Chloramphenicol-mobilized *Bacillus subtilis* transiently expresses resistance to multiple antibiotics, including the glycopeptides phleomycin and bleomycin

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Running Head: Antibiotic-induced resistance in sliding *B. subtilis*

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ABSTRACT

Antibiotic resistance is a global crisis that stems from the use of antibiotics as an essential part of modern medicine. Understanding how antibiotic resistance is controlled among cells in bacterial populations will provide insights into how antibiotics shape microbial communities. Here, we describe patterns of *B. subtilis* gene expression that arise from growth on a surface either in isolation or under subinhibitory chloramphenicol exposure. We identified elevated expression of genes encoding five different antibiotic resistance functions. The expression of four of the five resistance functions is controlled by a combination of terminator attenuation and transcriptional regulation. Two of these, *vmlR* and *tlrB* provide resistance to lincosamides and tylosin, respectively. We found that *bmrCD* promotes resistance to glycopeptides, including phleomycin and bleomycin. Promoter fusions to luciferase were used to follow expression of *bmrCD, vmlR,* and *tlrB*. Subinhibitory chloramphenicol exposure induces sliding motility of *B. subtilis*, wherein the three antibiotic resistance functions are expressed heterogeneously in spatiotemporally segregated pattern. We found that their expression is transiently elevated even in the absence of antibiotic exposure. The data suggest that for some antibiotics, intrinsic resistance genes are entrained to changes in growth and metabolism. Antibiotic exposure amplifies their expression, potentially providing a subpopulation of cells elevated protection to multiple classes of antibiotic.

IMPORTANCE

Antibiotics are a natural component of bacterial communities that are vital to modern medicine and to combatting antibiotic-resistant pathogens. Understanding the

roles antibiotics play in microbial populations provides insights to the origins of antibiotic resistance. Many studies show that subinhibitory concentrations of antibiotics stimulate bacterial gene expression, including antibiotic resistance, and influence community dynamics. This study shows that bacteria on an agar surface transiently elevate expression of intrinsic resistance genes for multiple antibiotics. Subinhibitory chloramphenicol exposure stimulates the bacterial population both to expand across the surface and enhance expression of the resistance genes in a heterogeneous pattern. The results illustrate how exposure to some antibiotics substantially changes bacterial population dynamics that include the spatiotemporally controlled expression of endogenous antibiotic resistance.

INTRODUCTION

Antibiotic resistance has increasingly become a global concern due to the emergence of diverse resistance mechanisms in pathogenic bacteria. While the origins of antibiotic resistance function vary, many of them are thought to provide competitive fitness within natural microbial communities[1,2]. These intrinsic mechanisms of antibiotic resistance may have been selected from widespread exposure to antibiotics during interspecies interactions. Intrinsic resistance takes many forms, including expression of outer membrane porin proteins[3], efflux pumps, and antibiotic modification enzymes[4]. These gene products either reduce the concentration of toxic substances inside bacterial cells or prevent the access of toxin to cellular targets. Intrinsic resistances may be activated by environmental stressors such as iron limitation[5] and subinhibitory antibiotic exposure[6]. The induction of resistance gene expression may arise indirectly

70 from competition sensing, or directly from antibiotic-induced damage[7]. In either case,
71 encoding multiple antibiotic resistance functions enables bacteria to survive in the
72 presence of antibiotic producing competitors in microbial communities.

73 *Bacillus subtilis*, a model Gram-positive bacterium has been used to study
74 antibiotic resistance genes due to its homology to several pathogenic and antibiotic-
75 resistant relatives like MRSA and the abundance of antibiotic resistance genes it
76 contains. Multiple studies have demonstrated that some genes encoding antibiotic
77 resistance genes are upregulated when exposed to subinhibitory concentrations of
78 chloramphenicol[6,8]. For example, the *bmrCD* operon encodes a multidrug efflux pump
79 that is induced by chloramphenicol. The *bmrCD* genes are preceded by a leader
80 peptide, *bmrB*, which promotes *bmrCD* gene expression when the ribosome stalls on
81 the promoter sequence due to antibiotic interference[6,9]. This mechanism of ribosome-
82 mediated regulation via transcriptional attenuation is shared by many genes in the *B.*
83 *subtilis* genome[10].

84 A previous study from our lab used a competition model composed of *B. subtilis*
85 and *S. venezuelae*. *S. venezuelae* produces the translation inhibitor chloramphenicol,
86 which we identified to be an inducer of *B. subtilis* sliding motility[11,12]. In the current
87 study, we focused on chloramphenicol-induced changes in the expression of several
88 known and predicted antibiotic resistance genes during colony expansion on solid agar
89 media. We observed that none of these genes encode resistance to chloramphenicol or
90 influence chloramphenicol-induced sliding motility. However, a moderate elevation of
91 chloramphenicol resistance arose from pre-exposure to subinhibitory chloramphenicol.
92 We found that BmrCD provides resistance to phleomycin and bleomycin. Thus, while

93 VmlR and TlrB provide resistance to ribosome-targeting antibiotics, BmrCD is specific to
94 DNA-damaging agents, despite its induction by chloramphenicol exposure. Using
95 luciferase promoter fusions, our data shows that *vmlR*, *tlrB*, and *bmrCD* are transiently
96 expressed during growth without any antibiotic. Chloramphenicol exposure results in
97 persistent expression of the genes, resulting in a heterogeneous pattern of resistance
98 gene expression in a sliding population of cells. These results suggest a model wherein
99 *B. subtilis* gains a competitive advantage by responding to antibiotic exposure through
100 sustained intrinsic resistance to multiple antibiotics and induction of sliding motility in the
101 presence of antibiotic-producing competitors.
102

103 **RESULTS**

104 **Deletion of antibiotic resistance genes does not change the sensitivity to**
105 **chloramphenicol**

106 Among the genes (~800) changing expression after chloramphenicol exposure,
107 we identified a specific subset of genes either known or predicted to be involved in
108 antibiotic resistance[12]. The genes *vmlR*, *tlrB, bmrCD*, *mdr*, and *ytbDE,* all of which have
109 different resistance specificity for antibiotics, are induced by chloramphenicol exposure
110 but encode resistance that is unrelated (**Table 1**)[13–19]. The *vmlR* gene encodes an ABC
111 transporter that binds to the ribosome and mediates the resistance to lincomycin and
112 virginiamycin M[19,20]. The *tlrB* gene encodes a 23S rRNA methyltransferase that confers
113 resistance to tylosin[16]. The *mdr* gene encodes a multidrug-efflux pump that is predicted
114 to provide resistance to fluoroquinolones like nerfloxacin, tosufloxacin and puromycin[17].
115 The *bmrCD* genes encode an efflux pump that was shown to function as a heterodimer

116 to transport multiple fluorescent molecules in a vesicle assay[6]. The function of *ytbDE* is
117 unknown but predicted to be an antibiotic resistance protein. Importantly, these genes
118 are not all controlled by a single regulatory protein, nor do they belong to any of the
119 known stress response pathways under sigma factor control[6,10,16,21]. However, all but
120 *mdr* share a terminator-antiterminator regulatory mechanism that is dependent upon
121 protein synthesis.

122 We hypothesized that one or more of the antibiotic resistance genes could
123 mediate the *B. subtilis* response to subinhibitory chloramphenicol. Since sliding motility
124 induced by chloramphenicol is a dose-dependent phenomenon[11], subtle changes in the
125 sensitivity may lead to differences in the sliding pattern. We deleted each of the five
126 antibiotic resistance genes that are transcriptionally induced by chloramphenicol
127 exposure. We assayed sliding induction using different concentrations of
128 chloramphenicol (0-8 μM). We observed no difference between *B. subtilis* NCIB 3610
129 wild type strain and the deletion strains, including a strain which has all five of the genes
130 deleted (Δ5 strain) (**Figure 1A and Figure S1**). We concluded that none of the
131 resistance functions has a direct role in *B. subtilis* sensitivity to chloramphenicol.
132 Previously it was shown that tylosin resistance via *tlrB* is elevated by pre-exposure to
133 subinhibitory tylosin[16]. We tested whether preexposure to subinhibitory concentrations
134 of chloramphenicol would increase the survival of *B. subtilis* to inhibitory concentrations
135 of chloramphenicol. A wild-type culture pre-exposed to chloramphenicol reached
136 moderately elevated density in the presence of inhibitory amounts of chloramphenicol,
137 relative to the unexposed control (**Figure 1B**). Therefore, while the five induced
138 antibiotic resistance functions are not chloramphenicol specific, pre-exposure may

139 induce unidentified chloramphenicol resistance. If the improved growth from pre-
140 exposure induces resistance to chloramphenicol, we hypothesized the same would be
141 observed for lincomycin and tylosin due to the induction of *vmlR and tlrB*. We assayed
142 growth, following pre-exposure to chloramphenicol, in the presence of lincomycin and
143 tylosin (**Figure S2**). We found no consistent pattern of enhanced resistance to
144 lincosamine or tylosin. Thus, while chloramphenicol exposure may induce the
145 expression of these antibiotic resistance genes, it is not clear whether the increase in
146 transcript abundance leads to elevated resistance for the population.
147

148 **Chloramphenicol treatment leads to increased phleomycin and bleomycin**
149 **resistance via *BmrCD***

150 Previous studies show that *vmlR* and *tlrB* provide resistance to lincomycin and
151 tylosin, respectively, both of which inhibit protein synthesis[16,19]. We hypothesized that
152 efflux via BmrCD or resistance via YtbDE may similarly provide resistance to an
153 unidentified antibiotic that targets protein synthesis. To identify a substrate antibiotic, we
154 tested relative sensitivity of wild type, Δ*bmrCD*, and Δ*ytbDE* strains to a panel of
155 antibiotics. In the case of *ytbDE*, no resistance was identified for the many types of
156 antibiotics tested (**Table S1**). In comparison of wild type with Δ*bmrCD*, we observed
157 differential sensitivity to phleomycin, a glycopeptide antibiotic that causes DNA
158 damage[22] (**Figure 2A**). We found that growth inhibition with phleomycin was
159 suppressed in the presence of chloramphenicol, consistent with the induction of *bmrCD*
160 expression. The chloramphenicol-induced resistance was lost in the Δ*bmrCD* strain,
161 indicating the resistance is specific to BmrCD. To demonstrate that induction of *bmrCD*

162 expression leads to phleomycin resistance, we placed expression of *bmrCD* under the
163 control of an IPTG-inducible promoter (P*hyperspank*). Even in the absence of IPTG, we
164 observed elevated resistance to phleomycin, which we suspect arises from background
165 activity of the IPTG-inducible promoter. Therefore, we engineered a strain that
166 expresses alleles of *bmrCD* that carry point mutations in the Walker A motifs of each
167 gene[14], resulting in dysfunctional BmrCD due to loss of nucleotide binding (**Figure S3**).
168 Expression of the mutant BmrC*D* was incapable of generating phleomycin resistance
169 either in the presence or absence of IPTG (**Figure 2B**).
170 The BmrCD-dependent resistance to phleomycin is moderate in our plate
171 assays. Because phleomycin belongs to the glycopeptide class of antibiotics, we asked
172 whether BmrCD can transport other structurally similar antibiotics. We selected
173 representatives of two types of glycopeptide antibiotic, bleomycin and vancomycin,
174 bleomycin being more closely related to phleomycin. Filters embedded with each
175 antibiotic revealed that BmrCD confers resistance to both phleomycin and bleomycin,
176 but not vancomycin (**Figure 2A**). The level of antibiotic resistance to bleomycin is
177 greater than that to phleomycin, suggesting possible selectivity of the BmrCD
178 transporter. Consistent with the phleomycin result, chloramphenicol exposure led to
179 increased bleomycin resistance, and overexpression using the P*hy-bmrCD* strain led to
180 bleomycin resistance with or without IPTG induction. Vancomycin remained sensitive
181 under identical conditions of chloramphenicol exposure and *bmrCD* overexpression.
182

183 **Spatiotemporal separation of resistance gene expression in a population exposed**
184 **to subinhibitory concentrations of chloramphenicol**

185 A shared feature of four of the chloramphenicol-induced resistance functions is
186 their regulation using transcriptional attenuation controlled by translational pausing of an
187 upstream leader peptide[6,10,16,21]. Using the upstream ORFs described in previous
188 publications, we input the nucleotide sequences upstream of *bmrCD*, *vmlR*, and *tlrB* into
189 RNAfold to display the similarities and differences of the uORFs of these three genes
190 (**Figure 3**). The *bmrCD* genes contain two regulatory regions with the 351 bp before the
191 *bmrC* start site: *bmrB* which contains the anti-anti-terminator, pause hairpin (anti-
192 terminator), and terminator hairpins[6] and *bmrX* which contains a cleavage site for an
193 endoribonuclease[9]. Like the uORF of *bmrCD*, the uORF of *vmlR,* which starts 219 nt
194 before the start site, also contains and anti-anti-terminator hairpin, pause hairpin, and
195 terminator hairpin[21]. Unlike *bmrCD* however, *vmlR* also contains another hairpin which
196 blocks the Shine-Dalgarno sequence of *vmlR*. The uORF of *tlrB* on the other hand
197 forms one long hairpin starting 178 nt upstream of the translation start site and contains
198 both the Shine-Dalgarno and transcription start site to block all transcription and
199 translation[16]. In each case, upon antibiotic exposure the ribosomes stall at upstream
200 ORFs, which generates promoter structures permissive for transcription of the
201 downstream gene or operon (**Figure 3**). Although each system provides resistance to a
202 specific class of antibiotic, chloramphenicol exposure leads to the induction of all three
203 through a general reduction in translation activity.
204 *B. subtilis* exhibits different spatiotemporal patterns of gene expression in
205 biofilms, swarming populations, and in chloramphenicol-induced sliding populations on
206 solid media[12,23,24]. We hypothesized that the induced expression of the antibiotic
207 resistance genes may occur in only a subpopulation, transiently, or both. If true,

208 changes in resistance may not be clearly observable as changes in growth pattern but
209 instead be a transient property of a subset of cells in the population. To characterize the
210 spatiotemporal expression pattern for these genes in a mobile colony, promoter fusions
211 were created using the native promoter of each gene fused to the luciferase reporter,
212 *luxABCDE* (*lux*). We first monitored the luciferase activity in liquid media over the course
213 of 30 hours (**Figure 4A**). Each strain shows a transient elevation of promoter activity
214 with subinhibitory (1 μM) chloramphenicol exposure relative to the untreated control.
215 The induced activity peaks near 5 hours of culture, which is a pattern consistent with
216 previous reports[6,10]. To monitor expression on a solid surface, we plated strains carrying
217 the reporter fusions to agar media with or without chloramphenicol and monitored
218 luciferase activity over time (**Figure 4, B-E**). Each strain exhibits increasing promoter
219 activity over times monitored (6, 9, 24, and 48 hours). At 24 hours after inoculation,
220 colony expansion is visible. Within these strains, the elevated promoter activity is
221 associated with the center of the population and is diminished in the expanding
222 population (**Figure 4D**). This pattern reverses as the population expands to encompass
223 nearly the entire surface of the agar media. At 48 hours, the most intense signal is
224 observed at the periphery of the expanding population, with little to no signal among the
225 interior cells (**Figure 4E**). Unexpectedly, under the control condition without
226 chloramphenicol, the activity of all three promoters is elevated at 24 and 48 hours,
227 initially in the center of the population and subsequently on the periphery of the non-
228 expanding population. A similar luciferase fusion was used to follow activity of the *ytbD*
229 promoter on solid agar (**Figure S4**). The *ytbD* promoter activity was also elevated in the
230 absence of chloramphenicol, but subsequently the activity was diminished, relative to

231 the other promoter fusions. Activation of the promoters in the absence of
232 chloramphenicol exposure suggests that an endogenous stalling of ribosomes and
233 translation activity occurs in spatiotemporally separated regions of the population.
234

235 **DISCUSSION**

236 Competition between *B. subtilis* and *S. venezuelae* activates defense
237 mechanisms in *B. subtilis*. In response to subinhibitory levels of chloramphenicol, *B.*
238 *subtilis* cells protect themselves from external stresses in part by enhancing the
239 expression of antibiotic resistance genes, and in part by initiating sliding motility[12,25]. Of
240 the five antibiotic resistance functions we selected for study, none confer resistance to
241 chloramphenicol. Here we have studied the expression of a subset of antibiotic
242 resistance genes that are induced directly from reduced translation efficiency. By
243 culturing the bacteria on solid agar media, we found that the expression of three of
244 these functions, *vmlR*, *tlrB*, and *bmrCD*, is naturally and transiently activated without
245 chloramphenicol. This observation suggests that expression of these genes may be
246 influenced by pauses in translation that occur during colony growth or the result of other
247 regulatory functions. Two of the genes, *vmlR* and *tlrB*, convey resistance to antibiotics
248 that bind the ribosome[16,19]. The biological substrate for BmrCD had not been identified.
249 Using a panel of antibiotics, we found that BmrCD provides resistance to the
250 glycopeptides, phleomycin and bleomycin, which target DNA, not protein synthesis.
251 Although the specificity of function is unknown, we suspect that two other induced
252 resistance functions, *mdr* and *ytbDE*, are specific to other antibiotics. Thus, during the
253 growth of a population of *B. subtilis*, changes in translation efficiency result in

254 heterogeneous and transient elevation of resistance to multiple antibiotics. The
255 heterogeneous pattern for induction of intrinsic resistance is sustained when exposure
256 to chloramphenicol, and presumably other protein synthesis inhibitors, further elevates
257 expression of the resistance genes.

258 Intrinsic resistance induced by one antibiotic is often related to the regulation of
259 the same antibiotic, while some antibiotics induce cross-resistance to other antibiotics.
260 For example, in the case of *ermC* expression induced by erythromycin, the product,
261 methylase C, methylates one adenine in the 23S rRNA to block the binding of not only
262 erythromycin, but also lincomycin and streptomycin[26]. In the case of chloramphenicol,
263 we found that none of the induced genes promoted resistance to chloramphenicol.
264 However, pre-exposure to subinhibitory chloramphenicol did provide moderate
265 resistance to inhibitory amounts of the drug by an unknown mechanism. We
266 hypothesized that perhaps the induced genes may all converge on resistance to protein
267 synthesis inhibitors. BmrCD functions as an efflux pump and has been reported to
268 transport fluorescent dyes and drugs out of vesicles[14]. However, a physiological
269 substrate for resistance was not known. The resistance we identify to phleomycin and
270 bleomycin from induced *bmrCD* reveals a subclass of glycopeptide antibiotics that are
271 substrates for BmrCD. These glycopeptides cause DNA damage and are not related to
272 protein synthesis. Thus, although BmrCD is induced by chloramphenicol and other
273 protein synthesis inhibitors, it appears to function specifically on a subclass of DNA-
274 damaging natural products. Recently, Meirelles et al. also reported that phenazine
275 produced from *P. aeruginosa* increased the tolerance to ciprofloxacin and levofloxacin in
276 *P. aeruginosa* by upregulating the expression of *metGHI-opmD* efflux system[27]. These

277 data suggest that cross-resistance induced by different chemical stimuli may be a
278 common phenomenon, which may provide a protective effect for bacteria against
279 competitors.

280 Physiological adaptions provide *B. subtilis* a complementary way to compete with
281 other microbes. The expression of antibiotic resistance genes is coupled to changes in
282 growth and physiology of bacterial cells. These changes have been observed in
283 heterogeneous populations of bacteria that show differential patterns of resistance gene
284 expression[28–31]. In response to environmental cues, *B. subtilis* can differentiate into
285 diverse cell types to adapt to local settings through a complex gene regulation network.
286 In our bacterial competition model, *B. subtilis* cells initiate a sliding response to evade *S.*
287 *venezuelae* [12,25]. Our data reveal that the changing resistance profile induced by
288 chloramphenicol exposure is also intrinsically elevated in the population during growth.
289 Although this study focused on a few antibiotic resistance genes induced early after
290 chloramphenicol exposure (6 hours), we suspect that the induced sliding population,
291 which shows elevated expression of hundreds of genes relative to uninduced controls,
292 will exhibit increased resistance to other antibiotics by mechanisms yet to be
293 characterized. A *B. subtilis* population using sliding motility resembles a mobile biofilm,
294 based on its rough surface morphology and dependence upon surfactin and EPS-
295 dependent[32,33]. Overall, when *B. subtilis* is exposed to chloramphenicol or other protein
296 synthesis inhibitors from competitors [12,25], our data suggest the population promotes
297 competitive fitness through mobilizing a biofilm that exhibits dynamic patterns of
298 resistance to multiple antibiotics.
299

300

301 **MATERIALS AND METHODS**

302 **Strains, primers, and media**

303 The strains of *Bacillus subtilis* used in this study are listed in **Table S2**. *Bacillus subtilis*
304 mutant strains in 168 or PY79 background were transduced to NCIB3610 by SPP1
305 phage transduction [34]. The primers are listed in **Table S3**. *Bacillus subtilis* strains were
306 cultured at 37℃ in lysogeny broth (LB) and were inoculated onto GYM7 plates (0.4%
307 [w/v] D-glucose, 0.4% [w/v] yeast extract, 1.0% [w/v] malt extract, pH7.0) with 1.5%
308 [w/v] agar when grown to an OD600 of 1.0.

309 **Construction of luciferase reporter strains**

310 To construct luciferase reporter strains, we used primers for each promoter region
311 (listed in **Table S3**) to amplify the target promoter from *B. subtilis* NCIB 3610 genomic
312 DNA, primers *lux*-For and *lux*-Rev to amplify the *luxABCDE* fragment from pBS3Klux,
313 primers *amyE*-back-For and *amyE*-front-Rev to amplify the plasmid backbone (including
314 the origin site, ampicillin resistance cassette, and front and back partial *amyE*
315 fragments) from pDR111, and primers *kan*-For and *kan*-Rev to amplify the kanamycin
316 resistance cassette from pDG780 [35]. These 4 fragments will be assembled to a
317 functional plasmid by Gibson assembly [36]. The plasmid was transformed to *B. subtilis*
318 PY79 wild type and then the construct containing the target promoter, *lux* operon, and
319 kanamycin resistance cassette was inserted into the *amyE* locus. The inserted construct
320 was verified by PCR. Once confirmed, the construct was moved to PDS0066 (*B. subtilis*
321 NCIB 3610 wild type) using SPP1 phage transduction.

322 **Sliding motility assay**

323 *B. subtilis* cells grown in 4 mL LB broth were diluted to an OD600 of 0.08. When grown
324 to an OD600 of 1.0, 1.5 µL of *B. subtilis* cells were spotted on the GYM7 plate with or
325 without 1 µM chloramphenicol. Pictures were taken with Nikon D60 digital camera. For
326 the luciferase reporter assays, images were captured with an Amersham imager 600, or
327 Canon 5D Mark IV [37].

328 **Preexposure Assay**

329 *B. subtilis* cells grown overnight in 4 mL LB broth were diluted to an OD600 of 0.08 with
330 subinhibitory concentrations of antibiotics and grown to an OD600 of 1. Cultures were
331 then diluted once again to and OD600 of 0.08 and grown in triplicates with media
332 containing varying inhibitory concentrations of drug as indicated per figure. OD was
333 measured every 30 minutes using the Agilent BioTek Synergy H1 plate reader was used
334 to record data for OD over 18 hours.

335
336 **Plate reader Assay**

337 Strains were inoculated in LB and grown overnight at 37°C. The overnight was then
338 diluted and grown to an OD600 of 1. Cultures were then diluted to an OD600 of 0.085
339 using liquid GYM7 as diluent, and 200µL of each dilution was dispensed in three sets of
340 triplicates. Control triplicates were untreated with antibiotic and the remaining two sets
341 of triplicates were treated with 1µM and 4µM chloramphenicol respectively. A Tecan
342 plate reader was used to record data for OD and luminescence signal over 18 hours.

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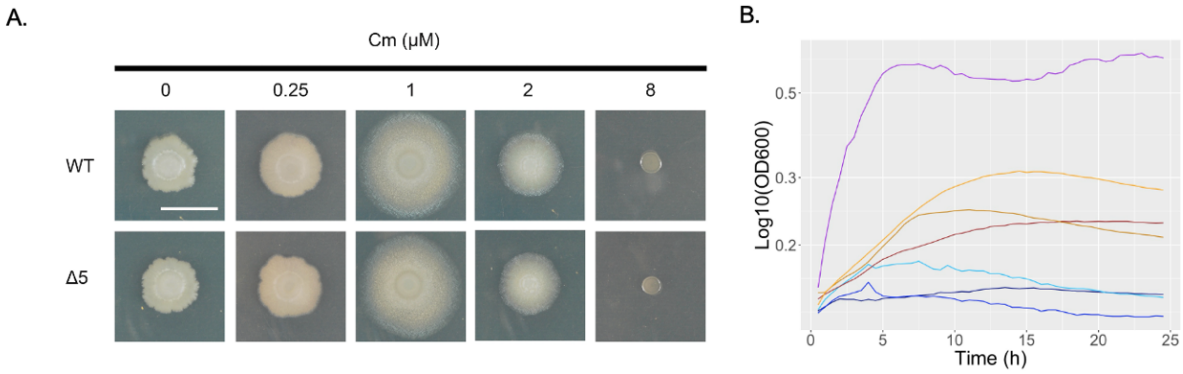


Figure 1. Deletion of resistance genes does not change the sensitivity to chloramphenicol.

Five resistance elements (*bmrCD*, *vmlR*, *tlrB*, *mdr* and *ytbDE*) that respond to chloramphenicol (Cm) at 6 h were deleted (refer to as Δ5 strain). (**A**) Wild type (WT) and Δ5 strains were plated on GYM7 agar with a range of subinhibitory chloramphenicol (0-8 μM). Pictures were taken at 24 h. Bar, 1 cm. (**B**) Growth inhibition assay of *B. subtilis* in chloramphenicol with and without preexposure to subinhibitory concentrations of chloramphenicol. *B. subtilis* (purple), *B. subtilis* without preexposure grown in chloramphenicol (light blue: 12.5, medium blue: 37.5 and dark blue: 100 μM) and *B. subtilis* pre-exposed to 1 μM chloramphenicol and then grown in chloramphenicol (orange:12.5, red:37.5 and brown:100 μM).

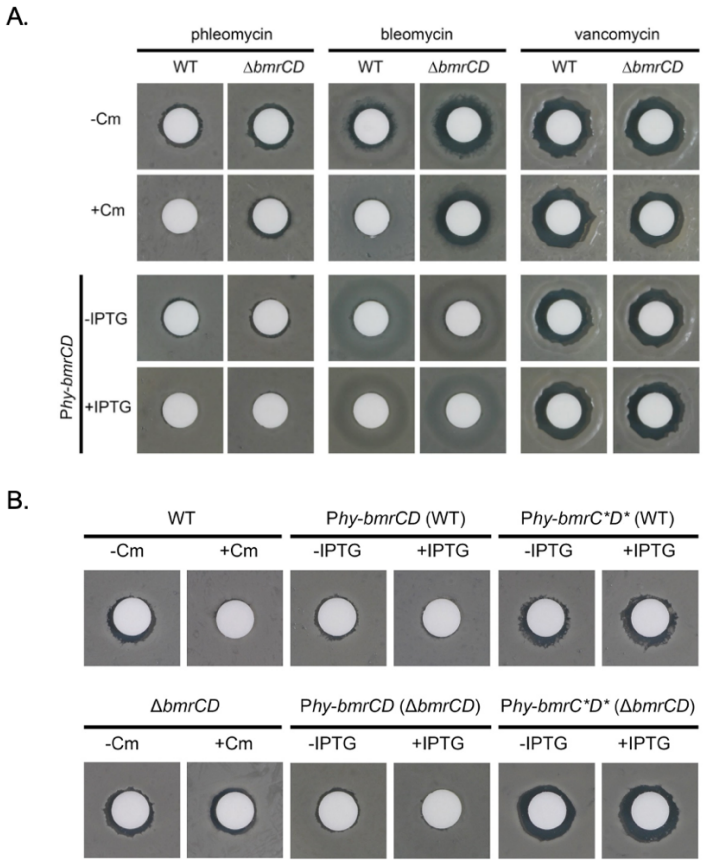


Figure 2. BmrCD provides resistance to phleomycin and bleomycin.
Deletions of bmrCD have increased sensitivity to phleomycin and bleomycin. (**A**) WT and Δ*bmrCD* strains (OD600=1.0, 100 μL) (top) and WT and Δ*bmrCD* strains with P*hy-bmrCD* inserted at the *amyE* locus (OD600=1.0, 100 μL) (bottom) were spread on the GYM7 agar medium in the absence (-) and presence of 1 μM Cm. Then, 10 μL of 125 μg/mL phleomycin (left), 10 μL of 1 mg/mL bleomycin (middle) and 10 μL of 25 μg/mL vancomycin (right) were added onto each individual paper disc. Pictures were taken at 24 h. Diameter of paper disc, 6 mm. (**B**) Left: WT and Δ*bmrCD* strains (OD600=1.0, 100 μL) were spread on the GYM7 agar medium in the absence (-) and presence of 1 μM Cm. Then, 10 μL of 125 μg/mL phleomycin was added onto the paper disc. Middle: Complementation of Δ*bmrCD* with *bmrCD* under the control IPTG-inducible promoter (*hyperspank*) restored the phleomycin resistance in the presence of 0.5 mM IPTG, with an observation of reduced inhibitory zone. Right: point mutations introduced to Walker A motif of BmrC and BmrD in both WT and Δ*bmrCD* strains abolished the BmrCD-dependent resistance, restoring the inhibitory zone. Pictures were taken at 24 h. Diameter of paper disc, 6 mm.

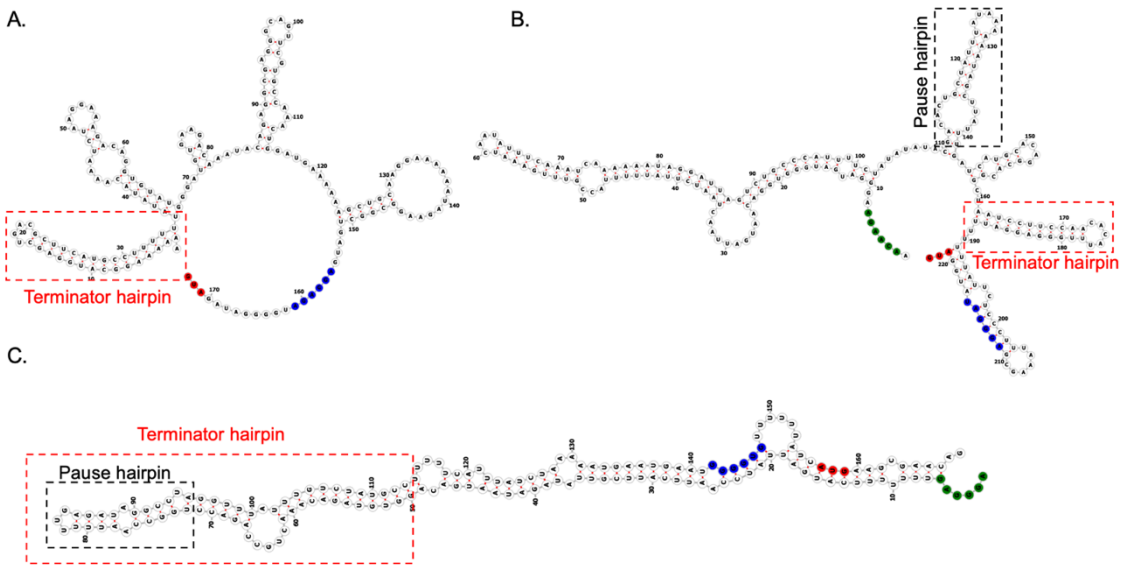


Figure 3. Predicted repressed conformations of the upstream regions of *tlrB*, *vmlR*, and *bmrCD*.

Secondary structure of the repressed conformation of the mRNA region upstream of the start codon of (**A**) *bmrCD* (170-nt), (**B**) *vmlR* (220-nt), and (**C**) *tlrB* (190-nt) predicted by RNA Fold web server. Shine-Dalgarno (SD) sequences for the uORF/leader peptide are shown in green along with the SD sequence for each gene shown in blue and the start codon in red. The pause and terminator hairpins are indicated with black and red boxes, respectively.

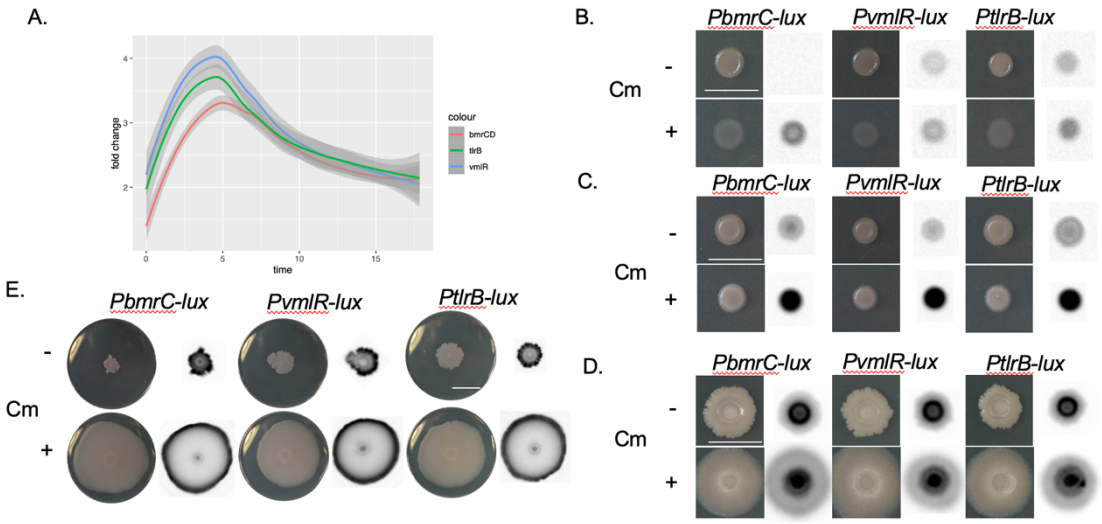


Figure 4. Gene expression of antibiotic resistance genes upon exposure to subinhibitory chloramphenicol using luciferase reporters.

(**A**) Reporter strains with luciferase operon *luxABCDE* fused to *bmrC, vmlR,* or *tlrB* promoter in the wild type *B. subtilis* strains were grown for 16 hours with 1 μM chloramphenicol. Luciferase signal is normalized to *B. subtilis* grown without chloramphenicol. (**B-E**) The strains were spotted on agar plates without (-) or with 1μM Cm (+). Pictures were taken with phase contrast (left) and chemiluminescence (right) at different time points: (**B**) 6 hr, (**C**) 9 hr, (**D**) 24 hr and (**E**) 48 hr. Images in B-E are inverted. The luminescence signal appears grey to black with increasing intensity.

Table 1. Antibiotic resistance genes upregulated upon exposure to subinhibitory concentrations of chloramphenicol.

	Function	**Resistance**	**6hr (log2fold)**
bmrCD	Heterodimer ABC transporter	‡Phleomycin, bleomycin (glycopeptides)	2.64, 2.26
tlrB	23S rRNA (guanine-N(1)-)-methyltransferase	Tylosin (macrolide)	2.12
vmlR	ABC transporter	virginiamycin M, lincomycin (glycopeptide)	2.27
mdr	multidrug-efflux transporter	Puromycin* (aminonucleoside), nerfloxacin*, tosufloxacin* (fluoroquinolone)	2.03
ytbDE	D - similar to antibiotic resistance E - putative aldo/keto reductase	Unknown	2.56, 2.48

‡Identified in this study.

*Predicted based on structure and sequence similarity but not experimentally demonstrated

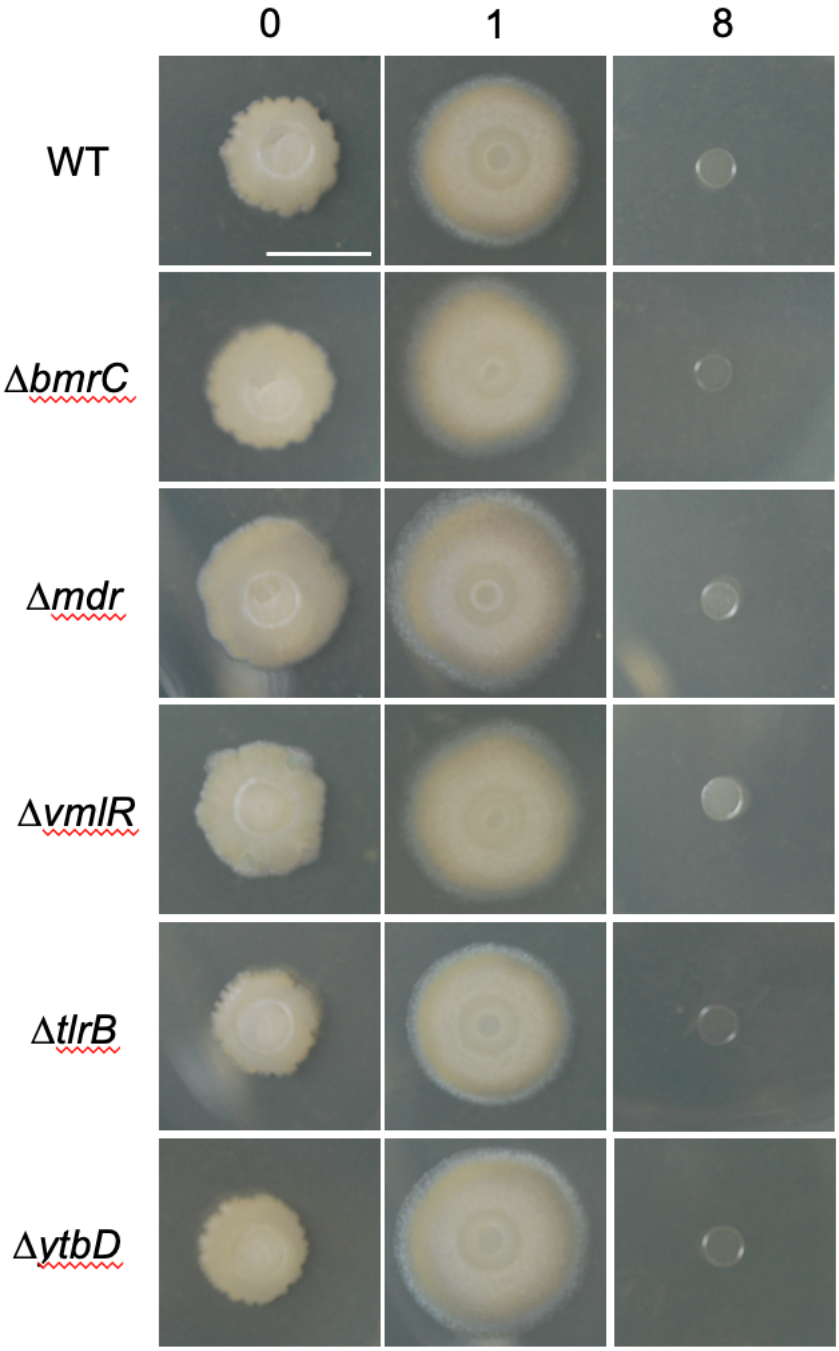


Figure S1. Five resistance elements (*bmrCD*, *vmlR*, *tlrB*, *mdr* and *ytbDE*) that respond to chloramphenicol (Cm) at 6 h were deleted. Wild type (WT) and each deletion strain were plated on the GYM7 plate with a range of subinhibitory concentrations of chloramphenicol (0-8 μM). Pictures were taken at 24 h.

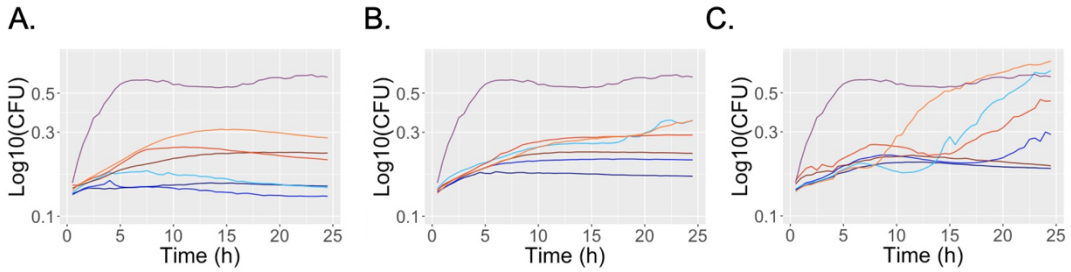


Figure S2. Preexposure to chloramphenicol provides slight increased resistance to antibiotics.

Growth inhibition assay of *B. subtilis* in (**A**) chloramphenicol (**B**) tylosin and (**C**) lincomycin with and without preexposure to subinhibitory concentrations of chloramphenicol. *B. subtilis* (purple), *B. subtilis* without preexposure grown in (**A**) chloramphenicol (**B**) tylosin and (**C**) lincomycin (light blue: 12.5, medium blue: 37.5 and dark blue: 100 μM) and *B. subtilis* pre-exposed to 1 μM chloramphenicol and then grown in (**A**) chloramphenicol (**B**) tylosin and (**C**) lincomycin (orange:12.5, red:37.5 and brown:100 μM).

A.

BmrC

MFSVLKKLGW FFKAYWLRYT IAIVLLLAVN VIEMFPPKLL GNAIDDMKAG AFTAEGLLFY IGIFFVLTAA VYIMSYFWMH QLFGGANLME KILRTKLMGH
LLTMSPPFYE KNRTGDLMAR GTNDLQAVSL TTGFGILTLV DSTMFMMTIF LTMGFLISWK LTFAAIIPLP VMAIAISLYG SKIHERFTEA QNAFGALNDR
VLESVSGVRV IRAYVQETND VRRFNEMTAD VYQKNMKVAF IDSLFEPTVK LLVGASYLIG LGYGAFLVFR NELTLGELVS FNVYLGMMIW PMFAIGELIN
VMQRGNASLD RVNETLSYET DVTDPKQPAD LKEPGDIVFS HVSFTYPSST SDNLQDISFT VRKGQTVGIA GKTGSGKTTI IKQLLRQYPP GEGSITFSGV
PIQQIPLDRL RGWIGYVPQD HLLFSRTVKE NILYGKQDAT DKEVQQAIAE AHFEKDLHML PSGLETMVGE KGVALSGGQK QRISIARALM ANPEILILDD
SLSAVDAKTE AAIIKNIREN RKGKTTFILT HRLSAVEHAD LILVMDGGVI AERGTHQELL ANNGWYREQY ERQQLFTAEE GGAGA

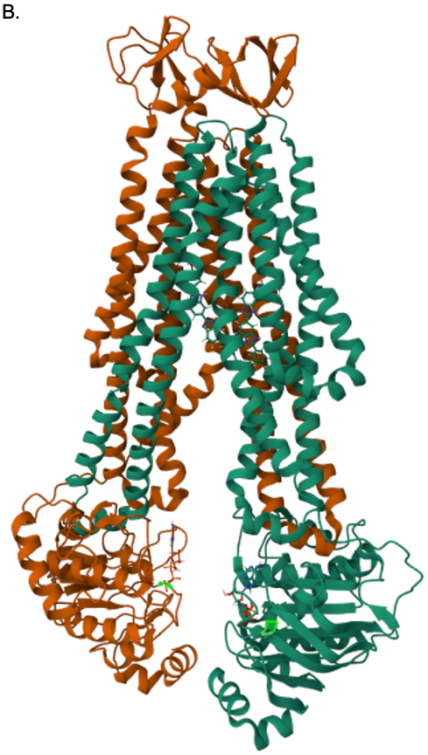
Walker A
*
Walker B

BmrD

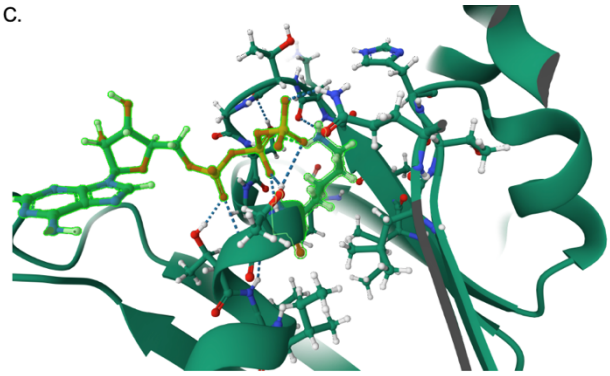
MKIGKTLWRY ALLYRKLLIT AVLLLTVAVG AELTGPFIGK KMIDDHILGI EKTWYEAAEK DKNAVQFHGV SYVREDRLQE PVSKAKEAHI YQVGMAFYFV
DQAVSFDGNR TVSDGKLTIT NGDKSRAYAA EKLTKQELFQ FYQPEIKGMV LLICLYGGLL VFSVFFQYGQ HYLLQMSANR IIQKMRQDVF SHIQKMPIRY
FDNLPAGKVV ARITNDTEAI RDLYVTVLST FVTSGIYMFG IFTALFLLDV KLAFVCLAIV PIIWLWSVIY RRYASYYNQK IRSINSDINA KMNESIQGMT
IIQAFRHQKE TMREFEELNE SHFYFQNRML NLNSLMSHNL VNVIRNLAFV CLIWHFGGAS LNAAGIVSIG VLYAFVDYLN RLFQPITGIV NQFSKLELAR
VSAGRVFELL EEKNTEEAGE PAKERALGRV EFRDVSFAYQ EGEEVLKHIS FTAQKGETVA LVGHTGSGKS SILNLLFRFY DAQKGDVLID GKSIYNMSRQ
ELRSHMGIVL QDPYLFSGTI GSNVSLDDER MTEEEIKNAL RQVGAEPLLK KLPKGINEPV IEKGSTLSSG ERQLISFARA LAFDPAILIL DEATAHIDTE
TEAVIQKALD VVKQGRTTFV IAHRLSTIRN ADQILVLDKG EIVERGNHEE LMALEGQYYQ MYELQKGQKH SIA

Walker A
*
Walker B

B.



C.



D.

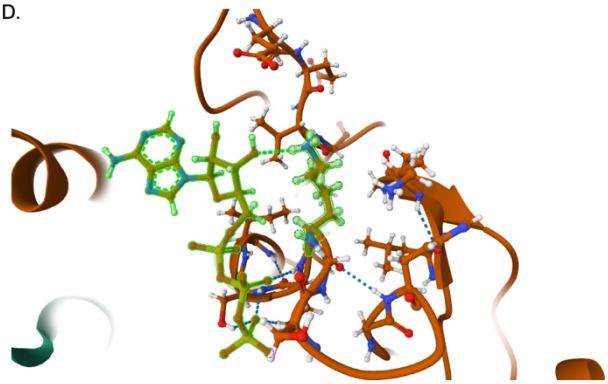


Figure S3. Walker A and B motifs of BmrCD

(**A**) BmrC and BmrD protein sequences with the Walker motifs highlighted in yellow. The sites mutated in this study (BmrC K377A; BmrD K469A) are denoted below the residue with and asterisk. (**B**) BmrCD protein structure via PDB:7M33. Zoomed in images of the ATP and edited lysine interaction in Walker motif A (**C**) and B (**D**).

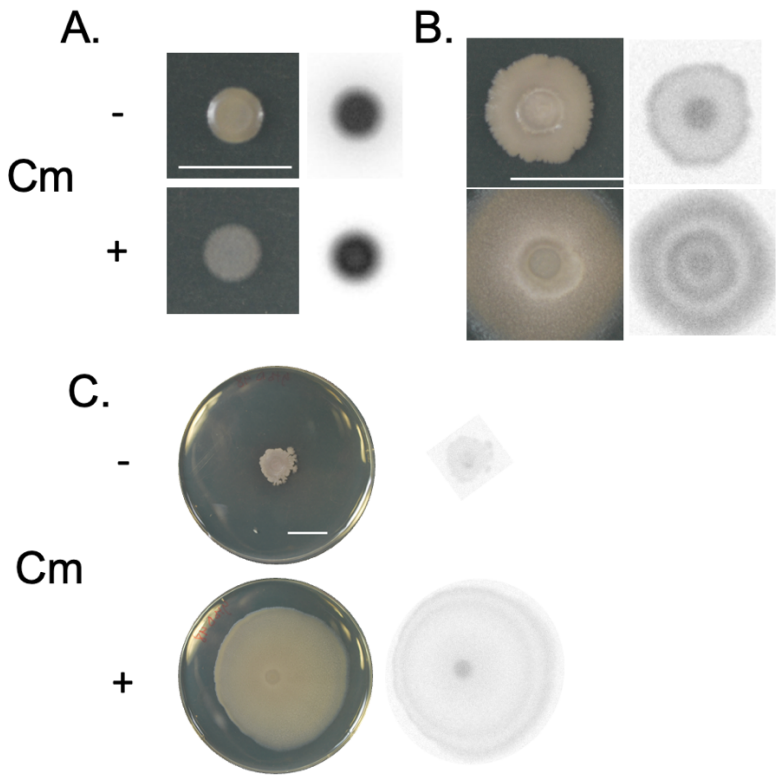


Figure S4. Gene expression of *ytbD* upon exposure to subinhibitory chloramphenicol using luciferase reporters.
Reporter strains with luciferase operon *luxABCDE* fused to the *ytbD* promoter in the wild type *B. subtilis* strains were spotted on an agar plate without (-) or with Cm (+). Pictures were taken with phase contrast (left) and chemiluminescence (right) mode at different time points: (**A**) 6 hr, (**B**) 24 hr and (**C**) 48 hr.

Table S1. Antibiotics tested for resistance via *bmrCD and ytbDE*.

Antibiotic	Class
Apramycin	Aminoglycoside
Kanamycin	Aminoglycoside
Hygromycin B*	Aminoglycoside
Erythromycin	Macrolide
Tylosin*	Macrolide
Spectinomycin	Aminocyclitol
Ampicillin*	Penicillin
Carbenicillin*	Penicillin
Lincomycin	Lincosamide
Tetracycline	Tetracycline
Chloramphenicol	Amphenicol
Rifamycin**	Ansamycin
Phleomycin**	Glycopeptide

**Only tested for ytbDE*

***Only tested for bmrCD*

Table S2. Bacterial strains used in this study

Strain	Genotype	Source
PDS0066	*B. subtilis* NCIB 3610 wild type	Laboratory collection
PDS0934	*B. subtilis* NCIB 3610 Δ*bmrCD:kan*	This study
PDS0986	*B. subtilis* NCIB 3610 Δ*vmlR*	This study
PDS0987	*B. subtilis* NCIB 3610 Δ*yxjB*	This study
PDS0988	*B. subtilis* NCIB 3610 Δ*mdr*	This study
PDS0989	*B. subtilis* NCIB 3610 Δ*ytbD*	This study
PDS0991	*B. subtilis* NCIB 3610 Δ*5:: kan(Δ vmlR, ΔbmrCD::kan, ΔyxjB, Δmdr, ΔytbD)*	This study
PDS0992	*B. subtilis* NCIB 3610 Δ*amyE*::P*hy-bmrCD-spec*	This study
PDS0993	*B. subtilis NCIB 3610 ΔbmrCD::kan ΔamyE::Phy-bmrCD-spec*	This study
PDS0994	*B. subtilis NCIB 3610 ΔamyE::Phy-bmrC*D*-spec (For bmrC, mutate AAA to GCA; K377A; For bmrD, mutate AAA to GCT; K469A)*	This study
PDS0995	*B. subtilis NCIB 3610 ΔbmrCD::kan ΔamyE::Phy-bmrC*D*-spec (For bmrC, mutate AAA to GCA; K377A For bmrD, mutate AAA to GCT;K469A)*	This study
PDS1204	*B. subtilis NCIB 3610 ΔamyE::PbmrCD-luxABCDE-kan*	This study
PDS1268	*B. subtilis NCIB 3610 ΔamyE::PvmlR-luxABCDE-kan*	This study
PDS1213	*B. subtilis NCIB 3610 ΔamyE::PtlrB-luxABCDE-kan*	This study

Table S3. Primers used in this study.

Primer	**Sequence (5'-3')**
lux-For	AGGAGGCTAGCCTATGAAATTTGGAAACTTTTTGC
lux-Rev (overhang paired with kan cassette)	CACCTCAAATGGTTCGCTGTCAACTATCAAACGCTTC
amyE-back-For	ATCCGTTTAGGCTGGGC
amyE-front-Rev (overhang)	CATAGGCTAGCCTCCTGGATCCCTCGAGGTCGACGAATTCTGTACTATTTCGATCAGACC
PbmrCD-For	GTCTGATCGAAATAGTACAATACGGTCAGCATGGAACAT
P*bmrCD*-Rev (overhang)	ATTTCATAGGCTAGCCTCCTTTGATAAACACGCAAATTCC
kan-For-new	CAGCGAACCATTTGAGGTGA
kan-Rev-new	CGATACAAATTCCTCGTAGGCG
P*yxjB*-For	GGTCTGATCGAAATAGTACAGAAACAATCAGACCATATTAC
P*yxjB*-Rev	ATTTCATAGGCTAGCCTCCTCTGGTATCCCGCCAAC-3
P*vmlR*-For	GGTCTGATCGAAATAGTACAGACTCAAAACTCCTGCCTCA
P*vmlR*-Rev	ATTTCATAGGCTAGCCTCCTTTTGATTACAAACACTATAG
spec-fwd	CAACGTTCTTGCCATTGC
spec-rev	GATCCCCCTATGCAAGGGT
RBS+*bmrC*-fwd	aggaggctagcctATGTTTTCAGTTTTGAAAAAGC
bmrD-rev	CAGCAATGGCAAGAACGTTGTTATGCAATGGAATGTTTCTG
bmrC1-mutation-rev	TTGTCGTTGCTCCGCTCCCGGTTTTAC
bmrC2-mutation-fwd	CGGGAGCGGAGCAACGACAATTATTAAG CAGC
bmrD1-mutation-rev	TCGAGCTAGCTCCTGATCCGGTATGGC
bmrD2-mutation-fwd	CGGATCAGGAGCTAGCTCGATTTTGAATCTTC
lacI-fwd	GGTCTGATCGAAATAGTACACATGCAAGCTAATTCGGTGG
lacI-rev	TGCTCACATTTaccctcgagTAATGGATTTCCTTACGCGA
P*hy*-fwd	ctcgagggtAAaTGTGAGCa
P*hy*-rev	AAAACATAGGCTAGCCTCCTAAGCTTAATTGTTATCCGCTC
amyE-Back-fwd	AACCCTTGCATAGGGGGATCATCCGTTTAGGCTGGGCTGTACTATTTCGATCAGACC
amyE-Front-rev	TGTACTATTTCGATCAGACC