Genome location dictates the transcriptional response to sub-inhibitory concentrations of PolC-inhibitors 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. ACX362E (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* Pol IIIC 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 ACX362E 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. ACX362E **(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). PolC-inhibitors can cause a stress response and cell death after
74 prolonged exposure. In *Bacillus subtilis*, this stress is characterized by a combination of DNA damage
75 (SOS) response, and an SOS-independent pathway dependent on the DNA replication initiator DnaA (21,
76 22). In *Streptococcus pneumoniae* cells, devoid of an SOS-response, competence for genetic
77 transformation is induced upon replication stress (23). The response of *C. difficile* to this particular class
78 of compounds is unknown.

79 In this study, we characterized aspects of the action of PolC-inhibitors towards *C. difficile*.
80 Minimal inhibitory concentrations for HPUra and ACX362E were determined using agar dilution for a
81 large collection of clinical isolates. Next, we investigated the effects of sub-inhibitory levels of PolC-
82 inhibitors on growth of *C. difficile* in liquid medium and performed RNAseq analyses to determine the
83 transcriptional response to PolC-inhibitors in our laboratory strain 630Δ*erm.* Finally, marker frequency
84 analysis and transcriptional reporters were used to provide a mechanistic explanation for the observed
85 up-regulation of origin-proximal genes under conditions of replication inhibition.

86

87 **Results**

88 *ACX362E is a potent inhibitor of diverse clinical isolates of C. difficile*

89 To date, reports on activity of PolC-inhibitors towards *C. difficile* are limited. For only 4 and 23 *C. difficile*
90 strains minimal inhibitory concentrations were published (19, 20), and no analysis was performed on
91 possible differences in efficacy between various phylogenetic groups (24, 25). Therefore, we assessed
92 the sensitivity of a diverse collection of *C. difficile* clinical isolates towards PolC-inhibitors and
93 determined if ACX362E was indeed superior to the general PolC–inhibitor HPUra.

94 HPUra and ACX362E were tested by the agar dilution method, according Clinical and Laboratory
95 Standards Institute (CLSI) guidelines for testing of antimicrobial susceptibility of anaerobes (26, 27),
96 against 364 *C. difficile* clinical isolates collected earlier in the framework of a pan-European study (6, 28).
97 We found that ACX362E (MIC_{50}: 2 µg/ml; MIC_{90}: 4 µg/ml) demonstrates lower inhibitory
98 concentrations than the general gram-positive PolC-inhibitors HPUra (MIC_{50}: 16 µg/ml; MIC_{90}: 32 µg/ml)
99 (**Figure 2, Supplemental Table 1),** consistent with previous *in vitro* activities against purified PolC (19).
100 We observed no significant difference in ACX362E susceptibility between clades **(Table 1)** and the
101 different PCR ribotypes demonstrated a similar distribution in MIC values (data not shown). No
102 resistance against either one of the PolC-inhibitors was among the clinical isolates tested (N=363),
103 suggesting that resistance against PolC-inhibitors is rare or absent in *C. difficile*. In contrast, the gram-
104 negative obligate anaerobe *Bacteroides fragilis* was resistant to both polymerase inhibitors under the
105 conditions tested (MIC >265 µg/ml), as expected for an organism lacking PolC. The gram-positive
106 bacterium *Staphylococcus aureus*, which was included as a control for the activity of HPUra against this
107 group of bacteria (16, 29), was highly sensitive to both HPUra and ACX362E, with MIC values of 2 µg/mL
108 and 1 µg/mL, respectively.
109 We conclude that ACX362E is highly active against diverse clinical isolates of *C. difficile* and
110 resistance is not a concern in currently circulating strains.
111
112 *Treatment with ACX362E leads to a pleiotropic transcriptional response*
113 In order to determine the transcriptional response of PolC-inhibitors, we established the optimal
114 concentration of sub-MIC levels of both inhibitors which affected growth of *C. difficile* in liquid medium.
115 The laboratory strain *C. difficile* 630*Δerm* (PCR ribotype 012, MLST Clade 1)(30, 31) was grown in
116 medium with varying amounts of HPUra (10-80 µg/mL) or ACX362E (0.25-8 µg/mL). We found that the
117 effect on growth was more prominent from 4 hours post inoculation (**Supplemental Figure 1**). The

118 growth kinetics of *C. difficile* under the influence of varying concentrations of HPUra was marginally
119 affected when using concentrations from 10-40 µg/ml, at approximately 80 percent of the non-treated
120 culture. Growth kinetics of cultures containing PolC-inhibitors ACX362E ≥ 1 µg/ml were similar and
121 resulted in about 30 percent reduced growth compared to the untreated culture. We note that
122 concentrations up to the MIC (as determined by agar dilution) did not lead to a complete growth arrest.
123 We found that growth was abolished at >2-fold MIC in liquid culture (data not shown). Such a difference
124 between MIC values from agar dilution and (micro)broth has been observed before (32).

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

130 *C. difficile* 630*Δerm* was grown for 5h in medium with HPUra (35 µg/mL) or ACX362E (4 µg/mL)
131 starting from an OD_{600} of 0.05 after which cells were harvested for RNA isolation. Purified RNA was
132 converted to cDNA and used for RNA-Seq as described in the Materials and Methods. For ACX362E, 722
133 genes were differentially expressed, of which 438 genes were up-regulated and 284 genes were down-
134 regulated. The number of differentially expressed genes in HPUra treated samples was approximately 2-
135 fold lower: 360, of which 124 genes were up-regulated and 236 genes were down-regulated. The full list
136 of differentially regulated genes is available as **Supplementary Table 2** and the top 10 of up- and down-
137 regulated genes are shown in **Table 2** (HPura) and **Table 3** (ACX362E). Here, we highlight three aspects
138 of the results.

139 First, we performed a Gene Set Enrichment Analysis (GSEA)(33) via the Genome2D web server
140 (34) using the locus tags of the differentially regulated genes (**Supplementary Table 1**) as input. Among
141 the genes up-regulated by ACX362E, there is a strong overrepresentation of those involved in

142 translation, ribosome structure and ribosome biogenesis. Not unexpectedly, also replication,
143 recombination and repair processes are affected. This suggests that genes from these pathways show a
144 coordinated response in the presence of ACX362E. Among the genes down-regulated in the presence of
145 ACX362E, the levels of significance for specific processes are generally much lower, suggesting that there
146 is a more heterogeneous response among genes from the same pathway. Nevertheless, metabolic
147 pathways (especially carbon metabolism and coenzyme A transfer) and tellurite resistance were found
148 to be significantly affected. Strikingly, a GSEA analysis on lists of genes that are differentially expressed
149 in the presence of HPUra revealed similar processes to be affected.

150 The findings from the GSEA analysis prompted us to evaluate the overlap in the lists of
151 differentially regulated genes between the ACX362E and HPUra datasets in more detail. If both
152 compounds act via a similar mechanism, we expect a conserved response. Indeed, we observe that
153 >90% of the genes that are up-regulated in the presence of HPUra compared to the non-treated
154 condition, are also identified as up-regulated in the presence of ACX362E (**Figure 3A**). Though the
155 overlap is not as strong for the down-regulated genes, we find that >30% of the genes affected by HPUra
156 are also identified as affected by ACX362E (**Figure 3B**). Notably, the directionality of the response is
157 conserved, as no genes were found to be up-regulated in one but down-regulated in the other
158 condition. Based on these observations, we believe that the differentially expressed genes identified in
159 this study are representative for a typical response to inhibition of PolC in *C. difficile*.

160 Finally, we related the changes in transcription to genome location. *C. difficile* has a single
161 circular chromosome and one origin of replication (*oriC*) from which the process of DNA replication
162 occurs bi-directionally towards the terminus (*terC*) (**Figure 4A**). Though neither *oriC* nor *terC* has been
163 definitively defined for *C. difficile*, it is assumed that *oriC* is located at or near *dnaA* (CD0001;
164 CD630DERM_RS00005). The terminus region is generally located at the inflection point of a GC skew ([G
165 - C]/[G + C]) plot. Such a plot places the *terC* region around 2.2Mb from CD0001, near the CD1931

166 (CD630DERM_RS10465) open reading frame (**Figure 4A**) (35). We noted that the differential expression
167 appeared to correlate with genome location (**Table 2, Table 3** and **Supplemental Table 2**): many of the
168 up-regulated genes have either low or high gene identifiers (CD numbers) indicative of an origin
169 proximal location and, conversely, many of the down-regulated genes appear to be located away from
170 *oriC*. Though this correlation is not absolute, we observed a clear trend when plotting the mean fold-
171 change against genome location for all genes (**Figure 4B**).

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

174

175 *Gene dosage shift occurs at sub-inhibitory concentration of ACX362E PolC-inhibitor*

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

182 We designed qPCR probes against the CD0001 and CD1931 regions, representing *oriC* and *terC*,
183 respectively (31, 35). Using these probes, we could show that *C. difficile* demonstrates multi-fork
184 replication in exponential growth phase and that the MFA assay detects the expected decrease in
185 *oriC*:*terC* ratio when cells enter stationary growth phase (data not shown). Next, we analyzed the effects
186 of PolC-inhibitors on the *oriC*:*terC* ratio. When cells were treated with HPUra (35 µg/mL), the MFA
187 showed a modest increase in *oriC:terC* ratio of 2,6-fold compared to untreated cells. However, when
188 cells were treated with ACX362E (4 µg/ml), the MFA showed a >8-fold increase in the *oriC*:*terC* ratio
189 compared to untreated cells. In contrast, such an increase was not observed for cells treated with

190 metronidazole (0.25 ug/mL; a DNA damaging agent), fidaxomicin (0.00125 ug/mL; an RNA polymerase
191 inhibitor) or surotomycin (0.625 ug/mL; a cell-wall synthesis inhibitor) (**Figure 5**) or chloramphenicol (2
192 µg/mL; a protein synthesis inhibitor)(data not shown).

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

195

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

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

202 First, we compared the list of differentially regulated genes from our study (**Supplemental Table**
203 **2**) to those under the control of σ^B (41). In contrast to most anaerobic gram-positive organisms, *C.*
204 *difficile* encodes a homolog of the general stress response sigma factor, σ^B (40, 42). A transcriptome
205 analysis comparing *sigB* mutant versus wild type cells was recently published (41). Strikingly, we find
206 ~40% of the genes (21/58) identified as involved in stress response under the control of σ^B to be
207 differentially expressed in our ACX362E dataset (**Supplemental Table 3**). Similarly, we observed that
208 7/20 (~35%) of the genes containing a transcriptional start site with a σ^B consensus sequence are
209 differentially expressed in our ACX362E dataset (**Supplemental Table 3**). These data suggest that the
210 response to DNA replication inhibition is at least partially dependent on σ^B.

211 Next, a *sigB* mutant was constructed using Allele-Coupled Exchange (43) as described in the
212 Materials and Methods. The chromosomal deletion of *sigB* and absence of σ^B protein was verified by
213 PCR and Western Blot, respectively (**Supplemental Figure 2**). To directly demonstrate a role for *sigB* in

214 the regulation of genes with altered transcription upon PolC-inhibition, we fused the predicted
215 promoter regions of selected genes to a secreted luciferase reporter (44) and evaluated luminescence in
216 wild type and *sigB* mutant backgrounds, after 5-hour growth in the presence and absence of 4 µg/mL
217 ACX362E. All genes tested demonstrated a significant increase in promoter activity in a wild type
218 background in the presence of ACX362E compared to the non-treated control, validating the results
219 from the RNA-Seq analysis (**Figure 6**). Three distinct patterns were observed. For CD0350 (encoding a
220 hypothetical protein) and CD3963 (encoding a putative peptidoglycan-binding exported protein) there
221 was virtually no expression in a *sigB* mutant background (**Figure 6A** and **B**). We conclude that these
222 genes are strictly dependent on σ^B for their expression under the conditions tested. CD3614 (encoding a
223 hypothetical protein) shows a basal level of expression, but no significant increase in transcription levels
224 in the presence of ACX362E in a *sigB* mutant background compared to the non-treated control (**Figure**
225 **6C**). This suggests that the transcriptional up-regulation under these conditions is σ^B dependent, and
226 indicates that the basal level of transcription observed is likely independent of σ^B. Finally, CD3412 (*uvrB*,
227 encoding a subunit of an excinuclease) shows reduced transcriptional up-regulation in ACX362E treated
228 cells compared to non-treated controls (**Figure 6D**). Thus, the transcription of this particular gene under
229 conditions of ACX362E exposure is brought about by both a σ^B-dependent and a σ^B-independent
230 regulatory pathway.

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

233 **Discussion**

234

235 *Resistance to PolC-inhibitors and specificity of ACX362E*

236 Limited treatment options and reports of reduced susceptibility to current treatment (11, 12, 45)
237 emphasize the necessity for the development of novel antimicrobials. More broadly, reducing the
238 widespread and prolonged use of vancomycin, first-line therapy of severe CDI (9, 10), may also
239 contribute in minimizing the risk of inducing and shedding of vancomycin-resistant enterococci and
240 staphylococci (19, 20). As CDI can be induced by use of broad spectrum antibiotics (7), new
241 antimicrobials ideally should only target *C. difficile*, thereby maintaining integrity of the colonic
242 microbiota. In this study, we have tested inhibitors HPUra and ACX362E which specifically target the
243 PolC enzyme, essential for DNA replication. The majority of PolC-inhibitors target gram-positive bacteria
244 with low G+C content, but ACX362E has been reported to demonstrate increased specificity towards *C.*
245 *difficile* PolC *in vitro* and showed promising results for the efficacy *in vivo* based on a limited set of *C.*
246 *difficile* strains (19, 20). In our study, we have tested HPUra and ACX362E against a large collection of
247 clinical isolates consisting of many PCR ribotypes. We have established that ACX362E demonstrated
248 lower inhibitory concentrations than the general gram-positive PolC-inhibitor HPUra. The MIC_{50} and
249 MIC_{90} of ACX362E are similar to those of antimicrobials commonly used to treat *C. difficile* infection
250 (metronidazole: MIC_{50} = 2 ug/mL and MIC_{90} = 4 ug/mL; vancomycin MIC_{50} = 2 ug/mL and MIC_{90} = 4 ug/mL
251 (20); fidaxomicin = MIC_{50} = 0.125 ug/mL and MIC_{90} = 0.5 ug/mL (46)). We did not detect a significant
252 difference in MICs between clades and ribotypes, demonstrating that PolC-inhibitors have the potential
253 to be used as treatment for the majority - if not all - circulating *C. difficile* strains. This includes the
254 epidemic types of PCR ribotype 027 and 078 (8, 47). These results are in line with other work that
255 demonstrated only 2- to 4-fold differences in antimicrobial susceptibility between different clades for
256 metronidazole, fidaxomicin and semi-synthetic thiopeptide antibiotic LFF571 (28). Importantly, we did

257 not find any strains resistant to either HPUra or ACX362E. PolC-inhibitors are competitive inhibitors of
258 polymerase activity by binding in the active site. Mutations that abolish binding of HPUra or ACX362E
259 are likely to affect the essential enzymatic activity of the polymerase and for that reason unlikely to
260 occur *in vivo*. A single mutation (*azp-12*) has been described in *B. subtilis* that confers resistance to
261 HPUra (48). This T>G transversion results in the replacement of a serine with an alanine in the highly
262 conserved PFAM07733 domain of the polymerase (49). To our knowledge, it is unknown whether this
263 mutation prevents binding of HPUra to PolC of *B. subtilis*. Few other mutations have been described that
264 confer resistance against other PolC-inhibitors (50, 51). It will be of interest to see if similar mutations in
265 *C. difficile* result in resistance to HPUra and/or ACX362E and what the effect on binding of these
266 compounds to *C. difficile* PolC is.

267 In our experiments, we included *S. aureus* as a control for the activity of HPUra against gram-
268 positive bacteria. Surprisingly, we found that *S. aureus* was highly sensitive to both HPUra and ACX362E
269 and even more so than *C. difficile*. The reasons for this sensitivity are unknown. If ACX362E targets DNA
270 replication in *S. aureus*, we would also expect to find an increase in *oriC*:*terC* ratio upon ACX362E
271 exposure in this organism. It is conceivable that ACX362E also targets PolC of *S. aureus* and if so, this
272 information could be used to further characterize the interaction of ACX362E with PolC. Alternatively,
273 ACX362E may also affect the activity of the other PolIII-type polymerase, DnaE, in *S. aureus*. PolIII-
274 inhibitors can affect PolC, DnaE or both (51), though *in vivo* activity appears to correlate with PolC-
275 inhibition. Both C*. difficile* and *S. aureus* possess PolC and DnaE polymerase, but the DnaE enzymes are
276 of different families (DnaE1 in *C. difficile* and DnaE3 in *S. aureus*). The phylogenetic split coincides with
277 the taxonomic division as DnaE3-polymerases are found in the class Bacilli (which includes *S. aureus*),
278 whereas the DnaE1 polymerase are present in the classes Clostridia and Negativicutes (52). To test
279 whether a difference in activity of ACX362E towards different DnaE-type polymerases explain the
280 increased sensitivity of *S. aureus* compared to *C. difficile*, the activity of ACX362E towards purified DnaE

281 and PolC from both organisms should be determined. Finally, ACX362 may have off-target effects in *S.*
282 *aureus* unrelated to its replication-inhibitory activity.

283 Though it is clear that ACX362E inhibits *C. difficile* efficiently and shows limited activity towards
284 certain other anaerobes (19), these findings highlight the necessity to perform additional (microbiome)
285 studies to more clearly define the antimicrobial spectrum of this compound. It also shows that ACX362E
286 may have therapeutic potential outside treatment of CDI.

287

288 *Regulators of the transcriptional response to PolC-inhibitors*

289 The present study is the first to describe the transcriptional response of *C. difficile* to inhibition of DNA
290 replication. We find that ~200 genes show differential expression under conditions of PolC-inhibition by
291 both HPUra and ACX362E, when compared to non-treated cells (**Supplemental Table 2, Figure 3**). When
292 considering only ACX362E, approximately 13% of all genes in the genome show statistically significant
293 altered transcription. We demonstrate that this large reprogramming of transcription is likely to be
294 caused directly by a gene dosage shift (**Figure 4 and 5**).

295 In addition to direct effects, it is conceivable that at least part of the transcriptional response is
296 indirect. Our list of differentially regulated genes includes several putative regulators; sigma factors
297 (including *sigE*, *sigG* and *sigH*), transcription factors and anti-terminators. The relatively long time (5h) at
298 sub-MIC levels of antimicrobials may have contributed to secondary effects, through one or more of
299 these regulators. Though shorter induction times are thought to provoke more compound-specific
300 responses (53), we did observed a highly consistent transcriptional signature with both HPura and
301 ACX362E (**Supplemental Table 2**, **Figure 3**).

302 Major stress response pathways are poorly characterized in *C. difficile*. On the basis of
303 experiments in other organisms (21-23, 54-56), we expect that inhibition of DNA replication inhibition
304 might possibly induce an SOS response (LexA)(57), a DnaA-dependent transcriptional response (21),a

305 heat shock response (HrcA/CtsR)(58) and/or a general stress response (σ^B)(42). Of these, the best
306 characterized stress response pathway in *C. difficile* is the one governed by σ^B (41). We noted a
307 significant overlap in putatively σ^B-dependent genes and those differentially expressed upon exposure
308 to PolC-inhibitors (**Supplemental Table 2**). In addition, our luciferase reporter fusions directly implicate
309 *sigB* in the expression and/or up-regulation of some of these (**Figure 6**). It should be noted that the *sigB*-
310 operon itself was not differentially expressed. This phenomenon has been observed before, and is
311 attributed to a transient up-regulation of *sigB* followed by a persistent response of σ^B-dependent gene
312 expression (58-60). In our experiment, cells were grown for 5h at sub-MIC levels of inhibitors, making it
313 unlikely that altered *sigB* transcription would still be detected. Though we do not favor this hypothesis,
314 σ^B activity might alternatively be regulated post-translationally.

315 To date, no genes have been identified that are regulated by DnaA in *C. difficile* and direct
316 regulation of genes through the other stress response pathways has not been demonstrated. Putative
317 LexA-regulated genes of *C. difficile* were identified *in silico* (61) and some of these (such as the *uvr*
318 excinuclease and 30S ribosomal protein S3) were differentially expressed in our dataset (**Supplemental**
319 **Table 2**). Indeed, we find that only part of the transcriptional up-regulation of *uvrB* as a result of
320 ACX362E exposure could be explained by *sigB* (**Figure 6D**), and LexA-dependent regulation might play a
321 role. Pleiotropic phenotypes have been described for a *C. difficile lexA* mutant (62) and it is likely that
322 other LexA targets than those identified *in silico* exist. No mutants of *hrcA* or *ctsR* have been described
323 for *C. difficile*, but transcriptome and proteome analyses have been performed with heat shocked cells
324 (42°C (60), or 41°C (59, 63, 64)). Similarities between these datasets and ours include genes encoding
325 proline racemase (*prdF*), chaperones (*groEL*, *groES*), thioredoxin systems (*trxA*, *trxB*) and Clp-proteases
326 (*clpC*, *clpP*).

327 Many parameters (such as the medium used, cell density, concentration of antibiotics, and
328 protocol used to arrest transcription between cell harvest and lysis) can influence overall transcription

329 signatures, and can also govern an incomplete overlap between our data and the stress regulons
330 determined by others (53).
331
332 *Genome location contributes to the transcriptional response to PolC-inhibition*
333 Our analysis of differential regulation in relation to genome location revealed a striking pattern of
334 relative up-regulation for *oriC*-proximal genes, and down-regulation for *terC*-proximal genes under
335 conditions of PolC inhibition (**Figure 4**). Antimicrobials directed at DNA replication in bacteria have a
336 profound negative effect on the processivity of replication forks, though initiation of DNA replication is
337 not or only marginally affected (23, 39). As a consequence, the presence of multiple replication forks
338 simultaneously increases the copy numbers of genes located in close proximity of the origin of
339 replication and such a gene dosage differences can result in functionally relevant transcriptional
340 differences, either directly or indirectly (15). We found an increase of *oriC:terC* ratio when performing
341 MFA on chromosomal DNA of cells subjected to a sub-inhibitory concentration of ACX362E (and HPUra,
342 albeit less pronounced) (**Figure 5**), consistent with findings in other organisms (23).
343 An example of direct regulation by gene dosage can be found in *Vibrio*, for instance, where the
344 location of ribosomal protein clusters close to the origin is crucial for fast growth, because increased
345 copy number under condition of multi-fork replication allows for higher expression levels (65). We note
346 that ribosomal gene clusters are up-regulated when DNA replication is inhibited in our experiments
347 (**Table 2**, **Table 3**, **Supplemental Table 2**), suggesting that a similar mechanism may be active in *C.*
348 *difficile*.
349 An example of indirect regulation as a result of gene dosage is the induction of competence
350 genes in *S. pneumoniae* (23). Competence is believed to be a stress response in this organism, that lacks
351 a canonical (σ^B-dependent) stress response pathway. Key regulatory genes for competence
352 development are located close to the origin, and replication inhibition therefore leads to the induction

353 of origin distal competence genes (23). In our experiments, the large overlap with the proposed σ^B
354 regulon (41), the origin proximal location of the *sigB* operon (8.5kb-10kb)(40) and the σ^B-dependence of
355 selected differentially expressed genes suggests that at least part of the transcriptional response to
356 PolC-inhibition can be explained by an indirect gene dosage effect. The positioning of stress-response
357 regulators close to *oriC* may therefore be a conserved strategy in bacteria to respond to DNA replication
358 insults that is independent of the nature of the regulator.

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

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

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

376

377 **Materials and Methods**

378

379 *Bacterial strains and culture conditions*

380 Plasmids and bacterial strains used in this study can be found in **Table 4**. Note that this table only
381 contains laboratory strains; the clinical isolates used for the agar dilution experiments (see below) are
382 listed in **Supplemental Table 1**. *E. coli* was cultured aerobically at 37°C (shaking at 200 rpm) in Luria-
383 Bertani (LB) broth supplemented with 20 µg/ml chloramphenicol and 50 µg/ml kanamycin when
384 appropriate. *C. difficile* strains were cultured in Brain Heart Infusion (BHI) broth (Oxoid) supplemented
385 with 0,5% yeast extract (Sigma-Aldrich), *Clostridium difficile* Selective Supplement (CDSS, Oxoid) and
386 20µg/ml thiamphenicol when appropriate. *C. difficile* was grown anaerobically in a Don Whitley VA-1000
387 workstation in an atmosphere of 10% CO_2, 10% H_2 and 80% N_2. Liquid cultures were grown under gentle
388 agitation (120 rpm).

389

390 *Agar dilution*

391 HPUra and ACX362E were tested against a collection of *C. difficile* clinical isolates. 375 clinical isolates
392 have been collected during the ECDIS study (6). All strains were characterized by PCR ribotyping (67) and
393 by PCR to confirm the presence of genes encoding toxins A , B and binary toxin (68-70). Of the 375
394 clinical isolates, we excluded stocks that were found to contain more than one strain and isolates that
395 could not be recultured. Testing was therefore performed on 363 isolates (**Supplemental Table 1**). *C.*
396 *difficile* ATCC 700057, *B. fragilis* ATCC 25285 and *S. aureus* ATCC 29213 were used as controls (71).

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

401 0.5 McFarland in phosphate buffered saline (PBS). The strains were inoculated onto BBA solid media
402 containing the PolC-inhibitors using multipoint inoculators to a final concentration of 10^4 CFU per spot.
403 Each series of antimicrobial agents was tested from the lowest concentration to the highest
404 concentration. Two control plates without antibiotics were inoculated to control for aerobic
405 contamination and purity of anaerobic growth. At the end of the final series, two control plates were
406 inoculated to verify the final organism viability and purity. Plates were incubated anaerobically in a Don
407 Whitley VA-1000 workstation in an atmosphere of 10% CO_2, 10% H_2 and 80% N_2 and the MICs were
408 determined after 24 and 48 hours.
409
410 *Sub-MIC determination*
411 *C. difficile* 630Δ*erm* (30, 31) was grown in 20 mL Brain Heart Infusion (Oxoid) supplemented with 0.5%
412 yeast extract (Sigma-Aldrich) (BHI/YE) starting from an optical density measured at 600nm (OD_{600}) of
413 0.05 using an exponentially growing starter culture (3 biological replicates per concentration). To
414 determine the effects on growth at sub-inhibitory concentrations of ACX362E, cells were cultured in the
415 presence of the following concentrations; 0.25, 0.5, 1, 2, 4, and 8 µg/mL ACX362E and compared to an
416 untreated culture. To determine the effects on growth at sub-inhibitory concentrations of HPUra, cells
417 were cultured in the presence of the following concentrations: 10, 20, 40, 80 µg/mL HPUra and
418 compared to an untreated culture. The OD_{600} was monitored every hour until stationary phase was
419 reached.
420
421 *Marker Frequency analysis*
422 *C. difficile* 630Δ*erm* (30, 31) was grown in 20 mL BHI supplemented with 0.5% yeast extract with sub-
423 MIC amounts of antimicrobials (HPUra: 35 µg/mL; ACX362E: 4 µg/mL; metronidazole: 0.25 µg/mL;
424 fidaxomicin: 0.00125 µg/mL, surotomycin: 0.625 µg/mL), starting from an OD_{600} of 0.05 using an

425 exponentially growing starter culture. We confirmed that these concentrations did not lead to a >30%
426 reduction in growth compared to non-treated cultures (**Supplemental Figure 1** and data not shown). In
427 parallel, cultures were grown without inhibitors from the same starter culture. All conditions were
428 performed in biological triplicates. After 5 hours, 1mL cells was harvested (OD_{600} ~ 0.5), and stored at -
429 20°C. Isolation of chromosomal DNA was performed the next day with the QIAamp DNA Blood Mini kit
430 (Qiagen) according to the instructions of the manufacturer. Marker frequency analysis (MFA) was
431 performed to assess the relative abundance of origin proximal genes relative to terminus proximal
432 genes. As a proxy for *oriC*, a probe was designed that targets the CD0001 region (CD0001-probe-FAM).
433 By using plots of the GC skew ([G - C]/[G + C]) generated using DNAPlotter (72), the approximate
434 location of the terminus region for the *C. difficile* chromosome was determined and a probe targeting
435 this region (CD1931) was designed (CD-1931-probe-TXR). Probe design was performed with Beacon
436 Designer™ (Premier Biosoft, Palo Alto CA, USA). Real-time PCR reactions were performed on a Biorad
437 CFX96™ real-time PCR detection system (95°C 15 m, 39 × (94°C 30 s + 55°C 30 s + 72°C 30 s). Sequences
438 for primers and probes are listed in **Table 5**. For each biological replicate, three technical replicates were
439 performed. Amplification efficiency was determined using standard curves obtained from DNA late
440 stationary phase cells of strain 630Δ*erm*, for which an *oriC*:*terC* ratio of 1 was assumed. RT-PCR results
441 from antibiotic treated cells were normalized to the *oriC*:*terC* ratio of DNA samples (3 biological
442 replicates) from non-treated cells. Calculations were performed in Microsoft Office Excel 2010, plotted
443 using Prism 7 (GraphPad) and prepared for publication in Corel Draw Suite X8. Significance was
444 determined using a One-way ANOVA and a Tukey's test for multiple comparisons (GraphPad).
445
446 *Growth and RNA isolation for RNA-Seq*
447 For RNA-Seq analysis, *C. difficile* 630*Δerm* (30, 31) was grown for 5h in BHI medium with HPUra (35
448 ug/mL) or ACX362E (4 µg/mL) starting from an OD_{600} of 0.05 using an exponentially growing starter

449 culture, after which cells (3mL) were harvested for RNA isolation. RNA isolation was performed with
450 NucleoSpin© RNA kit (Macherey-Nagel). Although the kit includes on column rDNAse digestion, a
451 second treatment was performed in solution and RNA was precipitated and recovered by NaAc
452 precipitation to remove residual DNA. Concentration determination and quality control (16S/23S ratio
453 and RNA integrity number [RIN]) was performed with a fragment analyzer (Agilent bio-analyzer),
454 according to the instructions of the manufacturer. Samples with a RIN>9 and 16S/23S ratio >1.4 were
455 submitted for analysis by RNA-Seq.
456
457 *RNA-Seq*
458 RNA-Seq was performed at a commercial provider (GenomeScan, Leiden, The Netherlands). In short,
459 the NEBNext Ultra Directional RNA Library Prep Kit for Illumina was used to process the samples. Sample
460 preparation was performed according to the protocol "NEBNext Ultra Directional RNA Library Prep Kit
461 for Illumina" (NEB #E7420S/L). Briefly, after selective removal of rRNA (Ribo-Zero rRNA Removal Kit for
462 gram-positive Bacteria) and fragmentation of the mRNA, cDNA synthesis was performed. cDNA was
463 ligated to the sequencing adapters and the resulting product was PCR amplified. Clustering and DNA
464 sequencing using the Illumina NextSeq 500 platform was performed according to manufacturer's
465 protocols. A concentration of 1.5 pM of DNA was used. Image analysis, base calling, and quality check
466 was performed with the Illumina data analysis pipeline RTA vl.18.64 and Bcl2fastq v2.17. Per sample,
467 four technical replicates were included in the RNA-Seq experiment. In case of insufficient reads, the
468 sample was re-run on another flow cell to reach satisfactory quantities (≥ 20 M).
469
470 *Analysis of RNA-Seq data*
471 Analysis of the data was performed using T-REx, a user-friendly webserver which has been optimized for
472 the analysis of prokaryotic RNAseq-derived expression data (73). The pipeline requires raw RNA

473 expression level data as an input for RNA-Seq data analysis. For data normalisation and determination of
474 the genes, the factorial design statistical method of the RNA-Seq analysis R-package EdgeR is
475 implemented in the T-Rex pipeline. Some samples displayed incomplete rRNA depletion and rRNA
476 mapping reads had to be removed manually prior to analysis.

477 To analyse the genome-wide pattern in differential gene expression a sliding window analysis
478 was performed essentially as described (23). In short, genome locations (start of the locus tag) were
479 coupled to the locus tags in the T-Rex output. Next, the median log(FC) was calculated for bins of 51 loci
480 with a stepsize of 1. For each bin of [X_1, X_2…X_{51}] the median absolute deviation of the median
481 (MAD=median(|X_i-median(X)|) was calculated as an robust indication of the distribution around
482 calculated median values. Calculations were performed and three curves (median, median-MAD and
483 median+MAD) were plotted in Microsoft Office Excel 2010 and the graph was prepared for publication
484 using Adobe Photoshop CC and Corel Draw Suite X8.

485 A GSEA analysis (33) was performed via the Genome2D webserver (73), using our reference
486 genome sequence for *C. difficile* 630Δ*erm*, Genbank identifier LN614756.1 (listed in Genome2D as
487 "Clostridioides_difficile_630Derm")(31). As input a single list of locus tags was used of either up- or
488 down regulated genes. The output was copied to Microsoft Excel 2010. The single_list column was split,
489 and a column was inserted to calculate the significance of the overrepresentation using the formula "(#
490 hits in list/ClassSize)*-log(p-value; 2)" to allow for sorting of the output of the GSEA analysis by
491 significance.

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

494

495 *General molecular biological techniques*

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

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

505

506 *Construction of luciferase-reporter fusion plasmids*

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

510 The P_{CD3412} luciferase reporter plasmid was created by restriction-ligation using the restriction
511 enzymes *Kpn*I and *Sac*I. P_{CD3412} was amplified using primers oIB-26 and oIB-27 (**Table 5**). The resulting
512 dsDNA fragment was digested and ligated into KpnI-SacI digested pAP24 (44), yielding plasmid pIB27.
513 Plamids pIB68 (P_{CD0350}), pIB69 (P_{CD2963}) and pIB74 (P_{CD3614}) were constructed using a Gibson assembly (77).
514 The plasmid backbone of pAP24 was linearized by PCR using primers oWKS-1580/oWKS-1582, and the
515 predicted promoter areas of CD0350, CD2963 and CD3614 were amplified with primers oIB-80/oIB-94,
516 oIB-82/oIB-95, and oIB-92/oIB-100 respectively. Primers were designed using the NEBuilder assembly
517 tool v.1.12.17 (New England Biolabs) using a 30 bp overlap. For the assembly, 100 ng of vector DNA was
518 assembled to a five-fold molar excess of the PCR fragment of the desired promoter using a homemade
519 Gibson Assembly Master Mix at 50°C for 30 minutes (final concentration: 4 U/µl Taq Ligase (Westburg),

520 0.004 U/µl T5 exonuclease (New England Biolabs), 0.025 U/µl Phusion polymerase (Bioké), 5%
521 polyethyleneglycol (PEG-8000),10 mM $MgCl_2$, 100 mM Tris-Cl pH=7.5, 10 mM dithiothreitol, 0.2 mM
522 dATP, 0.2 mM dTTP, 0.2 mM dCTP, 0.2 mM dGTP, and 1 mM β-nicotinamide adenine dinucleotide) and
523 transformed into *E. coli* DH5α. Transformants were screened by colony PCR using primers oWKS-1240
524 and oWKS-1241. Transformants yielding PCR fragments of the correct size were verified by Sanger
525 sequencing.
526
527 *Construction of* C. difficile *IB54 (*ΔsigB*)*
528 The up- and downstream regions (both 950 bp each) of the *sigB* coding sequence were amplified with
529 primers oIB-44/oIB-45, and oIB-46/oIB-47, respectively. Vector pMTL-SC7315 (43) was linearized by PCR
530 using primers oWKS-1537/oWKS-1538. Assembly was done according to the method of Gibson (44, 77).
531 The assembled plasmid was transformed into *E. coli* DH5α and verified using PCR and Sanger
532 sequencing. Generation of the unmarked *sigB* deletion mutant was performed using Allele-Coupled
533 Exchange essentially as described (43, 78). Briefly, pIB54 was introduced into *C. difficile* 630Δ*erm* (31) by
534 conjugation. Transconjugants were grown for two days on BHI agar plates supplemented with yeast
535 extract, thiamphenicol and CDSS, struck onto fresh pre-reduced plates and incubated anaerobically at
536 37°C for two days. Single-crossover integration was confirmed using PCR, and those clones were plated
537 onto non-selective BHI agar plates to allow the second-crossover event to occur. Colonies were then
538 serially diluted and plated onto minimal agar supplemented with 50 µg/ml 5-fluorocytosine (Sigma) as
539 described (43). DNA was isolated from thiamphenicol-susceptible colonies and the chromosomal
540 deletion was verified by PCR using primers oIB-78/oIB-79 (**Supplemental Figure 1**) as well as Sanger
541 sequencing of the PCR product using primers oIB-53/oIB-76/oIB-78.
542
543 *Luciferase reporter assay*

544 Strains containing luciferase reporter plasmids were inoculated to an OD_{600}=0.05 from an exponentially
545 growing starter culture. Fresh inoculums were grown in BHI broth supplemented with yeast extract,
546 with or without 4 µg/ml ACX362E for 5 hours. The supernatants from 1mL of culture (harvested by
547 centrifugation for 10 min, 4°C, 8000 rpm) were analyzed in a GloMax®-Multi Microplate Multimode
548 Reader (Promega) as described before (44). Statistical significance of the data ($p<0.05$) was determined
549 by two-way analysis of variance (ANOVA) and a pairwise Tukey-Kramer test using Prism 7 (GraphPad).
550

551 ***Accession numbers***

552 GEO GSE116503; GenBank LN614756.1

553 **Author's contributions**

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

557

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563 the sliding window analysis.

564

565 **Conflict of interest**

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

571

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788 **Tables**

789

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

791

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

793 non-treated cells.

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795 **Table 3.** List of the genes most highly up- and down-regulated in the presence of ACX362E compared to

796 non-treated cells.

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

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

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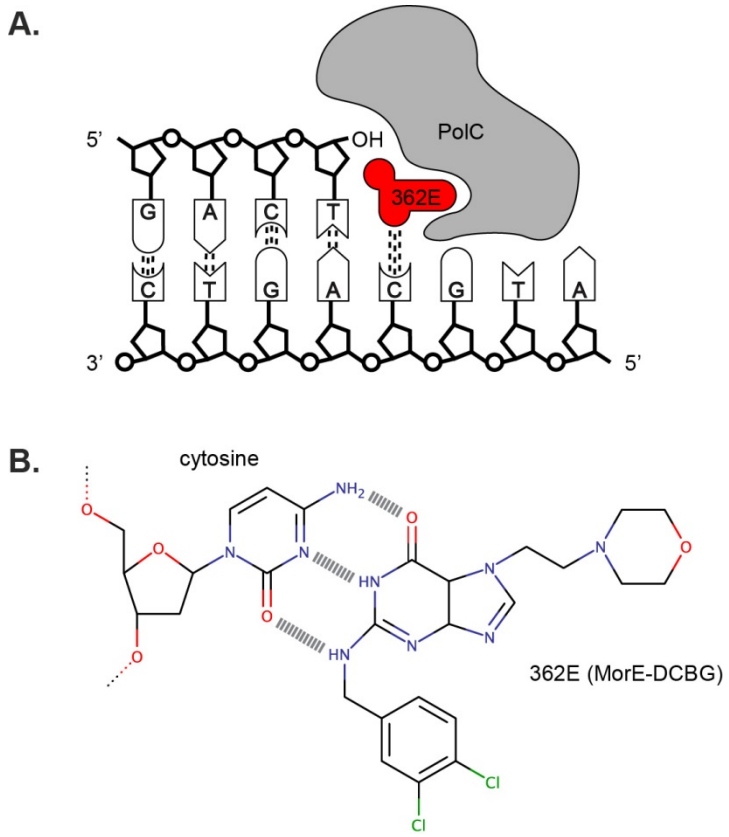
Figures

Figure 1. Mechanism of action of the PolC-inhibitors ACX362E. A. Ternary complex of inhibitor ACX362E, DNA ,and PolC**. B.** H-bonding between inhibitor molecule ACX362E and a cytosine residue of DNA.



820 **Figure 2. Minimal inhibitory concentrations of PolC-inhibitors.** MIC was determined by agar dilution

821 according to CLSI standards (26) and is expressed in µg/mL. The distribution in the MIC for the collection

822 of clinical isolates (N=363) is given for the PolC-inhibitors HPUra (blue) and ACX362E (red).

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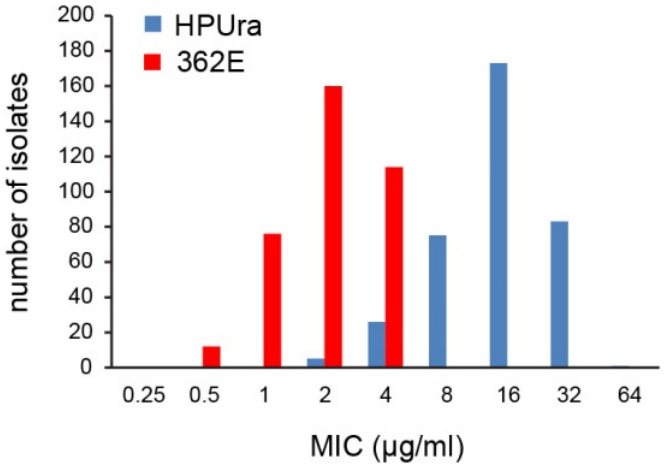

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826 **Figure 3. Overlap in the transcriptional response to different PolC-inhibitors. A.** Venn diagram of the
827 number of genes up-regulated in the presence of ACX362E (red), in the presence of HPUra (blue) or
828 under both conditions (overlapping region). **B.** Venn diagram of the number of genes down-regulated in
829 the presence of ACX362E (red), in the presence of HPUra (blue) or under both conditions (overlapping
830 region). The size of the circles is proportional to the number of genes that showed differential
831 expression.

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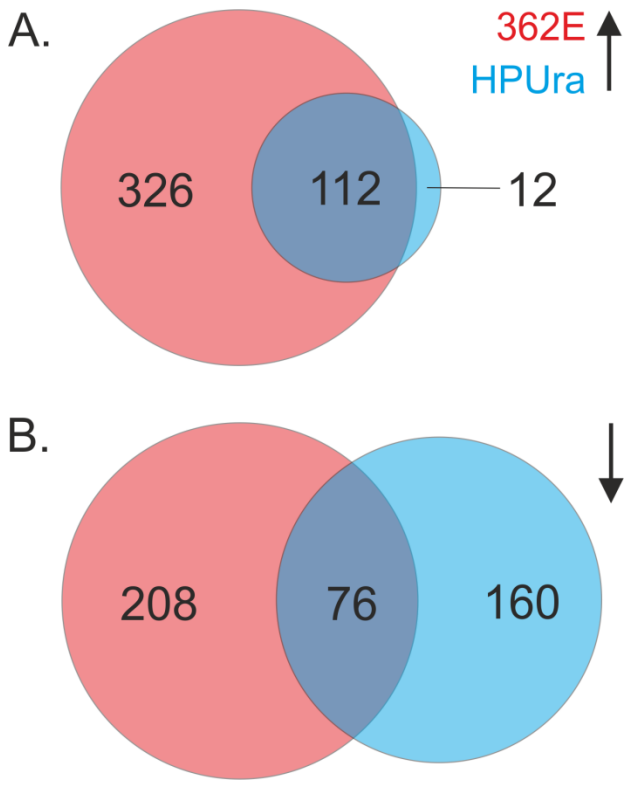

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836 **Figure 4. Genome location correlates with differential expression upon PolC inhibition. A.** Schematic
837 representation of the chromosome of *C. difficile*. Higher than average GC skew ([G - C]/[G + C]) (red) and
838 lower than average GC skew (blue) were calculated with DNAplotter (72). Vertical lines indicate the
839 position of the predicted origin (*oriC*) and terminus (*terC*) of replication. Arrows indicate the direction of
840 replication. **B.** Sliding window analysis (bins of 51 loci, stepsize 1) of the median log fold change (FC)
841 projected on a linear genome map. The *oriC* of the circular chromosome is located on either size of the
842 linear graph (0/4.29Mb), whereas *terC* is indicated with a vertical red line. The trend in log(FC) is
843 highlighted using a green line. Light blue shading indicates the median absolute deviation of the mean
844 (23).

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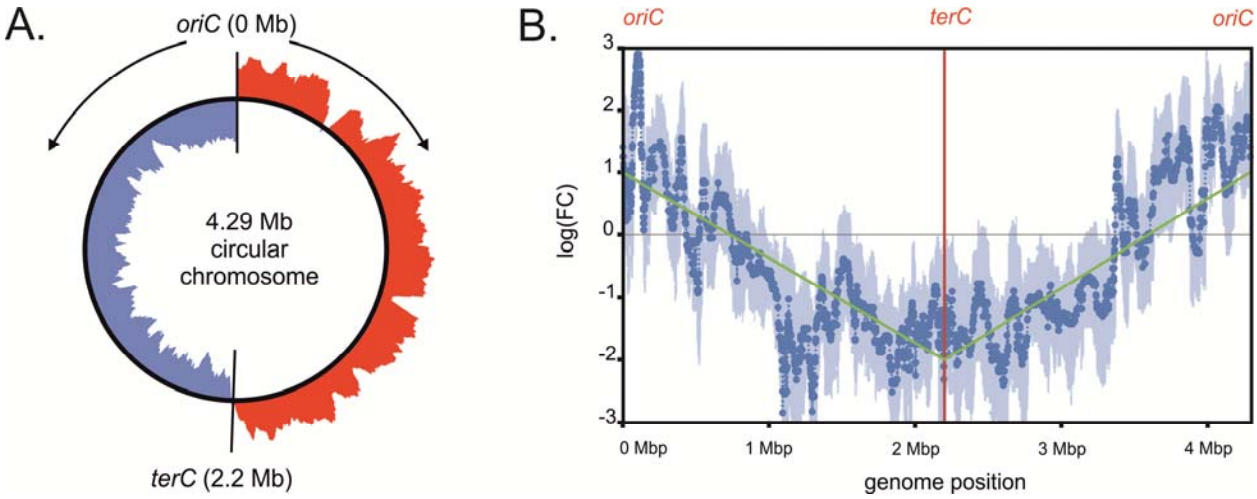

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849 **Figure 5. Polymerase inhibitors lead to an increase in *oriC*:*terC* ratio.** A marker frequency analysis of
850 the effects of sub-inhibitory amounts of polymerase inhibitors (red; HPUra: 35 ug/mL; ACX362E: 4
851 ug/mL) and three antibiotics with different modes of action (blue; metronidazole: 0.25 ug/mL;
852 fidaxomicin: 0.00125 ug/mL, surotomycin: 0.625 ug/mL) compared to non-treated cells (black).
853 Datapoints are averages of technical replicates (N=3). Black lines behind the datapoints indicate the
854 average of the biological replicates (N=3) and whiskers indicate the standard deviation of the mean.
855 Data have been normalized compared to the non-treated control. The mean of HPUra and ACX362E
856 treated samples is statistically different from the other samples (p<0.0001).

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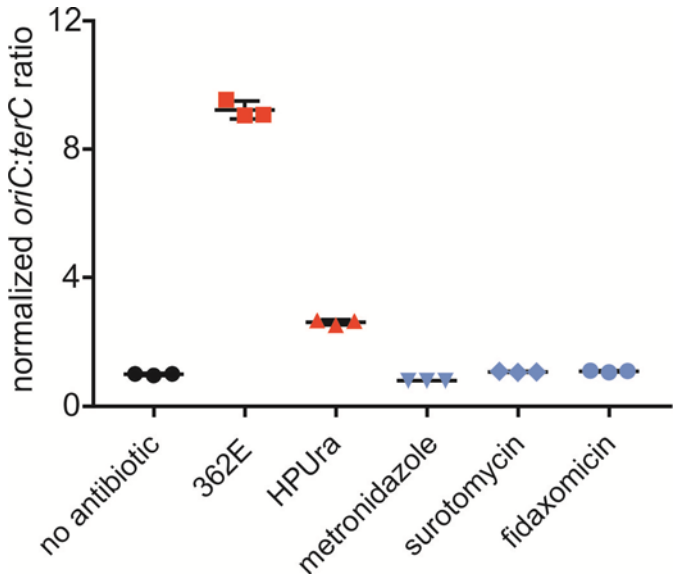

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860 **Figure 6. Genes differentially expressed due to polymerase inhibitors are regulated by σ^B**. The putative
861 promoters of the indicated genes were fused transcriptionally to a plasmid-based luciferase reporter
862 (44). Luciferase activity was measured after 5 h of growth in liquid medium in the presence (+362E) or
863 absence (no Ab) of polymerase inhibitor ACX365. N.S. = non-significant, *=p<0.05, **=p<0.005,
864 ***=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}.

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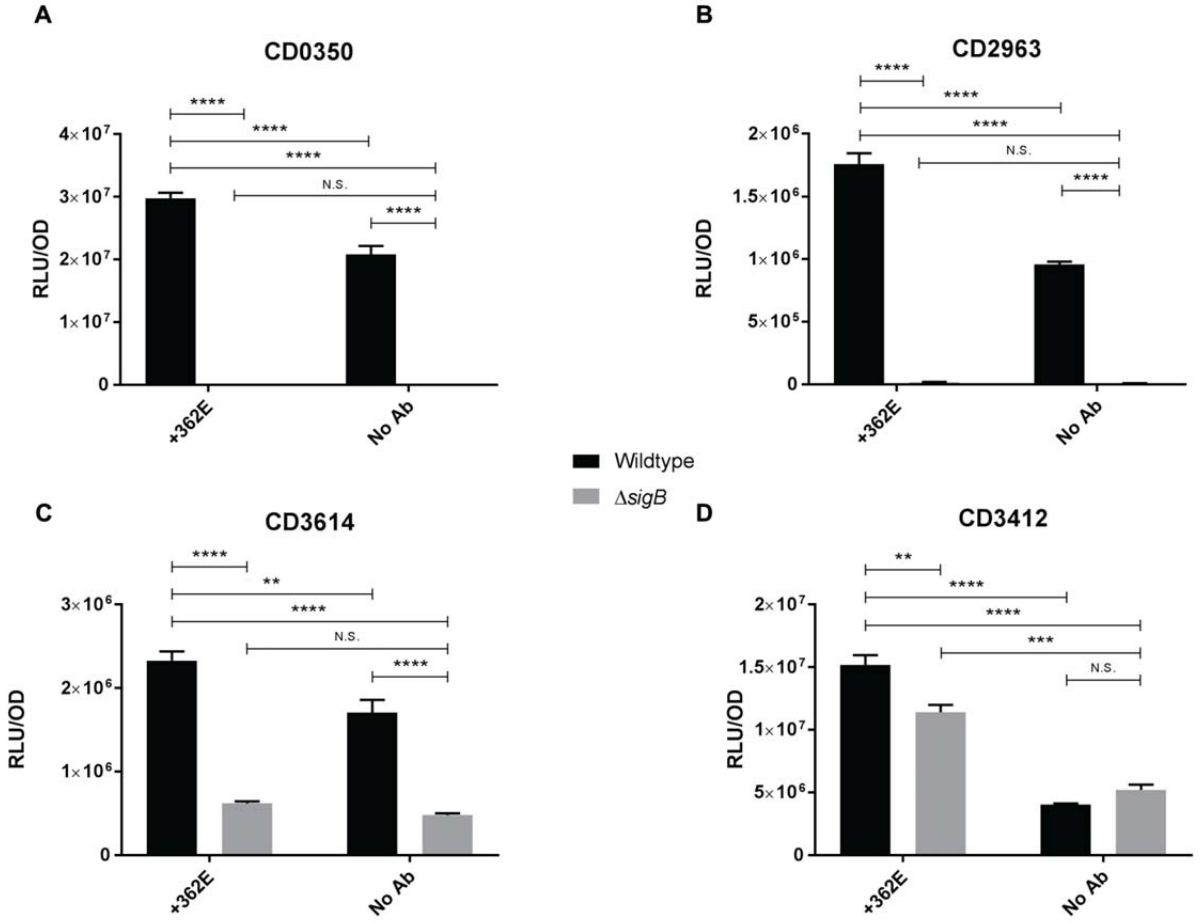

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867 **Supplemental information**

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

871 **Supplemental Table 2**. Lists of the genes that are differentially expressed in the presence of PolC-
872 inhibitors compared to non-treated cells. **A**. HPUra differentially expressed genes. **B**. ACX362E
873 differentially expressed genes.

874 **Supplemental Table 3**. Overlap with published σ^B regulon. **A**. σ^B -dependent stress response genes
875 differentially regulated in the ACX362E dataset. **B**. Genes containing a σ^B consensus promoter
876 differentially regulated in the ACX362E dataset.

877 **Supplemental Figure 1**. Relative inhibition of growth by varying concentrations of PolC-inhibitors.

878 **Supplemental Figure 2.** Confirmation of the *sigB* mutant strain (IB54).

879

880 Supplemental information is also available from Figshare (doi:10.6084/m9.figshare.6740771).

Table 1.

	MIC_{50}	MIC_{90}	Range	No. of isolates	PCR Ribotypes
Clade 1					
HPUra	16	32	2-64	230	001, 002, 003, 005, 009, 010, 011, 012, 014, 015, 018, 025, 026, 029, 031, 037, 050, 051, 053, 056, 057, 064, 070, 081, 084, 087, 106 and 118
362E	2	4	0,25-4		
Clade 2					
HPUra	16	32	2-32	24	016, 019, 027, 075 and 208.
362E	2	4	0,5-4		
Clade 3					
HPUra	16	32	4-32	7	023
362E	4	4	1-4		
Clade 4					
HPUra	8	16	2-16	9	017
362E	2	4	1-4		
Clade 5					
HPUra	16	32	4-32	43	033, 045, 078 and 126
362E	1	2	0,5-4		
Clade 6					
HPUra	4	4	4	1	131
362E	4	4	4		
Clade unknown					
HPUra	16	32	4-32	49	013, 024, 039, 046, 063, 090, 093, 097, 099, 101, 107, 110, 137, 139, 150, 154, 159, 161, 176, 202, 205, 207, 228, 229, 230, 231, 232, and 234
362E	2	4	0,5-4		

Table 2.

Top 10 significantly changed genes HPUra UP

RefSeq locus tag[1]	logFC	logCPM	LR	pvalue	adj_pvalue	Fold	minFDR	Old locus tag[2]	Gene name[3]	Product	Protein ID
CD630DERM_RS12480	11.16	3.84	9.8	1.8e-03	2.4e-02	2291.8	5.37	CD630DERM_23052	CD2305B	Hypothetical protein	WP_004454601.1
CD630DERM_RS15730	6.02	12.24	39.2	3.9e-10	1.9e-07	64.9	22.30	-	-	Hypothetical protein	WP_032506906.1
CD630DERM_RS17185	5.12	8.69	48.8	2.9e-12	3.8e-09	34.8	27.97	CD630DERM_32060	CD3206	Hypothetical protein	WP_003434302.1
CD630DERM_RS17015	4.64	8.61	45.7	1.3e-11	1.0e-08	25.0	26.55	CD630DERM_31740	CD3174	Type I glyceraldehyde-3-phosphate dehydrogenase	WP_003421962.1
CD630DERM_RS17020	4.62	8.68	54.7	1.4e-13	2.7e-10	24.6	31.77	CD630DERM_31750	CD3175	Transcriptional regulator	WP_003429564.1
CD630DERM_RS00700	4.37	10.08	33.9	5.8e-09	2.1e-06	20.7	18.85	CD630DERM_00801	CD0080A	50S ribosomal protein L29	WP_003421158.1
CD630DERM_RS00655	4.26	8.55	22.7	1.9e-06	1.4e-04	19.1	12.77	CD630DERM_00720	CD0072	30S ribosomal protein S10	WP_011860633.1
CD630DERM_RS16270	3.96	8.16	34.9	3.5e-09	1.4e-06	15.6	19.44	CD630DERM_30310	CD3031	Transcriptional antiterminator	WP_003432034.1
CD630DERM_RS19860	3.93	10.72	31.8	1.7e-08	4.1e-06	15.2	17.89	CD630DERM_36630	CD3663	30S ribosomal protein S6	WP_003420522.1
CD630DERM_RS00775	3.83	6.09	15.1	1.0e-04	2.7e-03	14.2	8.53	CD630DERM_00930	CD0093	50S ribosomal protein L14	WP_009895197.1

Top 10 significantly changed genes HPUra DOWN

RefSeq locus tag[1]	logFC	logCPM	LR	pvalue	adj_pvalue	Fold	minFDR	Old locus tag[2]	Gene name[3]	Product	Protein ID
CD630DERM_RS09945	-14.85	4.52	17.0	3.7e-05	1.2e-03	-29514.0	9.72	CD630DERM_17940	CD1794	Hypothetical protein	WP_003430297.1
CD630DERM_RS12730	-14.52	4.33	17.3	3.1e-05	1.1e-03	-23428.2	9.87	CD630DERM_23550	CD2355	Thiol reductase thioredoxin	WP_003416870.1
CD630DERM_RS00870	-14.39	4.91	21.8	3.0e-06	1.9e-04	-21483.7	12.35	-	-	tRNA-Asn	-
CD630DERM_RS00255	-14.35	5.48	45.8	1.3e-11	1.0e-08	-20874.2	26.55	-	-	tRNA-His	-
CD630DERM_RS00925	-14.18	4.91	23.1	1.5e-06	1.3e-04	-18514.8	12.89	-	-	tRNA-Ser	-
CD630DERM_RS17905	-14.02	5.11	26.3	3.0e-07	4.1e-05	-16646.0	14.57	-		Hypothetical protein	WP_042741280.1
CD630DERM_RS05300	-14.01	4.27	12.7	3.7e-04	7.3e-03	-16475.8	7.11	CD630DERM_09230	CD0923	Hypothetical protein, putative phage protein	WP_021362052.1
CD630DERM_RS00170	-13.98	4.82	13.8	2.0e-04	4.5e-03	-16156.5	7.78	-	-	tRNA-Asn	-
CD630DERM_RS03115	-13.96	5.24	56.8	4.8e-14	1.9e-10	-15908.4	32.28	CD630DERM_04981	CD0498A	Hypothetical protein	WP_011860839.1
CD630DERM_RS05160	-13.92	3.89	15.3	9.1e-05	2.5e-03	-15523.8	8.65	CD630DERM_08960	CD0896	Hypothetical protein	WP_003418667.1

[1] At time of analysis https://www.ncbi.nlm.nih.gov/nuccore/NZ_LN614756.1
[2] https://www.ncbi.nlm.nih.gov/nuccore/LN614756,1
[3] https://www.ncbi.nlm.nih.gov/nuccore/AM180355.1

Table 3.

362E	**top 10 significant changed genes up**										
RefSeq locus tag[1]	logFC	logCPM	LR	pvalue	adj_pvalue	Fold	minFDR	Old locus tag[2]	Gene name[3]	Product	Protein ID
CD630DERM_RS12480	11.31	3.84	9.9	1.6e-03	1.3e-02	2544.2	6.25	CD630DERM_23052	CD2305B	Hypothetical protein	WP_004454601.1
CD630DERM_RS17185	7.73	8.69	87.6	8.1e-21	3.3e-17	211.9	54.77	CD630DERM_32060	CD3206	Hypothetical protein	WP_003434302.1
CD630DERM_RS17955	6.88	7.78	28.5	9.5e-08	5.0e-06	117.8	17.62	CD630DERM_33520	CD3352	AraC family transcriptional regulator	WP_009891821.1
CD630DERM_RS17950	6.87	9.82	31.9	1.6e-08	1.0e-06	117.3	19.88	CD630DERM_33510	CD3351	ATP-dependent Clp protease proteolytic subunit	WP_003436212.1
CD630DERM_RS15730	6.67	12.24	45.3	1.7e-11	4.0e-09	101.9	27.89	-	-	Hypothetical protein	WP_032506906.1
CD630DERM_RS00990	5.92	8.75	50.1	1.4e-12	5.7e-10	60.4	30.70	CD630DERM_01090	CD0109	Anaerobic ribonucleoside-triphosphate reductase activating protein	WP_003432598.1
CD630DERM_RS00655	5.65	8.55	35.0	3.2e-09	2.7e-07	50.3	21.80	CD630DERM_00720	CD0072	30S ribosomal protein S10	WP_011860633.1
CD630DERM_RS00985	5.50	8.94	66.3	3.8e-16	7.6e-13	45.2	40.27	CD630DERM_01080	CD0108	Anaerobic ribonucleoside triphosphate reductase	WP_003436320.1
CD630DERM_RS05405	5.49	10.12	43.5	4.2e-11	8.0e-09	45.0	26.89	CD630DERM_09410	CD0941	Hypothetical protein	WP_009894743.1
CD630DERM_RS15695	5.24	10.08	41.5	1.2e-10	2.0e-08	37.9	25.61	CD630DERM_29250	CD2925	Hypothetical protein	WP_009894743.1

362E	**top 10 significant changed genes down**										
RefSeq locus tag[1]	logFC	logCPM	LR	pvalue	adj_pvalue	Fold	minFDR	Old locus tag[2]	Gene name[3]	Product	Protein ID
CD630DERM_RS05350	-14.17	5.04	40.4	2.1e-10	3.1e-08	-18382.7	24.94	CD630DERM_09310	CD0931	Hypothetical protein	WP_011861014.1
CD630DERM_RS05160	-13.92	3.89	15.0	1.1e-04	1.6e-03	-15523.8	9.33	CD630DERM_08960	CD0896	Hypothetical protein	WP_003418667.1
CD630DERM_RS05375	-13.72	4.09	14.3	1.5e-04	2.0e-03	-13454.4	8.95	CD630DERM_09360	CD0936	Endonuclease	WP_011861020.1
CD630DERM_RS15535	-13.57	4.30	23.5	1.3e-06	4.4e-05	-12181.6	14.47	CD630DERM_28950	CD2895	Membrane protein	WP_011861046.1
CD630DERM_RS13040	-13.55	3.91	11.4	7.5e-04	7.1e-03	-11987.2	7.15	CD630DERM_24140	CD2414	PTS sorbose transporter subunit IIB	WP_003430855.1
CD630DERM_RS05345	-13.55	4.41	16.5	4.9e-05	8.2e-04	-11969.7	10.25	CD630DERM_09301	CD0930A	Hypothetical protein	WP_011861013.1
CD630DERM_RS11925	-13.53	4.10	14.0	1.9e-04	2.3e-03	-11799.9	8.76	CD630DERM_21991	CD2199A	4Fe-4S ferredoxin	WP_003423406.1
CD630DERM_RS01060	-13.52	4.51	14.9	1.1e-04	1.6e-03	-11718.7	9.33	-	-	tRNA-Glu	-
CD630DERM_RS15350	-13.44	3.89	15.3	9.1e-05	1.4e-03	-11076.8	9.53	-	-	Membrane protein	WP_003426729.1
CD630DERM_RS06360	-13.43	4.12	23.9	1.0e-06	3.7e-05	-11012.2	14.72	CD630DERM_11160	CD1116	Transposase	WP_011861118.1

[1] At time of analysis https://www.ncbi.nlm.nih.gov/nuccore/NZ_LN614756.1
[2] https://www.ncbi.nlm.nih.gov/nuccore/LN614756,1
[3] https://www.ncbi.nlm.nih.gov/nuccore/AM180355.1

Table 4.

	Name	Relevant features	Source/reference
Plasmids	pAP24	*tetR* P*tet -sluc*^{opt} *catP* ; derived from pRPF185	Oliveira-Paiva
	pIB27	P_{CD3412}-*sluc*^{opt} *catP* ; derived from pAP24	This study
	pIB54	pMTL-SC7315 containing the up- and downstream area of the 630Δerm σB CDS (950 bp each)	This study
	pIB68	P_{CD0350}-*sluc*^{opt} *catP* ; derived from pAP24	This study
	pIB69	P_{CD2963}-*sluc*^{opt} *catP; derived from pAP24*	This study
	pIB74	P_{CD3614}-*sluc*^{opt} *catP; derived from pAP24*	This study
	pMTL-SC7315	pMTL83151 with *codA catP*	Cartman
Strains	DH5α	*Escherichia coli* F– *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20 φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(rK–mK+), λ–*	Laboratory stock
	CA434	*Escherichia coli* HB101 [*F- mcrB mrr hsdS20(rB-mB-) recA14 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rspL20(SmR)glnV44λ-*] R702	Purdy
	C. difficile 630Δ*erm*	MLS susceptible derivative of strain 630	Van Eijk, Hussain
	IB37	630Δ*erm* pIB27; thia^{R}	This study
	IB54	630Δ*erm* Δ*sigB*	This study
	IB95	630Δ*erm* pIB68; thia^{R}	This study
	IB96	630Δ*erm* pIB69; thia^{R}	This study
	IB98	IB54 pIB27; thia^{R}	This study
	IB99	IB54 pIB68; thia^{R}	This study
	IB100	IB54 pIB69; thia^{R}	This study
	IB108	630Δ*erm* pIB74; thia^{R}	This study
	IB111	IB54 pIB74; thia^{R}	This study

Table 5.

Restriction sites are underlined. Overlap with the vector backbone is indicated in lowercase letters.

Name	Sequence (5' –> 3')	Description
CD-0001- F	GAGACAAGAATTGCTATACTTA	Forward primer CD0001 MFA (*ori* C)
CD-0001- R	CAACCACTCTAGTTAATGC	Reverse primer CD0001 MFA (*ori* C)
CD-0001-probe-FAM	CTCAACTAGAACGTATAGATGTGCCAA	Probe CD0001 MFA (*ori* C)
CD-1931- F	GCAGGAATTTTAGATGAAGA	Forward primer CD1931 MFA (*ter* C)
CD-1931- R	GGCTGAAGTCTTATTAAATTTC	Reverse primer CD1931 MFA (*ter* C)
CD-1931-probe-TXR	CCTCTTAACTGTAGCAGATTCACCAT	Probe CD1931 MFA (*ter* C)
oIB-26	GGAAGGTACCGTTGAATAAAGTATTTATTTTCCATG	Forward primer for pCD3412 containing a kpnI restriction site
oIB-27	GGTAGAGCTCAGTATCACTCCTTTTTTCGAAC	Reverse primer for pCD3412 containing a SacI restriction site
oIB-44	CGTAGAAATACGGTGTTTTTTGTTACCCTACTTATAGTAGCAATTTATTTAGCTAAAAC	Forward primer for region 950 bp upstream of *sigB* CDS
oIB-45	TACTTTTTTTTATATTTTTTTTAAATATCAACTCCTAAATATTTAGTC	Reverse primer for region 950 bp upstream of *sigB* CDS
oIB-46	GGAGTTGATATTTAAAAAAATATAAAAAAAGTATTGACCTACTG	Forward primer for region 950 bp downstream of *sigB* CDS
oIB-47	GGGATTTTGGTCATGAGATTATCAAAAAGGGGACTACCAGGGTATCTAATC	Reverse primer for region 950 bp downstream of *sigB* CDS
oIB-53	CTTTAAAACAGTAGGTCAATACTTTTTTTATAT	Reverse primer to verify *sigB* CDS deletion
oIB-76	AAAATATAAAAAAAGTATTGACCTACTGTTTTAAAGATGGTATAGTATTAC	Forward primer to verify *sigB* deletion
oIB-78	GGAGATGTTAACTAATGAATGCATGG	Forward primer to verify *sigB* CDS deletion
oIB-79	CTAATGCACAGCGCCAGCAAACAAAC	Reverse primer to verify *sigB* CDS deletion
oIB-80	ctagcataaaaataagaagcctgcatttgcAAATTTACGAAAAGCTTGC	Forward primer for P_{CD0350}
oIB-82	ctagcataaaaataagaagcctgcatttgcTTGTGTTTAAGGGATTTTGAAAG	Forward primer for P_{CD2963}
oIB-92	ctagcataaaaataagaagcctgcatttgcGAATAAAAAAGGTGGTGTC	Forward primer for P_{CD3614}
oIB-94	agctattaataattttttacttggtctcatTTTTACCTCCATGTAACATTTATTG	Reverse primer for P_{CD0350}
oIB-95	agctattaataattttttacttggtctcatAATTAAATCCTTCCTTACATTGTAATTAC	Reverse primer for P_{CD2963}
oIB-100	agctattaataattttttacttggtctcatATAAACACCCTCCTATTCTTTG	Reverse primer for P_{CD3614}
oWKS-1240	CACCTCCTTTTTGACTTTAAGCCTACGAATACC	Forward primer on pAP24 backbone to amplify insert
oWKS-1241	CACCGACGAGCAAGGCAAGACCG	Reverse primer on pAP24 backbone to amplify insert
oWKS-1537	TAGGGTAACAAAAAACACCG	Reverse primer to amplify pMTL-SC7315 backbone
oWKS-1538	CCTTTTTGATAATCTCATGACC	Forward primer to amplify pMTL-SC7315 backbone
oWKS-1580	ATGAGACCAAGTAAAAAATTATTAATAGC	Forward primer to amplify pAP24 backbone
oWKS-1582	GCAAATGCAGGCTTCTTATTTTTATG	Reverse primer to amplify pAP24 backbone