted luciferase reporter (44) and evaluated luminescence in
227 wild type and *sigB* mutant backgrounds, after 5-hour growth in the presence and absence of 4 µg/mL
228 ACX-362E. All genes tested demonstrated a significant increase in promoter activity in a wild type
229 background in the presence of ACX-362E compared to the non-treated control, validating the results
230 from the RNA-Seq analysis (**Figure 7**). Three distinct patterns were observed. For CD0350 (encoding a
231 hypothetical protein) and CD3963 (encoding a putative peptidoglycan-binding exported protein) there
232 was virtually no expression in a *sigB* mutant background (**Figure 7A** and **B**). We conclude that these
233 genes are strictly dependent on σ^B for their expression under the conditions tested. CD3614 (encoding a
234 hypothetical protein) shows a basal level of expression, but no significant increase in transcription levels

235 in the presence of ACX-362E in a *sigB* mutant background compared to the non-treated control (**Figure**
236 **6C**). This suggests that the transcriptional up-regulation under these conditions is σ^B dependent, and
237 indicates that the basal level of transcription observed is likely independent of σ^B. Finally, CD3412 (*uvrB*,
238 encoding a subunit of an excinuclease) shows reduced transcriptional up-regulation in ACX-362E treated
239 cells compared to non-treated controls (**Figure 6D**). Thus, the transcription of this particular gene under
240 conditions of ACX-362E exposure is brought about by both a σ^B-dependent and a σ^B-independent
241 regulatory pathway.

242 Together, these results demonstrate that *sigB* controls the expression of at least a subset of
243 genes that are up-regulated under PolC-inhibition

244 **Discussion**

245

246 *Resistance to PolC-inhibitors and specificity of ACX-362E*

247 Limited treatment options and reports of reduced susceptibility to current treatment (11, 12, 45)
248 emphasize the necessity for the development of novel antimicrobials. As CDI can be induced by use of
249 broad spectrum antibiotics (7), new antimicrobials ideally should only target *C. difficile*, thereby
250 maintaining integrity of the colonic microbiota. In this study, we have tested inhibitors HPUra and ACX-
251 362E which specifically target the PolC enzyme, essential for DNA replication. The majority of PolC-
252 inhibitors target gram-positive bacteria with low G+C content, but ACX-362E has been reported to
253 demonstrate increased specificity towards *C. difficile* PolC *in vitro* and showed promising results for the
254 efficacy *in vivo* based on a limited set of *C. difficile* strains (19, 20). The compound will progress to
255 clinical trials in the near future (Acurx Pharmaceuticals, personal communication). The present study, is
256 the largest survey of the efficacy of HPUra and ACX-362E against a large collection of clinical isolates
257 consisting of many relevant PCR ribotypes to date. We have established that ACX-362E demonstrated
258 lower inhibitory concentrations than the general gram-positive PolC-inhibitor HPUra in agar dilution
259 experiments. The MIC_{50} and MIC_{90} of ACX-362E are similar to those of antimicrobials commonly used to
260 treat *C. difficile* infection (metronidazole: MIC_{50} = 2 ug/mL and MIC_{90} = 4 ug/mL; vancomycin MIC_{50} = 2
261 ug/mL and MIC_{90} = 4 ug/mL (20); fidaxomicin = MIC_{50} = 0.125 ug/mL and MIC_{90} = 0.5 ug/mL (46)). We did
262 not detect a significant difference in MICs between clades and ribotypes, demonstrating that PolC-
263 inhibitors have the potential to be used as treatment for the majority - if not all - circulating *C. difficile*
264 strains. This includes the epidemic types of PCR ribotype 027 and 078 (8, 47). These results are in line
265 with other work that demonstrated only 2- to 4-fold differences in antimicrobial susceptibility between
266 different clades for metronidazole, fidaxomicin and semi-synthetic thiopeptide antibiotic LFF571 (28). In
267 the course of our experiments, we did not find any strains tha grew at the highest concentrations of

268 either HPUra or ACX-362E tested. We cannot exclude rare pre-existing/intrinsic resistance, or the
269 development of resistance in strains over time, for instance through multi-drug exporters, but at present
270 there is no evidence for this. PolC-inhibitors are competitive inhibitors of polymerase activity by binding
271 in the active site. Mutations that abolish binding of HPUra or ACX-362E are likely to affect the essential
272 enzymatic activity of the polymerase and for that reason unlikely to occur *in vivo*. A single mutation
273 (*azp-12*) has been described in *B. subtilis* that confers resistance to HPUra (48). This T>G transversion
274 results in the replacement of a serine with an alanine in the highly conserved PFAM07733 domain of
275 the polymerase (49). To our knowledge, it is unknown whether this mutation prevents binding of HPUra
276 to PolC of *B. subtilis*. Few other mutations have been described that confer resistance against other
277 PolC-inhibitors (50, 51). It will be of interest to see if similar mutations in *C. difficile* result in resistance
278 to HPUra and/or ACX-362E and what the effect on binding of these compounds to *C. difficile* PolC is.

279 ACX362 may have off-target effects unrelated to its replication-inhibitory activity For *C. difficile*,
280 it has not been established that PolC inhibition is the sole mode of action of the inhibitors. In our
281 experiments, we found that *S. aureus* was sensitive to both HPUra and ACX-362E and even more so than
282 *C. difficile*. It should be established if this is due to inhibition of PolC or mediated by an alternative
283 mechanism. If ACX-362E targets DNA replication in *S. aureus*, we would also expect to find an increase in
284 *oriC*:*terC* ratio upon ACX-362E exposure in this organism.ACX-362EACX-362E Alternatively, ACX-362E
285 may also affect the activity of the other PolIII-type polymerase, DnaE, in *S. aureus*. PolIII-inhibitors can
286 affect PolC, DnaE or both (51), though *in vivo* activity appears to correlate with PolC-inhibition. Both C.
287 *difficile* and *S. aureus* possess PolC and DnaE polymerase, but the DnaE enzymes are of different families
288 (DnaE1 in *C. difficile* and DnaE3 in *S. aureus*) (52). To verify the mode of action, and whether a ACX-
289 362Edifferent DnaE-type polymerases explain the increased sensitivity of *S. aureus* compared to *C.*
290 *difficile*, the activity of ACX-362E towards purified DnaE and PolC from both organisms should be
291 determined..

292 Though it is clear that ACX-362E inhibits *C. difficile* efficiently and shows limited activity towards
293 certain other anaerobes (19), these findings highlight the necessity to perform additional (microbiome)
294 studies to more clearly define the antimicrobial spectrum of this compound. It also shows that ACX-362E
295 may have therapeutic potential outside treatment of CDI.
296
297 *Regulators of the transcriptional response to PolC-inhibitors*
298 The present study is the first to describe the transcriptional response of *C. difficile* to inhibition of DNA
299 replication. We find that ~200 genes show differential expression under conditions of PolC-inhibition by
300 both HPUra and ACX-362E, when compared to non-treated cells (**Supplemental Table 2, Figure 3**). When
301 considering only ACX-362E, approximately 13% of all genes in the genome show statistically significant
302 altered transcription. We demonstrate that this large reprogramming of transcription is likely to be
303 caused directly by a gene dosage shift (**Figure 4 and 5**).
304 In addition to direct effects, it is conceivable that at least part of the transcriptional response is
305 indirect. Our list of differentially regulated genes includes several putative regulators; sigma factors
306 (including *sigE*, *sigG* and *sigH*), transcription factors and anti-terminators. The relatively long time (5h) at
307 sub-MIC levels of antimicrobials may have contributed to secondary effects, through one or more of
308 these regulators. Though shorter induction times are thought to provoke more compound-specific
309 responses (53), we did observed a highly consistent transcriptional signature with both HPura and ACX-
310 362E (**Supplemental Table 2**, **Figure 3**).
311 Major stress response pathways are poorly characterized in *C. difficile*. On the basis of
312 experiments in other organisms (21-23, 54-56), we expect that inhibition of DNA replication inhibition
313 might possibly induce an SOS response (LexA)(57), a DnaA-dependent transcriptional response (21), and
314 possibly a heat shock response (HrcA/CtsR)(58) and/or a general stress response (σ^B)(42). Of these, the
315 best characterized stress response pathway in *C. difficile* is the one governed by σ^B (41). We noted a

316 significant overlap in putatively σ^B-dependent genes and those differentially expressed upon exposure
317 to PolC-inhibitors (**Supplemental Table 2**). In addition, our luciferase reporter fusions directly implicate
318 *sigB* in the expression and/or up-regulation of some of these (**Figure 7**). It should be noted that the *sigB*-
319 operon itself was not differentially expressed in our RNA-seq analysis. A *sigB*-reporter fusion suggests
320 that *sigB* is transiently up-regulated prior to the time point of sampling for the RNA-Seq analysis (**Figure**
321 **6**). Similar transient up-regulation of *sigB* followed by a persistent response of σ^B-dependent gene
322 expression has been observed in other organisms (58-60). To our knowledge, this is the first indication
323 that *sigB*- and SigB-dependent gene expression could be subject to a gene-dosage effect.

324 To date, no genes have been identified that are regulated by DnaA in *C. difficile* and direct
325 regulation of genes through the other stress response pathways has not been demonstrated. Putative
326 LexA-regulated genes of *C. difficile* were identified *in silico* (61) and some of these (such as the *uvr*
327 excinuclease and 30S ribosomal protein S3) were differentially expressed in our dataset (**Supplemental**
328 **Table 2**). Indeed, we find that only part of the transcriptional up-regulation of *uvrB* as a result of ACX-
329 362E exposure could be explained by *sigB* (**Figure 7D**), and LexA-dependent regulation might play a role.
330 Pleiotropic phenotypes have been described for a *C. difficile lexA* mutant (62) and it is likely that other
331 LexA targets than those identified *in silico* exist. No mutants of *hrcA* or *ctsR* have been described for *C.*
332 *difficile*, but transcriptome and proteome analyses have been performed with heat shocked cells (42°C
333 (60), or 41°C (59, 63, 64)). Similarities between these datasets and ours include genes encoding proline
334 racemase (*prdF*), chaperones (*groEL*, *groES*), thioredoxin systems (*trxA*, *trxB*) and Clp-proteases (*clpC*,
335 *clpP*).

336 Many parameters (such as the medium used, cell density, concentration of antibiotics, and
337 protocol used to arrest transcription between cell harvest and lysis) can influence overall transcription
338 signatures, and can also govern an incomplete overlap between our data and the stress regulons
339 determined by others (53).

340

341 *Genome location contributes to the transcriptional response to PolC-inhibition*

342 Our analysis of differential regulation in relation to genome location revealed a striking pattern of
343 relative up-regulation for *oriC*-proximal genes, and down-regulation for *terC*-proximal genes under
344 conditions of PolC inhibition (**Figure 4**). Antimicrobials directed at DNA replication in bacteria have a
345 profound negative effect on the processivity of replication forks, though initiation of DNA replication is
346 not or only marginally affected (23, 39). As a consequence, the presence of multiple replication forks
347 simultaneously increases the copy numbers of genes located in close proximity of the origin of
348 replication and such a gene dosage differences can result in functionally relevant transcriptional
349 differences, either directly or indirectly (15). We found an increase of *oriC:terC* ratio when performing
350 MFA on chromosomal DNA of cells subjected to a sub-inhibitory concentration of ACX-362E (and HPUra,
351 albeit less pronounced) (**Figure 5**), consistent with findings in other organisms (23). This is the first
352 demonstration of gene-dosage dependent transcriptional regulation in *C. difficile*.

353 An example of direct regulation by gene dosage can be found in *Vibrio*, for instance, where the
354 location of ribosomal protein clusters close to the origin is crucial for fast growth, because increased
355 copy number under condition of multi-fork replication allows for higher expression levels (65). We note
356 that ribosomal gene clusters are up-regulated when DNA replication is inhibited in our experiments
357 (**Table 2**, **Table 3**, **Supplemental Table 2**), suggesting that a similar mechanism may be active in *C.*
358 *difficile*.

359 An example of indirect regulation as a result of gene dosage is the induction of competence
360 genes in *S. pneumoniae* (23). Competence is believed to be a stress response in this organism, that lacks
361 a canonical (σ^B-dependent) stress response pathway. Key regulatory genes for competence
362 development are located close to the origin, and replication inhibition therefore leads to the induction
363 of origin distal competence genes (23). In our experiments, the large overlap with the proposed σ^B

364 regulon (41), the origin proximal location of the *sigB* operon (8.5kb-10kb) (40) and the σ^B-dependence of
365 selected differentially expressed genes suggests that at least part of the transcriptional response to
366 PolC-inhibition can be explained by an indirect gene dosage effect. The positioning of stress-response
367 regulators close to *oriC* may therefore be a conserved strategy in bacteria to respond to DNA replication
368 insults that is independent of the nature of the regulator.

369 Though it is likely that an increase in gene copy number leads to an increase in transcription of
370 these genes, it is less clear whether this is the case for the observed down-regulation. Most methods of
371 normalization for transcriptome analyses are based on the assumption that there is no overall change in
372 transcription or that the number of transcripts per cells is the same for all conditions and this may not
373 be the case when a global copy number shift occurs (15). Absolute transcript levels for down-regulated
374 genes might therefore be similar under both conditions (but lower relative to *oriC*-proximal transcripts).

375 It is interesting that certain processes are highly enriched in the list of genes up-regulated under
376 conditions of PolC-inhibition (most notably ribosome function and DNA-related functions), whereas this
377 is less so for the down-regulated genes. This suggests that pathways susceptible to replication
378 dependent gene-dosage effects demonstrate a functional clustering of genes near *oriC*, whereas
379 clustering of genes from specific pathways in the *terC*-proximal region is less pronounced. Indeed, most
380 ribosomal gene clusters in *C. difficile* are located close to the origin of replication (31, 40) and also many
381 genes involved in DNA replication and repair are located in these regions. Consistent with this,
382 positioning of genes involved in transcription and translation close to the origin appears to be under
383 strong selection as such genomes tend to be more stable (66).

384 In conclusion, both direct and indirect effects of gene dosage shifts are likely to contribute to the
385 transcriptional response of *C. difficile* to replication inhibition.

386

387 **Materials and Methods**

388

389 *Bacterial strains and culture conditions*

390 Plasmids and bacterial strains used in this study can be found in **Table 4**. Note that this table only
391 contains laboratory strains; the clinical isolates used for the agar dilution experiments (see below) are
392 listed in **Supplemental Table 1**. *E. coli* was cultured aerobically at 37°C (shaking at 200 rpm) in Luria-
393 Bertani (LB) broth supplemented with 20 µg/ml chloramphenicol and 50 µg/ml kanamycin when
394 appropriate. *C. difficile* strains were cultured in Brain Heart Infusion (BHI) broth (Oxoid) supplemented
395 with 0,5% yeast extract (Sigma-Aldrich), *Clostridium difficile* Selective Supplement (CDSS, Oxoid) and
396 20µg/ml thiamphenicol when appropriate. *C. difficile* was grown anaerobically in a Don Whitley VA-1000
397 workstation in an atmosphere of 10% CO_2, 10% H_2 and 80% N_2. Liquid cultures were grown under gentle
398 agitation (120 rpm).

399

400 *Agar dilution*

401 HPUra and ACX-362E were tested against a collection of *C. difficile* clinical isolates. 375 clinical isolates
402 have been collected during the ECDIS study (6). All strains were characterized by PCR ribotyping (67) and
403 by PCR to confirm the presence of genes encoding toxins A , B and binary toxin (68-70). Of the 375
404 clinical isolates, we excluded stocks that were found to contain more than one strain and isolates that
405 could not be recultured. Testing was therefore performed on 363 isolates (**Supplemental Table 1**). *C.*
406 *difficile* ATCC 700057, *B. fragilis* ATCC 25285 and *S. aureus* ATCC 29213 were used as controls (71).

407 The strains were tested for the different concentrations of antimicrobial using the agar dilution
408 method according to Clinical & Laboratory Standards Institute guidelines (26, 27). In short, the
409 antimicrobials were diluted into Brucella Blood Agar (BBA) supplemented with hemin and vitamin K1.
410 Bacterial isolates were cultured on blood agar plates and after 24 hours re-suspended to a turbidity of

411 0.5 McFarland in phosphate buffered saline (PBS). The strains were inoculated onto BBA solid media
412 containing the PolC-inhibitors using multipoint inoculators to a final concentration of 10^4 CFU per spot.
413 Each series of antimicrobial agents was tested from the lowest concentration to the highest
414 concentration. Two control plates without antibiotics were inoculated to control for aerobic
415 contamination and purity of anaerobic growth. At the end of the final series, two control plates were
416 inoculated to verify the final organism viability and purity. Only experiments where both positive and
417 negative controls performed according to expectations were included. Plates were incubated
418 anaerobically in a Don Whitley VA-1000 workstation in an atmosphere of 10% CO_2, 10% H_2 and 80% N_2
419 and the MICs were recorded after 24 and 48 hours and are presented in the manuscript with values at
420 48h, according to the CLSI guidelines (26).
421
422 *Sub-MIC determination*
423 *C. difficile* 630Δ*erm* (30, 31) was grown in 20 mL Brain Heart Infusion (Oxoid) supplemented with 0.5%
424 yeast extract (Sigma-Aldrich) (BHI/YE) starting from an optical density measured at 600nm (OD_{600}) of
425 0.05 using an exponentially growing starter culture (3 biological replicates per concentration). To
426 determine the effects on growth at sub-inhibitory concentrations of ACX-362E, cells were cultured in the
427 presence of the following concentrations; 0.25, 0.5, 1, 2, 4, and 8 µg/mL ACX-362E and compared to an
428 untreated culture. To determine the effects on growth at sub-inhibitory concentrations of HPUra, cells
429 were cultured in the presence of the following concentrations: 10, 20, 40 µg/mL HPUra and compared to
430 an untreated culture. The OD_{600} was monitored every hour until stationary phase was reached.
431
432 *Marker Frequency analysis*
433 *C. difficile* 630Δ*erm* (30, 31) was grown in 20 mL BHI supplemented with 0.5% yeast extract with sub-
434 MIC amounts of antimicrobials (HPUra: 35 µg/mL; ACX-362E: 4 µg/mL; metronidazole: 0.25 µg/mL;

435 fidaxomicin: 0.00125 µg/mL, surotomycin: 0.625 µg/mL), starting from an OD_{600} of 0.05 using an
436 exponentially growing starter culture. These samples were taken in the course of an independent, but
437 simultaneously performed, set of experiments for which we obtained surotomycin and fidaxomicin from
438 Cubist Pharmaceuticals. Metronidazole was commercially obtained (Sigma-Aldrich). We confirmed that
439 these concentrations did not lead to a >30% reduction in growth compared to non-treated cultures
440 (**Supplemental Figure 1** and data not shown). In parallel, cultures were grown without inhibitors from
441 the same starter culture. All conditions were performed in biological triplicates. Previous experiments
442 have shown that *ori*:*ter* differences are reliably detected >3h after dilution into fresh medium (23).
443 Therefore, after 5 hours, 1mL cells was harvested (OD_{600} ~ 0.5), and stored at -20°C. Isolation of
444 chromosomal DNA was performed the next day with the QIAamp DNA Blood Mini kit (Qiagen) according
445 to the instructions of the manufacturer. Marker frequency analysis (MFA) was performed to assess the
446 relative abundance of origin proximal genes relative to terminus proximal genes. As a proxy for *oriC*, a
447 probe was designed that targets the CD0001 region (CD0001-probe-FAM). By using plots of the GC skew
448 ([G - C]/[G + C]) generated using DNAPlotter (72), the approximate location of the terminus region for
449 the *C. difficile* chromosome was determined and a probe targeting this region (CD1931) was designed
450 (CD-1931-probe-TXR). Probe design was performed with Beacon Designer™ (Premier Biosoft, Palo Alto
451 CA, USA). Real-time PCR reactions were performed on a Biorad CFX96™ real-time PCR detection system
452 (95°C 15 m, 39 × (94°C 30 s + 55°C 30 s + 72°C 30 s). Sequences for primers and probes are listed in **Table**
453 **5**. For each biological replicate, three technical replicates were performed. Amplification efficiency was
454 determined using standard curves obtained from DNA late stationary phase cells of strain 630Δ*erm*, for
455 which an *oriC*:*terC* ratio of 1 was assumed. RT-PCR results from antibiotic treated cells were normalized
456 to the *oriC*:*terC* ratio of DNA samples (3 biological replicates) from non-treated cells. Calculations were
457 performed in Microsoft Office Excel 2010, plotted using Prism 7 (GraphPad) and prepared for

458 publication in Corel Draw Suite X8. Significance was determined using a One-way ANOVA and a Tukey's
459 test for multiple comparisons (GraphPad).
460
461 *Growth and RNA isolation for RNA-Seq*
462 For RNA-Seq analysis, *C. difficile* 630*Δerm* (30, 31) was grown for 5h in BHI medium with HPUra (35
463 ug/mL) or ACX-362E (4 µg/mL) starting from an OD_{600} of 0.05 using an exponentially growing starter
464 culture, after which cells (3mL) were harvested for RNA isolation. These concentrations were some of
465 the highest concentrations that resulted in a modest effects on growth (<30% reduction of growth
466 compared to wild type cells, **Supplemental Figure 1**). RNA isolation was performed with NucleoSpin©
467 RNA kit (Macherey-Nagel). Although the kit includes on column rDNAse digestion, a second treatment
468 was performed in solution and RNA was precipitated and recovered by NaAc precipitation to remove
469 residual DNA. Concentration determination and quality control (16S/23S ratio and RNA integrity number
470 [RIN]) was performed with a fragment analyzer (Agilent bio-analyzer), according to the instructions of
471 the manufacturer. Samples with a RIN>9 and 16S/23S ratio >1.4 were submitted for analysis by RNA-
472 Seq.
473
474 *RNA-Seq*
475 RNA-Seq was performed at a commercial provider (GenomeScan, Leiden, The Netherlands). In short,
476 the NEBNext Ultra Directional RNA Library Prep Kit for Illumina was used to process the samples. Sample
477 preparation was performed according to the protocol "NEBNext Ultra Directional RNA Library Prep Kit
478 for Illumina" (NEB #E7420S/L). Briefly, after selective removal of rRNA (Ribo-Zero rRNA Removal Kit for
479 gram-positive Bacteria) and fragmentation of the mRNA, cDNA synthesis was performed. cDNA was
480 ligated to the sequencing adapters and the resulting product was PCR amplified. Clustering and DNA
481 sequencing using the Illumina NextSeq 500 platform was performed according to manufacturer's

482 protocols. A concentration of 1.5 pM of DNA was used. Image analysis, base calling, and quality check
483 was performed with the Illumina data analysis pipeline RTA vl.18.64 and Bcl2fastq v2.17. Per sample,
484 four technical replicates were included in the RNA-Seq experiment. In case of insufficient reads, the
485 sample was re-run on another flow cell to reach satisfactory quantities (≥ 20 M).
486
487 *Analysis of RNA-Seq data*
488 Analysis of the data was performed using T-REx, a user-friendly webserver which has been optimized for
489 the analysis of prokaryotic RNAseq-derived expression data (73). The pipeline requires raw RNA
490 expression level data as an input for RNA-Seq data analysis. For data normalisation and determination of
491 the genes, the factorial design statistical method of the RNA-Seq analysis R-package EdgeR is
492 implemented in the T-Rex pipeline. Some samples displayed incomplete rRNA depletion and rRNA
493 mapping reads had to be removed manually prior to analysis.
494 To analyse the genome-wide pattern in differential gene expression a sliding window analysis
495 was performed essentially as described (23). In short, genome locations (start of the locus tag) were
496 coupled to the locus tags in the T-Rex output. Next, the median log(FC) was calculated for bins of 51 loci
497 with a stepsize of 1. For each bin of [X_1, X_2…X_{51}] the median absolute deviation of the median
498 (MAD=median(|X_i-median(X)|) was calculated as an robust indication of the distribution around
499 calculated median values. Calculations were performed and three curves (median, median-MAD and
500 median+MAD) were plotted in Microsoft Office Excel 2010 and the graph was prepared for publication
501 using Adobe Photoshop CC and Corel Draw Suite X8.
502 A GSEA analysis (33) was performed via the Genome2D webserver (73), using our reference
503 genome sequence for *C. difficile* 630Δ*erm*, Genbank identifier LN614756.1 (listed in Genome2D as
504 "Clostridioides_difficile_630Derm")(31). As input a single list of locus tags was used of either up- or
505 down regulated genes. The output was copied to Microsoft Excel 2010. The single_list column was split,

506 and a column was inserted to calculate the significance of the overrepresentation using the formula "(#
507 hits in list/ClassSize)*-log(p-value; 2)" to allow for sorting of the output of the GSEA analysis by
508 significance.

509 Data for the RNA-Seq experiment has been deposited in the Gene Expression Omnibus (GEO)
510 database (74), accession number GSE116503.

511

512 *General molecular biological techniques*

513 *E. coli* strain DH5α was used for maintenance of all plasmids. All plasmid transformations into *E. coli*
514 were performed using standard procedures (75). *E. coli* CA434 was used as a donor for conjugation of
515 plasmids into the recipient *C. difficile* (76). Conjugation was performed as previously described (76).
516 Briefly, 1 mL of an overnight culture of donor cells was mixed with 200 µl of the recipient and spotted
517 onto anaerobic BHI agar plates and incubated for 5-8 hours. After incubation cells were collected and
518 tenfold serial dilutions were plated onto fresh BHI plates containing thiamphenicol and CDSS.

519 Plasmid DNA was isolated using the Nucleospin Plasmid (Macherey-Nagel) mini prep kits per
520 manufacturer's instructions. *C. difficile* genomic DNA was isolated using the DNeasy Blood and Tissue kit
521 (Qiagen) with pre-treatment for gram-positives according to instructions of the manufacturer.

522

523 *Construction of luciferase-reporter fusion plasmids*

524 All PCR reactions for plasmid construction were carried out with Q5 polymerase (New England Biolabs).
525 Putative promoter regions were amplified using *C. difficile* 630Δ*erm* chromosomal DNA (31) as a
526 template.

527 The P_{CD3412} luciferase reporter plasmid was created by restriction-ligation using the restriction
528 enzymes *Kpn*I and *Sac*I. P_{CD3412} was amplified using primers oIB-26 and oIB-27 (**Table 5**). The resulting
529 dsDNA fragment was digested and ligated into KpnI-SacI digested pAP24 (44), yielding plasmid pIB27.

530 Plamids pIB68 (P_{CD0350}), pIB69 (P_{CD2963}), pIB74 (P_{CD3614}) and pPH28 (P_{CD0007}) were constructed using a
531 Gibson assembly (77). The plasmid backbone of pAP24 was linearized by PCR using primers oWKS-
532 1580/oWKS-1582, and the predicted promoter areas of CD0350, CD2963 and CD3614 were amplified
533 with primers oIB-80/oIB-94, oIB-82/oIB-95, oIB-92/oIB-100 and oPH-19/oPH-20, respectively. Primers
534 were designed using the NEBuilder assembly tool v.1.12.17 (New England Biolabs) using a 30 bp overlap.
535 For the assembly, 100 ng of vector DNA was assembled to a five-fold molar excess of the PCR fragment
536 of the desired promoter using a homemade Gibson Assembly Master Mix at 50°C for 30 minutes (final
537 concentration: 4 U/µl Taq Ligase (Westburg), 0.004 U/µl T5 exonuclease (New England Biolabs), 0.025
538 U/µl Phusion polymerase (Bioké), 5% polyethyleneglycol (PEG-8000),10 mM $MgCl_2$, 100 mM Tris-Cl
539 pH=7.5, 10 mM dithiothreitol, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dCTP, 0.2 mM dGTP, and 1 mM β-
540 nicotinamide adenine dinucleotide) and transformed into *E. coli* DH5α. Transformants were screened by
541 colony PCR using primers oWKS-1240 and oWKS-1241. Transformants yielding PCR fragments of the
542 correct size were verified by Sanger sequencing.
543
544 *Construction of* C. difficile *IB56 (*ΔsigB*)*
545 The up- and downstream regions (both 950 bp each) of the *sigB* coding sequence were amplified with
546 primers oIB-44/oIB-45, and oIB-46/oIB-47, respectively. Vector pMTL-SC7315 (43) was linearized by PCR
547 using primers oWKS-1537/oWKS-1538. Assembly was done according to the method of Gibson (44, 77).
548 The assembled plasmid was transformed into *E. coli* DH5α and verified using PCR and Sanger
549 sequencing. Generation of the unmarked *sigB* deletion mutant was performed using Allele-Coupled
550 Exchange essentially as described (43, 78). Briefly, pIB54 was introduced into *C. difficile* 630Δ*erm* (31) by
551 conjugation. Transconjugants were grown for two days on BHI agar plates supplemented with yeast
552 extract, thiamphenicol and CDSS, struck onto fresh pre-reduced plates and incubated anaerobically at
553 37°C for two days. Single-crossover integration was confirmed using PCR, and those clones were plated

554 onto non-selective BHI agar plates to allow the second-crossover event to occur. Colonies were then
555 serially diluted and plated onto minimal agar supplemented with 50 µg/ml 5-fluorocytosine (Sigma) as
556 described (43). DNA was isolated from thiamphenicol-susceptible colonies and the chromosomal
557 deletion was verified by PCR using primers oIB-78/oIB-79 (**Supplemental Figure 3**) as well as Sanger
558 sequencing of the PCR product using primers oIB-53/oIB-76/oIB-78.
559
560 *Luciferase reporter assay*
561 Strains containing luciferase reporter plasmids were inoculated to an OD_{600}=0.05 from an exponentially
562 growing starter culture. Fresh inoculums were grown in BHI broth supplemented with yeast extract,
563 with or without 4 µg/ml ACX-362E for 5 (for putative *sigB* target genes) or 5.5 (*sigB* promoter) hours.
564 The supernatants from 1mL of culture (harvested by centrifugation for 10 min, 4°C, 8000 rpm) were
565 analyzed in a GloMax®-Multi Microplate Multimode Reader (Promega) as described before (44).
566 Statistical significance of the data ($p<0.05$) was determined by two-way analysis of variance (ANOVA)
567 and a pairwise Tukey-Kramer test using Prism 7 (GraphPad) where appropriate.
568
569 ***Accession numbers***
570 GEO GSE116503; GenBank LN614756.1

571 **Author's contributions**

572 EVE, IMB, EJK and WKS designed experiments. GW and EJK provided reagents or strains. EVE, IMB and
573 IMGJBS performed experiments. EVE, IMB and WKS analyzed data. EVE, IMB and WKS wrote the
574 manuscript. All authors read and approved the manuscript.

575

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581 the sliding window analysis.

582

583 **Conflict of interest**

584 EVE and WKS have performed research for Cubist. EJK has performed research for Cubist, Novartis and
585 Qiagen, and has participated in advisory forums of Astellas, Optimer, Actelion, Pfizer, Sanofi Pasteur and
586 Seres Therapeutics and currently holds an unrestricted research grant from Vedanta Biosciences Inc. GW
587 is a consultant for Acurx Pharmaceuticals. The companies had no role in the design of the experiments
588 or the decision to publish. IMB and IMGJSB: none to declare.

589

590 **References**

591 1. Lawson PA, Citron DM, Tyrrell KL, Finegold SM. 2016. Reclassification of Clostridium difficile as
592 Clostridioides difficile (Hall and O'Toole 1935) Prevot 1938. Anaerobe 40:95-9.
593 2. Leffler DA, Lamont JT. 2015. Clostridium difficile infection. N Engl J Med 372:1539-48.
594 3. Smits WK, Lyras D, Lacy DB, Wilcox MH, Kuijper EJ. 2016. Clostridium difficile infection. Nat Rev
595 Dis Primers 2:16020.
596 4. Abt MC, McKenney PT, Pamer EG. 2016. Clostridium difficile colitis: pathogenesis and host
597 defence. Nat Rev Microbiol 14:609-20.
598 5. Anonymous (ed). 2006. Investigation into outbreaks of Clostridium difficile at Stoke Mandeville
599 Hospital, Buckinghamshire Hospitals NHS Trust. Commission for Healthcare Audit and
600 Inspection,
601 6. Bauer MP, Notermans DW, van Benthem BH, Brazier JS, Wilcox MH, Rupnik M, Monnet DL, van
602 Dissel JT, Kuijper EJ, Group ES. 2011. Clostridium difficile infection in Europe: a hospital-based
603 survey. Lancet 377:63-73.
604 7. Hensgens MP, Goorhuis A, Dekkers OM, Kuijper EJ. 2012. Time interval of increased risk for
605 Clostridium difficile infection after exposure to antibiotics. J Antimicrob Chemother 67:742-8.
606 8. He M, Miyajima F, Roberts P, Ellison L, Pickard DJ, Martin MJ, Connor TR, Harris SR, Fairley D,
607 Bamford KB, D'Arc S, Brazier J, Brown D, Coia JE, Douce G, Gerding D, Kim HJ, Koh TH, Kato H,
608 Senoh M, Louie T, Michell S, Butt E, Peacock SJ, Brown NM, Riley T, Songer G, Wilcox M,
609 Pirmohamed M, Kuijper E, Hawkey P, Wren BW, Dougan G, Parkhill J, Lawley TD. 2013.
610 Emergence and global spread of epidemic healthcare-associated Clostridium difficile. Nat Genet
611 45:109-13.
612 9. Debast SB, Bauer MP, Kuijper EJ, European Society of Clinical M, Infectious D. 2014. European
613 Society of Clinical Microbiology and Infectious Diseases: update of the treatment guidance
614 document for Clostridium difficile infection. Clin Microbiol Infect 20 Suppl 2:1-26.
615 10. McDonald LC, Gerding DN, Johnson S, Bakken JS, Carroll KC, Coffin SE, Dubberke ER, Garey KW,
616 Gould CV, Kelly C, Loo V, Shaklee Sammons J, Sandora TJ, Wilcox MH. 2018. Clinical Practice
617 Guidelines for Clostridium difficile Infection in Adults and Children: 2017 Update by the
618 Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of
619 America (SHEA). Clin Infect Dis 66:e1-e48.
620 11. Baines SD, O'Connor R, Freeman J, Fawley WN, Harmanus C, Mastrantonio P, Kuijper EJ, Wilcox
621 MH. 2008. Emergence of reduced susceptibility to metronidazole in Clostridium difficile. J
622 Antimicrob Chemother 62:1046-52.
623 12. Chong PM, Lynch T, McCorrister S, Kibsey P, Miller M, Gravel D, Westmacott GR, Mulvey MR,
624 Canadian Nosocomial Infection Surveillance P. 2014. Proteomic analysis of a NAP1 Clostridium
625 difficile clinical isolate resistant to metronidazole. PLoS One 9:e82622.
626 13. Moura I, Spigaglia P, Barbanti F, Mastrantonio P. 2013. Analysis of metronidazole susceptibility
627 in different Clostridium difficile PCR ribotypes. J Antimicrob Chemother 68:362-5.
628 14. Davies J, Spiegelman GB, Yim G. 2006. The world of subinhibitory antibiotic concentrations. Curr
629 Opin Microbiol 9:445-53.
630 15. Slager J, Veening JW. 2016. Hard-Wired Control of Bacterial Processes by Chromosomal Gene
631 Location. Trends Microbiol 24:788-800.
632 16. Tarantino PM, Jr., Zhi C, Wright GE, Brown NC. 1999. Inhibitors of DNA polymerase III as novel
633 antimicrobial agents against gram-positive eubacteria. Antimicrob Agents Chemother 43:1982-7.
634 17. Brown NC. 1970. 6-(p-hydroxyphenylazo)-uracil: a selective inhibitor of host DNA replication in
635 phage-infected Bacillus subtilis. Proc Natl Acad Sci U S A 67:1454-61.

636 18. Butler MM, Dudycz LW, Khan NN, Wright GE, Brown NC. 1990. Development of novel inhibitor
637 probes of DNA polymerase III based on dGTP analogs of the HPUra type: base, nucleoside and
638 nucleotide derivatives of N2-(3,4-dichlorobenzyl)guanine. Nucleic Acids Res 18:7381-7.
639 19. Torti A, Lossani A, Savi L, Focher F, Wright GE, Brown NC, Xu WC. 2011. Clostridium difficile DNA
640 polymerase IIIC: basis for activity of antibacterial compounds. Curr Enzym Inhib 7:147-153.
641 20. Dvoskin S, Xu WC, Brown NC, Yanachkov IB, Yanachkova M, Wright GE. 2012. A novel agent
642 effective against Clostridium difficile infection. Antimicrob Agents Chemother 56:1624-6.
643 21. Goranov AI, Katz L, Breier AM, Burge CB, Grossman AD. 2005. A transcriptional response to
644 replication status mediated by the conserved bacterial replication protein DnaA. Proc Natl Acad
645 Sci U S A 102:12932-7.
646 22. Goranov AI, Kuester-Schoeck E, Wang JD, Grossman AD. 2006. Characterization of the global
647 transcriptional responses to different types of DNA damage and disruption of replication in
648 Bacillus subtilis. J Bacteriol 188:5595-605.
649 23. Slager J, Kjos M, Attaiech L, Veening JW. 2014. Antibiotic-induced replication stress triggers
650 bacterial competence by increasing gene dosage near the origin. Cell 157:395-406.
651 24. Knetsch CW, Terveer EM, Lauber C, Gorbalenya AE, Harmanus C, Kuijper EJ, Corver J, van
652 Leeuwen HC. 2012. Comparative analysis of an expanded Clostridium difficile reference strain
653 collection reveals genetic diversity and evolution through six lineages. Infect Genet Evol
654 12:1577-85.
655 25. He M, Sebaihia M, Lawley TD, Stabler RA, Dawson LF, Martin MJ, Holt KE, Seth-Smith HM, Quail
656 MA, Rance R, Brooks K, Churcher C, Harris D, Bentley SD, Burrows C, Clark L, Corton C, Murray V,
657 Rose G, Thurston S, van Tonder A, Walker D, Wren BW, Dougan G, Parkhill J. 2010. Evolutionary
658 dynamics of Clostridium difficile over short and long time scales. Proc Natl Acad Sci U S A
659 107:7527-32.
660 26. CLSI. 2012. Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved
661 Standard - Eighth edition, p 56, 8 ed. Clinical and Laboratory Standards Institute, Wayne, PA.
662 27. Freeman J, Stott J, Baines SD, Fawley WN, Wilcox MH. 2005. Surveillance for resistance to
663 metronidazole and vancomycin in genotypically distinct and UK epidemic Clostridium difficile
664 isolates in a large teaching hospital. J Antimicrob Chemother 56:988-9.
665 28. Debast SB, Bauer MP, Sanders IM, Wilcox MH, Kuijper EJ, Group ES. 2013. Antimicrobial activity
666 of LFF571 and three treatment agents against Clostridium difficile isolates collected for a pan-
667 European survey in 2008: clinical and therapeutic implications. J Antimicrob Chemother
668 68:1305-11.
669 29. Pacitti DF, Barnes MH, Li DH, Brown NC. 1995. Characterization and overexpression of the gene
670 encoding Staphylococcus aureus DNA polymerase III. Gene 165:51-6.
671 30. Hussain HA, Roberts AP, Mullany P. 2005. Generation of an erythromycin-sensitive derivative of
672 Clostridium difficile strain 630 (630Deltaerm) and demonstration that the conjugative
673 transposon Tn916DeltaE enters the genome of this strain at multiple sites. J Med Microbiol
674 54:137-41.
675 31. van Eijk E, Anvar SY, Browne HP, Leung WY, Frank J, Schmitz AM, Roberts AP, Smits WK. 2015.
676 Complete genome sequence of the Clostridium difficile laboratory strain 630Deltaerm reveals
677 differences from strain 630, including translocation of the mobile element CTn5. BMC Genomics
678 16:31.
679 32. Hastey CJ, Dale SE, Nary J, Citron D, Law JH, Roe-Carpenter DE, Chesnel L. 2017. Comparison of
680 Clostridium difficile minimum inhibitory concentrations obtained using agar dilution vs broth
681 microdilution methods. Anaerobe 44:73-77.
682 33. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A,
683 Pomeroy SL, Golub TR, Lander ES, Mesirov JP. 2005. Gene set enrichment analysis: a knowledge-

684 based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A
685 102:15545-50.
686 34. Anonymous. Genome2D web server. http://genome2d.molgenrug.nl. Accessed
687 35. van Eijk E, Paschalis V, Green M, Friggen AH, Larson MA, Spriggs K, Briggs GS, Soultanas P, Smits
688 WK. 2016. Primase is required for helicase activity and helicase alters the specificity of primase
689 in the enteropathogen Clostridium difficile. Open Biol 6.
690 36. Sousa C, de Lorenzo V, Cebolla A. 1997. Modulation of gene expression through chromosomal
691 positioning in Escherichia coli. Microbiology 143 (Pt 6):2071-8.
692 37. Sauer C, Syvertsson S, Bohorquez LC, Cruz R, Harwood CR, van Rij T, Hamoen LW. 2016. Effect of
693 Genome Position on Heterologous Gene Expression in Bacillus subtilis: An Unbiased Analysis.
694 ACS Synth Biol 5:942-7.
695 38. Block DH, Hussein R, Liang LW, Lim HN. 2012. Regulatory consequences of gene translocation in
696 bacteria. Nucleic Acids Res 40:8979-92.
697 39. Samadpour AN, Merrikh H. 2018. DNA gyrase activity regulates DnaA-dependent replication
698 initiation in Bacillus subtilis. Mol Microbiol 108:115-127.
699 40. Sebaihia M, Wren BW, Mullany P, Fairweather NF, Minton N, Stabler R, Thomson NR, Roberts
700 AP, Cerdeno-Tarraga AM, Wang H, Holden MT, Wright A, Churcher C, Quail MA, Baker S, Bason
701 N, Brooks K, Chillingworth T, Cronin A, Davis P, Dowd L, Fraser A, Feltwell T, Hance Z, Holroyd S,
702 Jagels K, Moule S, Mungall K, Price C, Rabbinowitsch E, Sharp S, Simmonds M, Stevens K, Unwin
703 L, Whithead S, Dupuy B, Dougan G, Barrell B, Parkhill J. 2006. The multidrug-resistant human
704 pathogen Clostridium difficile has a highly mobile, mosaic genome. Nat Genet 38:779-86.
705 41. Kint N, Janoir C, Monot M, Hoys S, Soutourina O, Dupuy B, Martin-Verstraete I. 2017. The
706 alternative sigma factor sigma(B) plays a crucial role in adaptive strategies of Clostridium difficile
707 during gut infection. Environ Microbiol 19:1933-1958.
708 42. Hecker M, Pane-Farre J, Volker U. 2007. SigB-dependent general stress response in Bacillus
709 subtilis and related gram-positive bacteria. Annu Rev Microbiol 61:215-36.
710 43. Cartman ST, Kelly ML, Heeg D, Heap JT, Minton NP. 2012. Precise manipulation of the
711 Clostridium difficile chromosome reveals a lack of association between the tcdC genotype and
712 toxin production. Appl Environ Microbiol 78:4683-90.
713 44. Oliveira Paiva AM, Friggen AH, Hossein-Javaheri S, Smits WK. 2016. The Signal Sequence of the
714 Abundant Extracellular Metalloprotease PPEP-1 Can Be Used to Secrete Synthetic Reporter
715 Proteins in Clostridium difficile. ACS Synth Biol 5:1376-1382.
716 45. Lynch T, Chong P, Zhang J, Hizon R, Du T, Graham MR, Beniac DR, Booth TF, Kibsey P, Miller M,
717 Gravel D, Mulvey MR, Canadian Nosocomial Infection Surveillance P. 2013. Characterization of a
718 stable, metronidazole-resistant Clostridium difficile clinical isolate. PLoS One 8:e53757.
719 46. Goldstein EJ, Citron DM, Sears P, Babakhani F, Sambol SP, Gerding DN. 2011. Comparative
720 susceptibilities to fidaxomicin (OPT-80) of isolates collected at baseline, recurrence, and failure
721 from patients in two phase III trials of fidaxomicin against Clostridium difficile infection.
722 Antimicrob Agents Chemother 55:5194-9.
723 47. Knetsch CW, Kumar N, Forster SC, Connor TR, Browne HP, Harmanus C, Sanders IM, Harris SR,
724 Turner L, Morris T, Perry M, Miyajima F, Roberts P, Pirmohamed M, Songer JG, Weese JS, Indra
725 A, Corver J, Rupnik M, Wren BW, Riley TV, Kuijper EJ, Lawley TD. 2018. Zoonotic Transfer of
726 Clostridium difficile Harboring Antimicrobial Resistance between Farm Animals and Humans. J
727 Clin Microbiol 56.
728 48. Ott RW, Barnes MH, Brown NC, Ganesan AT. 1986. Cloning and characterization of the polC
729 region of Bacillus subtilis. J Bacteriol 165:951-7.

730 49. Barnes MH, Hammond RA, Foster KA, Mitchener JA, Brown NC. 1989. The cloned polC gene of
731 Bacillus subtilis: characterization of the azp12 mutation and controlled in vitro synthesis of
732 active DNA polymerase III. Gene 85:177-86.
733 50. Barnes MH, Hammond RA, Kennedy CC, Mack SL, Brown NC. 1992. Localization of the
734 exonuclease and polymerase domains of Bacillus subtilis DNA polymerase III. Gene 111:43-9.
735 51. Barnes MH, Butler MM, Wright GE, Brown NC. 2012. Antimicrobials targeted to the replication-
736 specific DNA polymerases of gram-positive bacteria: target potential of dnaE. Infect Disord Drug
737 Targets 12:327-31.
738 52. Timinskas K, Balvociute M, Timinskas A, Venclovas C. 2014. Comprehensive analysis of DNA
739 polymerase III alpha subunits and their homologs in bacterial genomes. Nucleic Acids Res
740 42:1393-413.
741 53. Wecke T, Mascher T. 2011. Antibiotic research in the age of omics: from expression profiles to
742 interspecies communication. J Antimicrob Chemother 66:2689-704.
743 54. Kazmierczak MJ, Mithoe SC, Boor KJ, Wiedmann M. 2003. Listeria monocytogenes sigma B
744 regulates stress response and virulence functions. J Bacteriol 185:5722-34.
745 55. Weber H, Polen T, Heuveling J, Wendisch VF, Hengge R. 2005. Genome-wide analysis of the
746 general stress response network in Escherichia coli: sigmaS-dependent genes, promoters, and
747 sigma factor selectivity. J Bacteriol 187:1591-603.
748 56. van Schaik W, van der Voort M, Molenaar D, Moezelaar R, de Vos WM, Abee T. 2007.
749 Identification of the sigmaB regulon of Bacillus cereus and conservation of sigmaB-regulated
750 genes in low-GC-content gram-positive bacteria. J Bacteriol 189:4384-90.
751 57. Kreuzer KN. 2013. DNA damage responses in prokaryotes: regulating gene expression,
752 modulating growth patterns, and manipulating replication forks. Cold Spring Harb Perspect Biol
753 5:a012674.
754 58. Helmann JD, Wu MF, Kobel PA, Gamo FJ, Wilson M, Morshedi MM, Navre M, Paddon C. 2001.
755 Global transcriptional response of Bacillus subtilis to heat shock. J Bacteriol 183:7318-28.
756 59. Ternan NG, Jain S, Srivastava M, McMullan G. 2012. Comparative transcriptional analysis of
757 clinically relevant heat stress response in Clostridium difficile strain 630. PLoS One 7:e42410.
758 60. Emerson JE, Stabler RA, Wren BW, Fairweather NF. 2008. Microarray analysis of the
759 transcriptional responses of Clostridium difficile to environmental and antibiotic stress. J Med
760 Microbiol 57:757-64.
761 61. Walter BM, Rupnik M, Hodnik V, Anderluh G, Dupuy B, Paulic N, Zgur-Bertok D, Butala M. 2014.
762 The LexA regulated genes of the Clostridium difficile. BMC Microbiol 14:88.
763 62. Walter BM, Cartman ST, Minton NP, Butala M, Rupnik M. 2015. The SOS Response Master
764 Regulator LexA Is Associated with Sporulation, Motility and Biofilm Formation in Clostridium
765 difficile. PLoS One 10:e0144763.
766 63. Jain S, Graham C, Graham RL, McMullan G, Ternan NG. 2011. Quantitative proteomic analysis of
767 the heat stress response in Clostridium difficile strain 630. J Proteome Res 10:3880-90.
768 64. Ternan NG, Jain S, Graham RL, McMullan G. 2014. Semiquantitative analysis of clinical heat
769 stress in Clostridium difficile strain 630 using a GeLC/MS workflow with emPAI quantitation.
770 PLoS One 9:e88960.
771 65. Soler-Bistue A, Mondotte JA, Bland MJ, Val ME, Saleh MC, Mazel D. 2015. Genomic location of
772 the major ribosomal protein gene locus determines Vibrio cholerae global growth and
773 infectivity. PLoS Genet 11:e1005156.
774 66. Couturier E, Rocha EP. 2006. Replication-associated gene dosage effects shape the genomes of
775 fast-growing bacteria but only for transcription and translation genes. Mol Microbiol 59:1506-
776 18.

777 67. Bidet P, Lalande V, Salauze B, Burghoffer B, Avesani V, Delmee M, Rossier A, Barbut F, Petit JC.
778 2000. Comparison of PCR-ribotyping, arbitrarily primed PCR, and pulsed-field gel electrophoresis
779 for typing Clostridium difficile. J Clin Microbiol 38:2484-7.
780 68. Kato H, Kato N, Watanabe K, Iwai N, Nakamura H, Yamamoto T, Suzuki K, Kim SM, Chong Y,
781 Wasito EB. 1998. Identification of toxin A-negative, toxin B-positive Clostridium difficile by PCR. J
782 Clin Microbiol 36:2178-82.
783 69. Paltansing S, van den Berg RJ, Guseinova RA, Visser CE, van der Vorm ER, Kuijper EJ. 2007.
784 Characteristics and incidence of Clostridium difficile-associated disease in The Netherlands,
785 2005. Clin Microbiol Infect 13:1058-64.
786 70. Stubbs S, Rupnik M, Gibert M, Brazier J, Duerden B, Popoff M. 2000. Production of actin-specific
787 ADP-ribosyltransferase (binary toxin) by strains of Clostridium difficile. FEMS Microbiol Lett
788 186:307-12.
789 71. Anonymous. American Type Culture Collection.
790 72. Anonymous. DNAPlotter. http://www.sanger.ac.uk/science/tools/dnaplotter. Accessed
791 73. de Jong A, van der Meulen S, Kuipers OP, Kok J. 2015. T-REx: Transcriptome analysis webserver
792 for RNA-seq Expression data. BMC Genomics 16:663.
793 74. Anonymous. Gene Expression Omnibus (GEO). https://www.ncbi.nlm.nih.gov/geo/. Accessed
794 75. Sambrook J, Russell DW. 2001. Molecular cloning : a laboratory manual. Cold Spring Harbor
795 Laboratory, Cold Spring Harbor, N.Y.
796 76. Purdy D, O'Keeffe TA, Elmore M, Herbert M, McLeod A, Bokori-Brown M, Ostrowski A, Minton
797 NP. 2002. Conjugative transfer of clostridial shuttle vectors from Escherichia coli to Clostridium
798 difficile through circumvention of the restriction barrier. Mol Microbiol 46:439-52.
799 77. Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA, 3rd, Smith HO. 2009. Enzymatic
800 assembly of DNA molecules up to several hundred kilobases. Nat Methods 6:343-5.
801 78. van Leeuwen PT, van der Peet JM, Bikker FJ, Hoogenkamp MA, Oliveira Paiva AM, Kostidis S,
802 Mayboroda OA, Smits WK, Krom BP. 2016. Interspecies Interactions between Clostridium
803 difficile and Candida albicans. mSphere 1.

804

805

806 **Tables**

807

808 **Table 1.** Minimal inhibitory concentrations of PolC-inhibitors towards *C. difficile* stratified by clade.

809

810 **Table 2.** List of the genes most highly up- and down-regulated in the presence of HPUra compared to

811 non-treated cells.

812

813 **Table 3.** List of the genes most highly up- and down-regulated in the presence of ACX-362E compared to

814 non-treated cells.

815

816 **Table 4.** Plasmids and strains used in this study.

817

818 **Table 5.** Oligonucleotides and probes used in this study.

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829 **Figures**

830

831 **Figure 1. Mechanism of action of the PolC-inhibitors ACX-362E. A.** Ternary complex of inhibitor ACX-
832 362E, DNA ,and PolC**. B.** H-bonding between inhibitor molecule ACX-362E and a cytosine residue of
833 DNA.

834

835 **Figure 2. Minimal inhibitory concentrations of PolC-inhibitors.** MIC was determined by agar dilution
836 according to CLSI standards (26) and is expressed in µg/mL. The distribution in the MIC for the collection
837 of clinical isolates (N=363) is given for the PolC-inhibitors HPUra (blue) and ACX-362E (red).

838

839 **Figure 3. Overlap in the transcriptional response to different PolC-inhibitors. A.** Venn diagram of the
840 number of genes up-regulated in the presence of ACX-362E (red), in the presence of HPUra (blue) or
841 under both conditions (overlapping region). **B.** Venn diagram of the number of genes down-regulated in
842 the presence of ACX-362E (red), in the presence of HPUra (blue) or under both conditions (overlapping
843 region). The size of the circles is proportional to the number of genes that showed differential
844 expression.

845

846 **Figure 4. Genome location correlates with differential expression upon PolC inhibition. A.** Schematic
847 representation of the chromosome of *C. difficile*. Higher than average GC skew ([G - C]/[G + C]) (red) and
848 lower than average GC skew (blue) were calculated with DNAplotter (72). Vertical lines indicate the
849 position of the predicted origin (*oriC*) and terminus (*terC*) of replication. Arrows indicate the direction of
850 replication. **B.** Sliding window analysis (bins of 51 loci, stepsize 1) of the median log fold change (FC)
851 projected on a linear genome map. The *oriC* of the circular chromosome is located on either size of the
852 linear graph (0/4.29Mb), whereas *terC* is indicated with a vertical red line. The trend in log(FC) is
853 highlighted using a green line. Light blue shading indicates the median absolute deviation of the mean
854 (23).

855

856 **Figure 5. Polymerase inhibitors lead to an increase in *oriC*:*terC* ratio.** A marker frequency analysis of
857 the effects of sub-inhibitory amounts of polymerase inhibitors (red; HPUra: 35 µg/mL; ACX-362E: 4
858 µg/mL) and three antibiotics with different modes of action (blue; metronidazole: 0.25 µg/mL;
859 fidaxomicin: 0.00125 ug/mL, surotomycin: 0.625 µg/mL) compared to non-treated cells (black).
860 Datapoints are averages of technical replicates (N=3). Black lines behind the datapoints indicate the
861 average of the biological replicates (N=3) and whiskers indicate the standard deviation of the mean.
862 Data have been normalized compared to the non-treated control. The mean of HPUra and ACX-362E
863 treated samples is statistically different from the other samples ($p<0.0001$).

864

865 **Figure 6. The *sigB* operon is transiently induced upon exposure to ACX-362E.** The putative promoter of
866 the *sigB* operon (41) was fused transcriptionally to a plasmid-based luciferase reporter (44). Luciferase
867 activity was measured regularly between 30 mins and 5.5h of growth in liquid medium in the presence
868 (+362E) or absence (no Ab) of polymerase inhibitor ACX-362E.

869

870 **Figure 7. Genes differentially expressed due to polymerase inhibitors are regulated by σ^B**. The putative
871 promoters of the indicated genes were fused transcriptionally to a plasmid-based luciferase reporter
872 (44). Luciferase activity was measured after 5 h of growth in liquid medium in the presence (+362E) or
873 absence (no Ab) of polymerase inhibitor ACX-362E. N.S. = non-significant, *=p<0.05, **=p<0.005,
874 ***=p<0.0005, ****=p<0.00005. **A**. P_{CD350}-*sLuc*^{opt}. **B**. P_{CD2963}-*sLuc*^{opt} **C**. P_{CD3614}-*sLuc*^{opt} **D**. P_{CD3412}-*sLuc*^{opt}.

875

876 **Supplemental information**

877

878 **Supplemental Table 1**. Characteristics and minimal inhibitory concentrations of the clinical isolates used
879 in the agar dilution experiments.

880 **Supplemental Table 2**. Lists of the genes that are differentially expressed in the presence of PolC-
881 inhibitors compared to non-treated cells. **A**. HPUra differentially expressed genes. **B**. ACX-362E
882 differentially expressed genes.

883 **Supplemental Table 3**. Overlap with published σ^B regulon. **A**. σ^B -dependent stress response genes
884 differentially regulated in the ACX-362E dataset. **B**. Genes containing a σ^B consensus promoter
885 differentially regulated in the ACX-362E dataset.

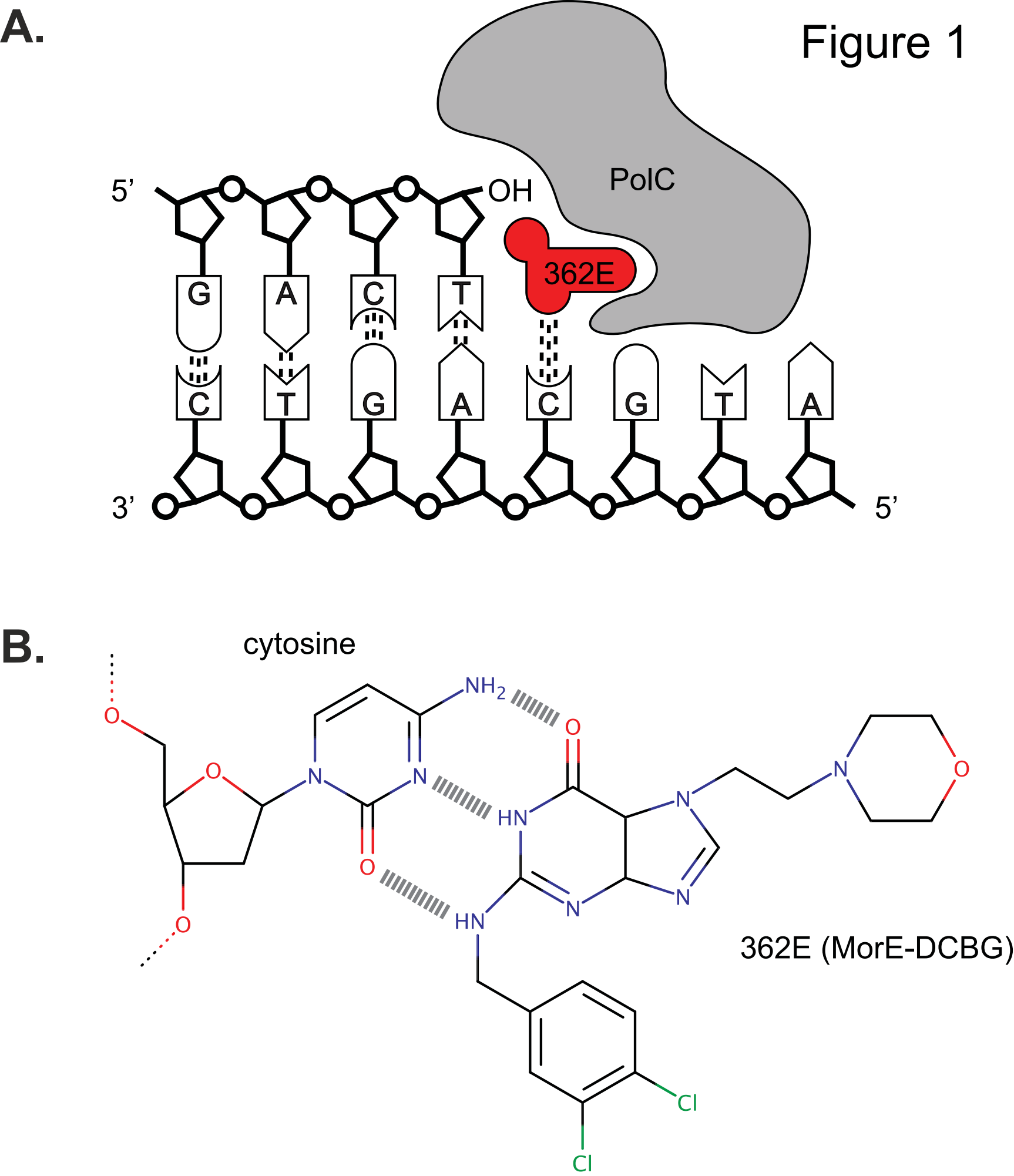
886 **Supplemental Figure 1**. Inhibition of growth by varying concentrations of PolC-inhibitors.

887 **Supplemental Figure 2**. Chloramphenicol does not result in a gene dosage shift.

888 **Supplemental Figure 3.** Confirmation of the *sigB* mutant strain (IB56).

889

890 Supplemental information is also available from Figshare (10.6084/m9.figshare.7117463).


A.
Figure 1
PolC
362E
5'
OH
G
A
C
T
C
T
G
A
C
G
T
A
3'
5'
B.
cytosine
NH2
O
N
HN
362E (MorE-DCBG)
Cl

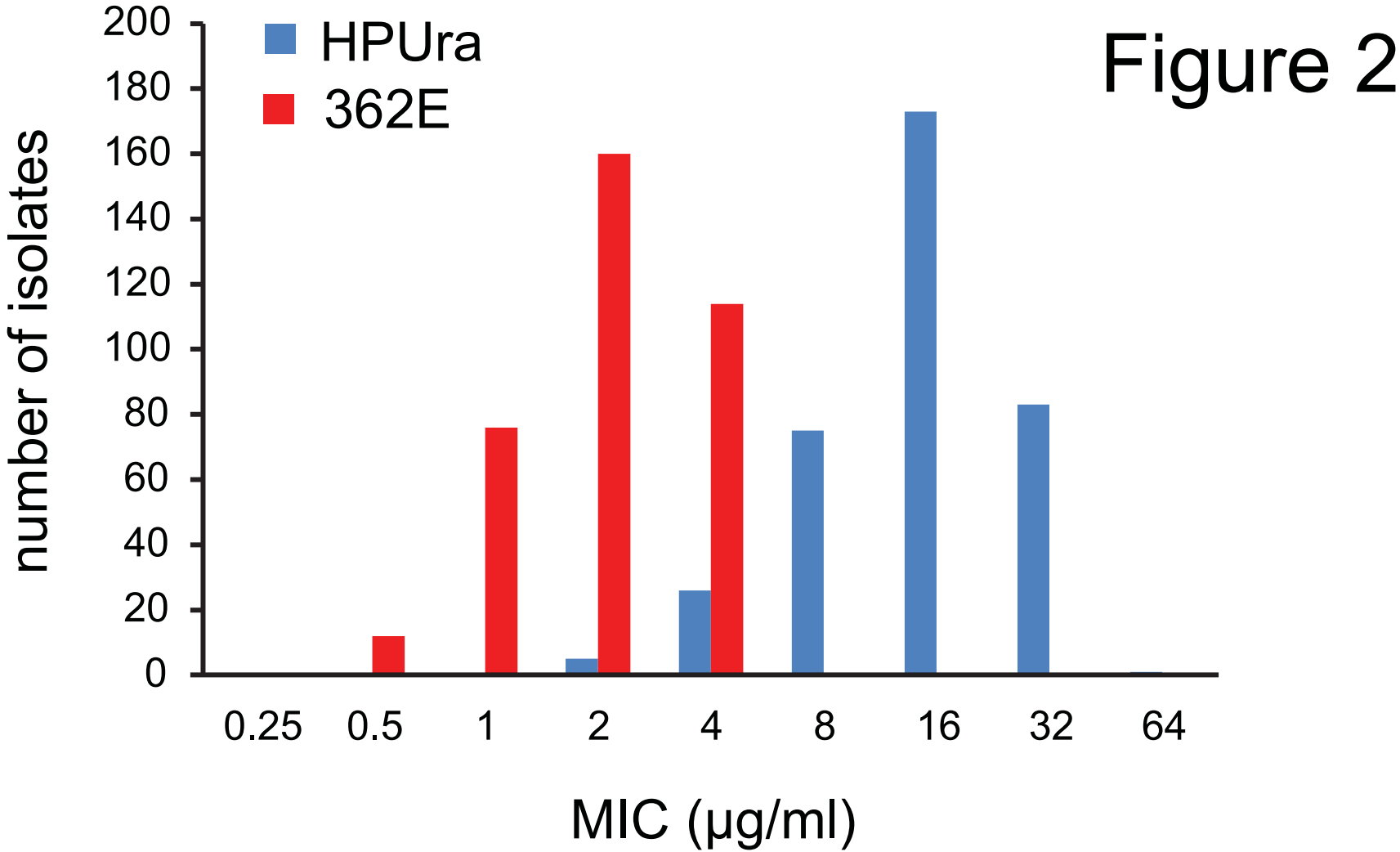


Figure 2
HPUra
362E
number of isolates
200
180
160
140
120
100
80
60
40
20
0
0.25
0.5
1
2
4
8
16
32
64
MIC (µg/ml)

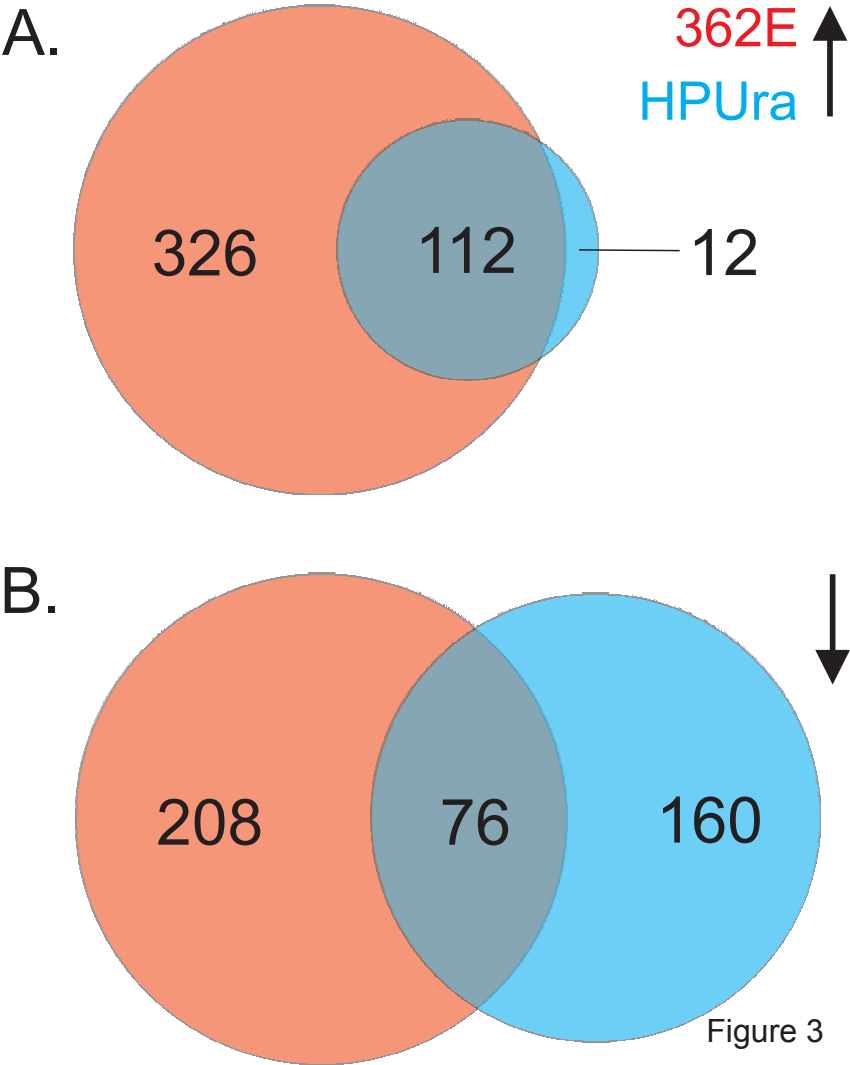


Figure 3

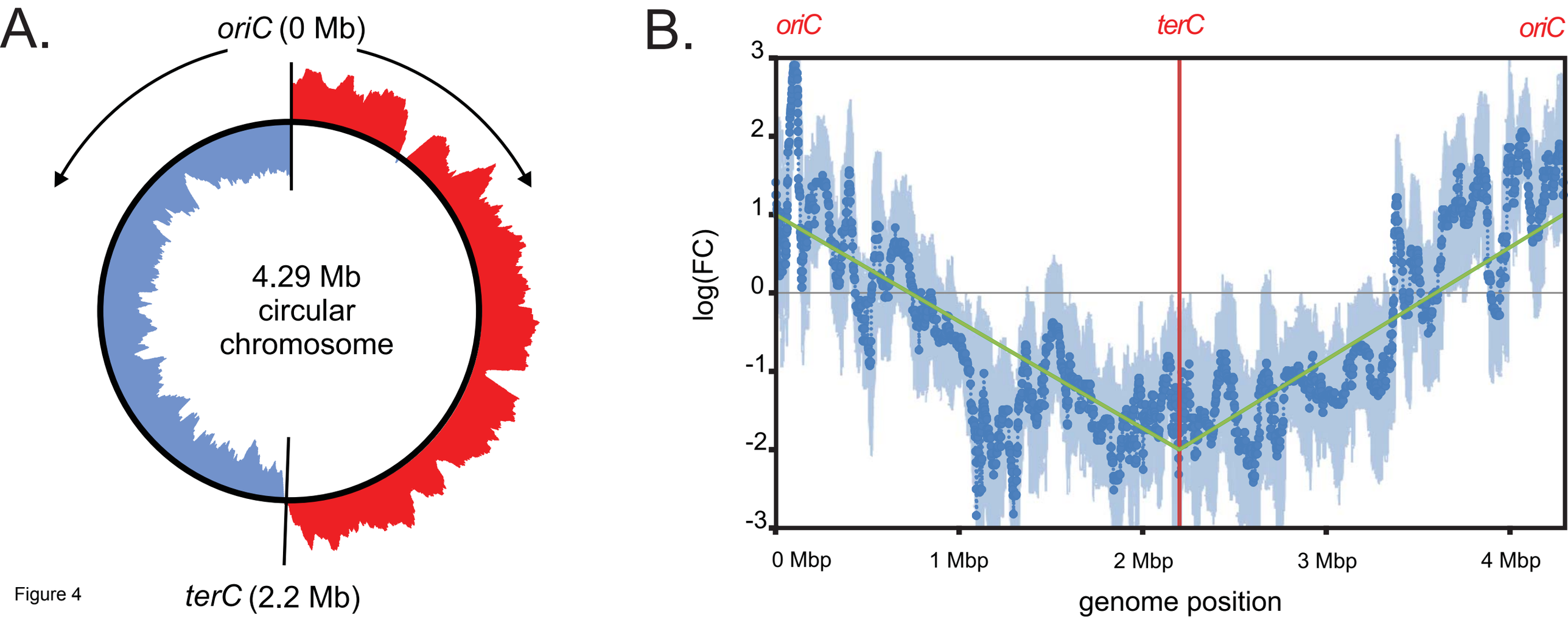

A.
oriC (0 Mb)
4.29 Mb
circular
chromosome
terC (2.2 Mb)
B.
oriC
terC
oriC
log(FC)
3
2
1
0
-1
-2
-3
0 Mbp
1 Mbp
2 Mbp
3 Mbp
4 Mbp
genome position

Figure 4

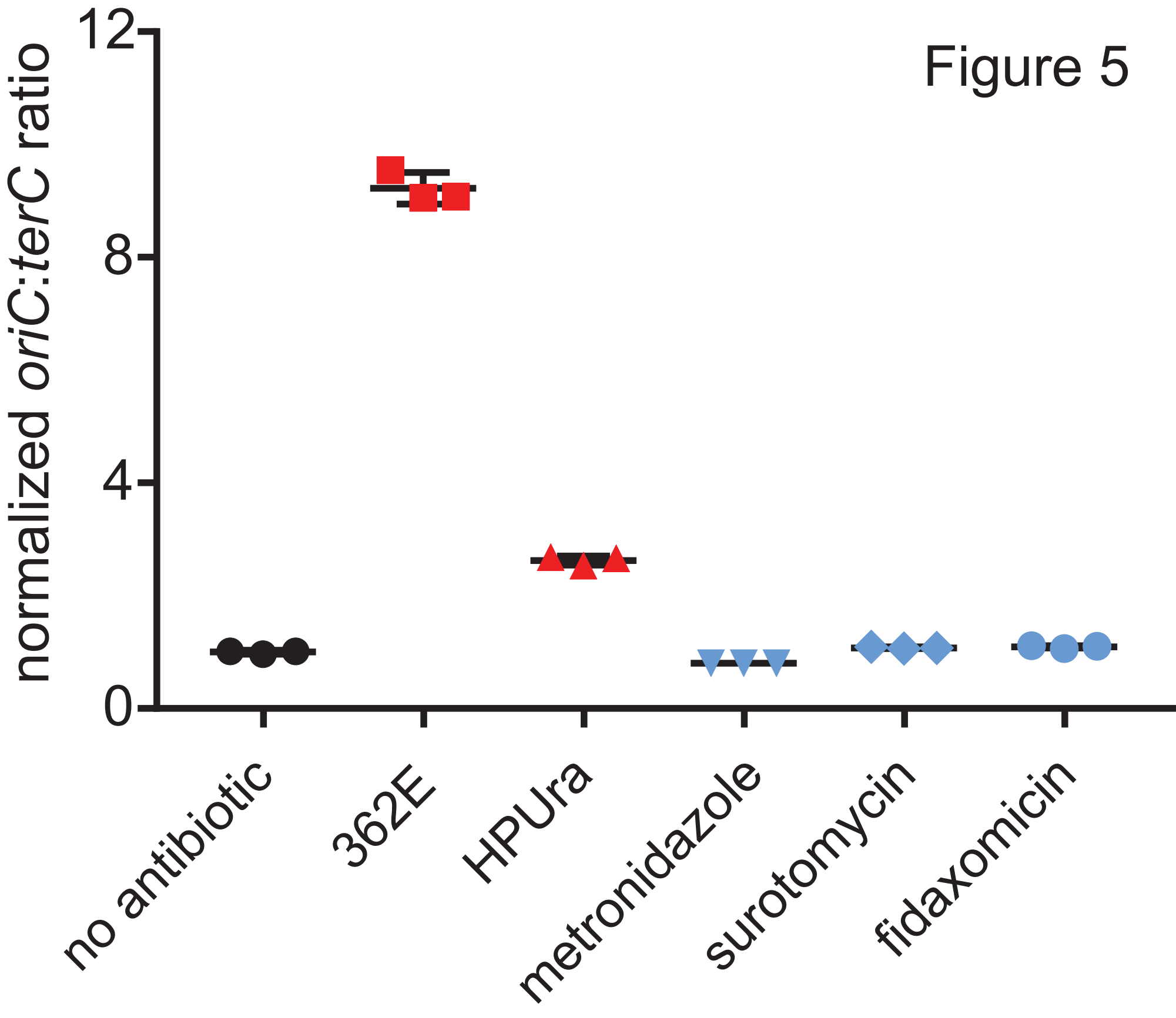


Figure 5
normalized oriC:terC ratio
12
8
4
0
no antibiotic
362E
HPUra
metronidazole
surotomycin
fidaxomicin

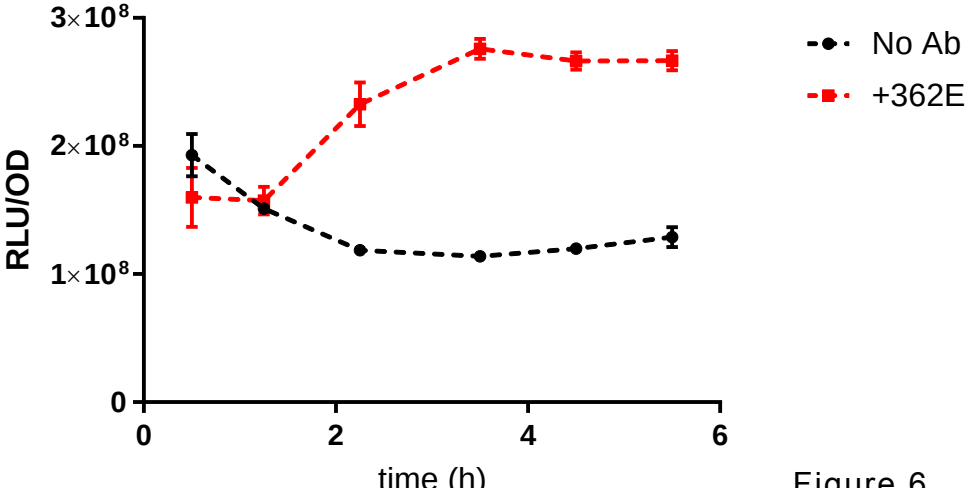
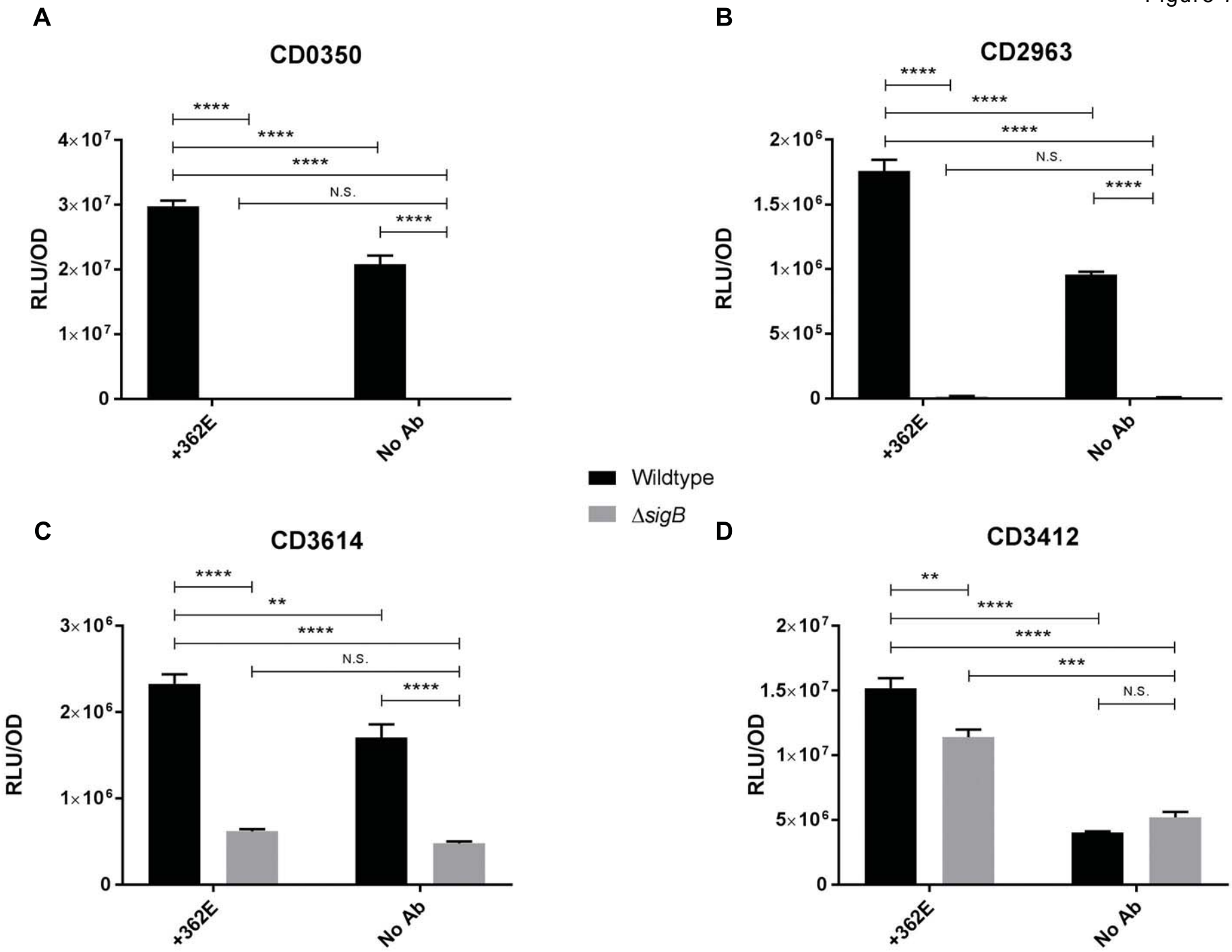


Figure 6

Figure 7


A
CD0350
RLU/OD
4×10^7
3×10^7
2×10^7
1×10^7
0
****
****
****
N.S.
****
+362E
No Ab
B
CD2963
RLU/OD
2×10^6
1.5×10^6
1×10^6
5×10^5
0
****
****
****
N.S.
****
+362E
No Ab
Wildtype
ΔsigB
C
CD3614
RLU/OD
3×10^6
2×10^6
1×10^6
0
****
**
****
N.S.
****
+362E
No Ab
D
CD3412
RLU/OD
2×10^7
1.5×10^7
1×10^7
5×10^6
0
**
****
****
***
N.S.
+362E
No Ab