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

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

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

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

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

RESULTS

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

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

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

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

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

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

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

expression leads to phleomycin resistance, we placed expression of *bmrCD* under the control of an IPTG-inducible promoter (P*hyperspank*). Even in the absence of IPTG, we observed elevated resistance to phleomycin, which we suspect arises from background activity of the IPTG-inducible promoter. Therefore, we engineered a strain that expresses alleles of *bmrCD* that carry point mutations in the Walker A motifs of each gene[14], resulting in dysfunctional BmrCD due to loss of nucleotide binding (**Figure S3**). Expression of the mutant BmrC*D* was incapable of generating phleomycin resistance either in the presence or absence of IPTG (**Figure 2B**).

The BmrCD-dependent resistance to phleomycin is moderate in our plate assays. Because phleomycin belongs to the glycopeptide class of antibiotics, we asked whether BmrCD can transport other structurally similar antibiotics. We selected representatives of two types of glycopeptide antibiotic, bleomycin and vancomycin, bleomycin being more closely related to phleomycin. Filters embedded with each antibiotic revealed that BmrCD confers resistance to both phleomycin and bleomycin, but not vancomycin (**Figure 2A**). The level of antibiotic resistance to bleomycin is greater than that to phleomycin, suggesting possible selectivity of the BmrCD transporter. Consistent with the phleomycin result, chloramphenicol exposure led to increased bleomycin resistance, and overexpression using the P*hy-bmrCD* strain led to bleomycin resistance with or without IPTG induction. Vancomycin remained sensitive under identical conditions of chloramphenicol exposure and *bmrCD* overexpression.

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

A shared feature of four of the chloramphenicol-induced resistance functions is their regulation using transcriptional attenuation controlled by translational pausing of an upstream leader peptide[6,10,16,21]. Using the upstream ORFs described in previous publications, we input the nucleotide sequences upstream of *bmrCD*, *vmlR*, and *tlrB* into RNAfold to display the similarities and differences of the uORFs of these three genes (**Figure 3**). The *bmrCD* genes contain two regulatory regions with the 351 bp before the *bmrC* start site: *bmrB* which contains the anti-anti-terminator, pause hairpin (anti-terminator), and terminator hairpins[6] and *bmrX* which contains a cleavage site for an endoribonuclease[9]. Like the uORF of *bmrCD*, the uORF of *vmlR,* which starts 219 nt before the start site, also contains and anti-anti-terminator hairpin, pause hairpin, and terminator hairpin[21]. Unlike *bmrCD* however, *vmlR* also contains another hairpin which blocks the Shine-Dalgarno sequence of *vmlR*. The uORF of *tlrB* on the other hand forms one long hairpin starting 178 nt upstream of the translation start site and contains both the Shine-Dalgarno and transcription start site to block all transcription and translation[16]. In each case, upon antibiotic exposure the ribosomes stall at upstream ORFs, which generates promoter structures permissive for transcription of the downstream gene or operon (**Figure 3**). Although each system provides resistance to a specific class of antibiotic, chloramphenicol exposure leads to the induction of all three through a general reduction in translation activity.

B. subtilis exhibits different spatiotemporal patterns of gene expression in biofilms, swarming populations, and in chloramphenicol-induced sliding populations on solid media[12,23,24]. We hypothesized that the induced expression of the antibiotic resistance genes may occur in only a subpopulation, transiently, or both. If true,

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

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

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

MATERIALS AND METHODS

Strains, primers, and media

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

Construction of luciferase reporter strains

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

Sliding motility assay

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

Preexposure Assay

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

Plate reader Assay

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

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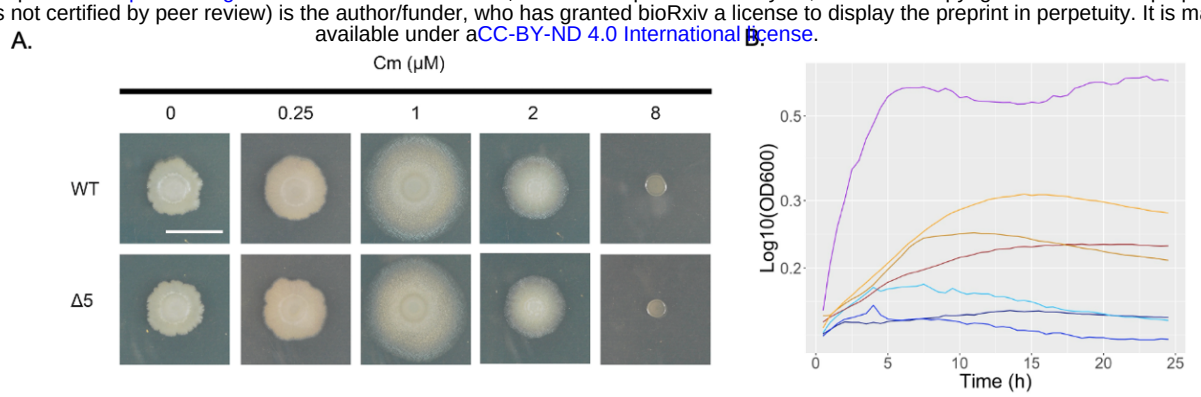


Figure 1. Deletion of resistance genes does not change the sensitivity to chloramphenicol.

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).

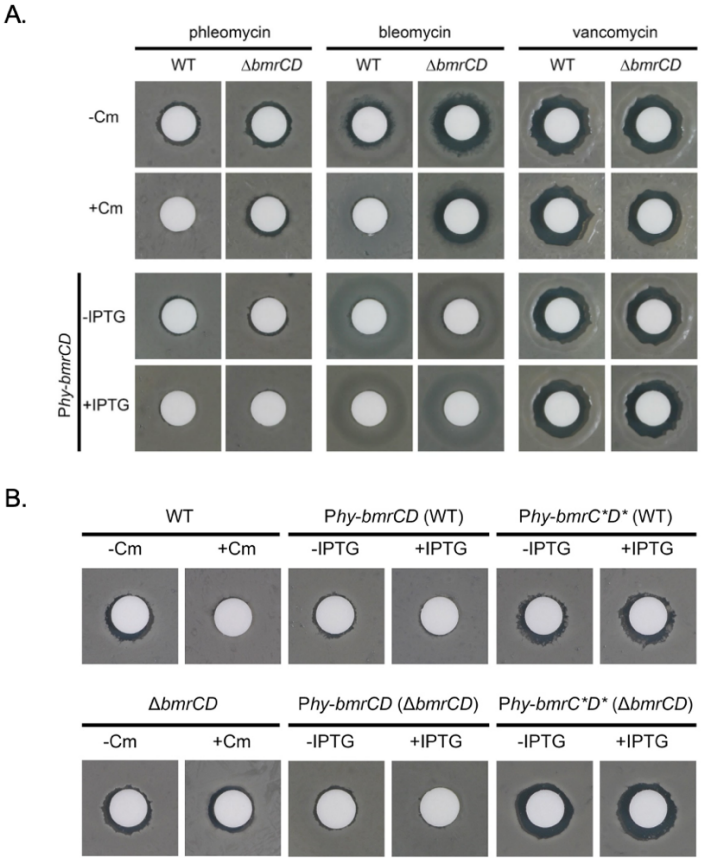


Figure 2. BmrCD provides resistance to phleomycin and bleomycin.
Deletions of bmrCD have increased sensitivity to phleomycin and bleomycin. (**A**) WT and Δ*bmrCD* strains (OD600=1.0, 100 µL) (top) and WT and Δ*bmrCD* strains with P*hy-bmrCD* inserted at the *amyE* locus (OD600=1.0, 100 µL) (bottom) 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.

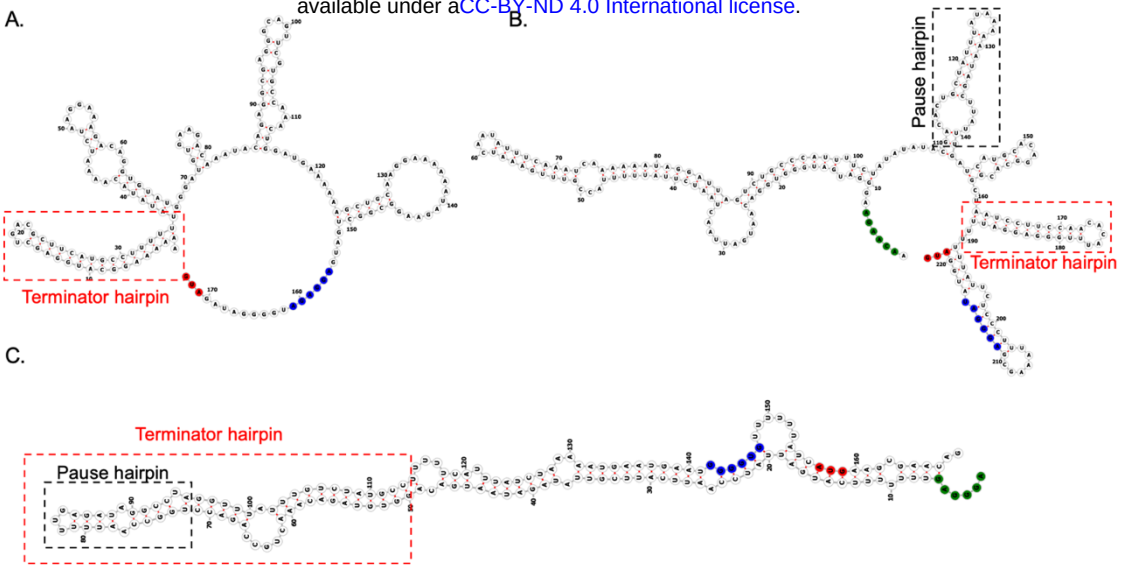


Figure 3. Predicted repressed conformations of the upstream regions of *tlrB*, *vmlR*, and *bmrCD*.

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.

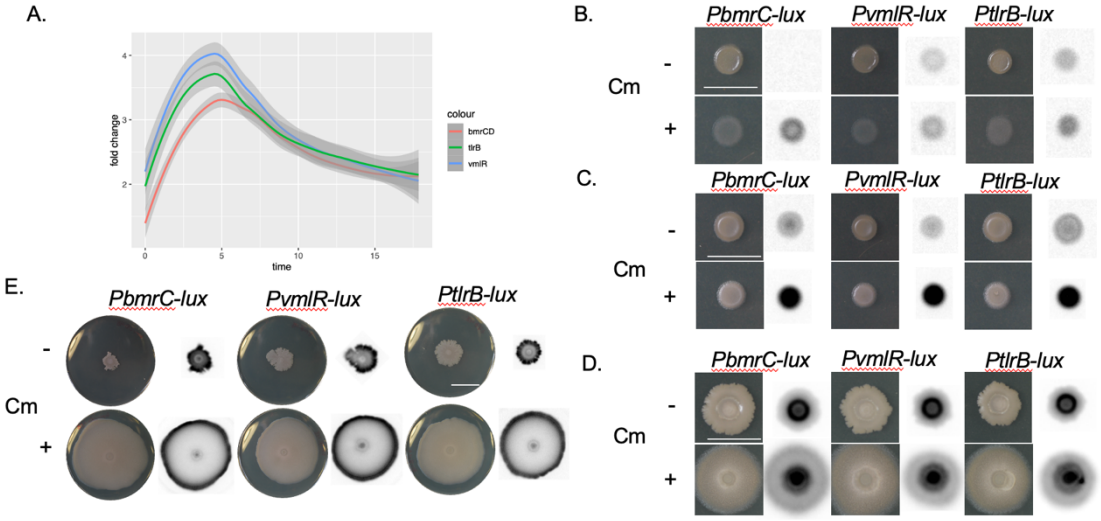


Figure 4. Gene expression of antibiotic resistance genes upon exposure to subinhibitory chloramphenicol using luciferase reporters.

(**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

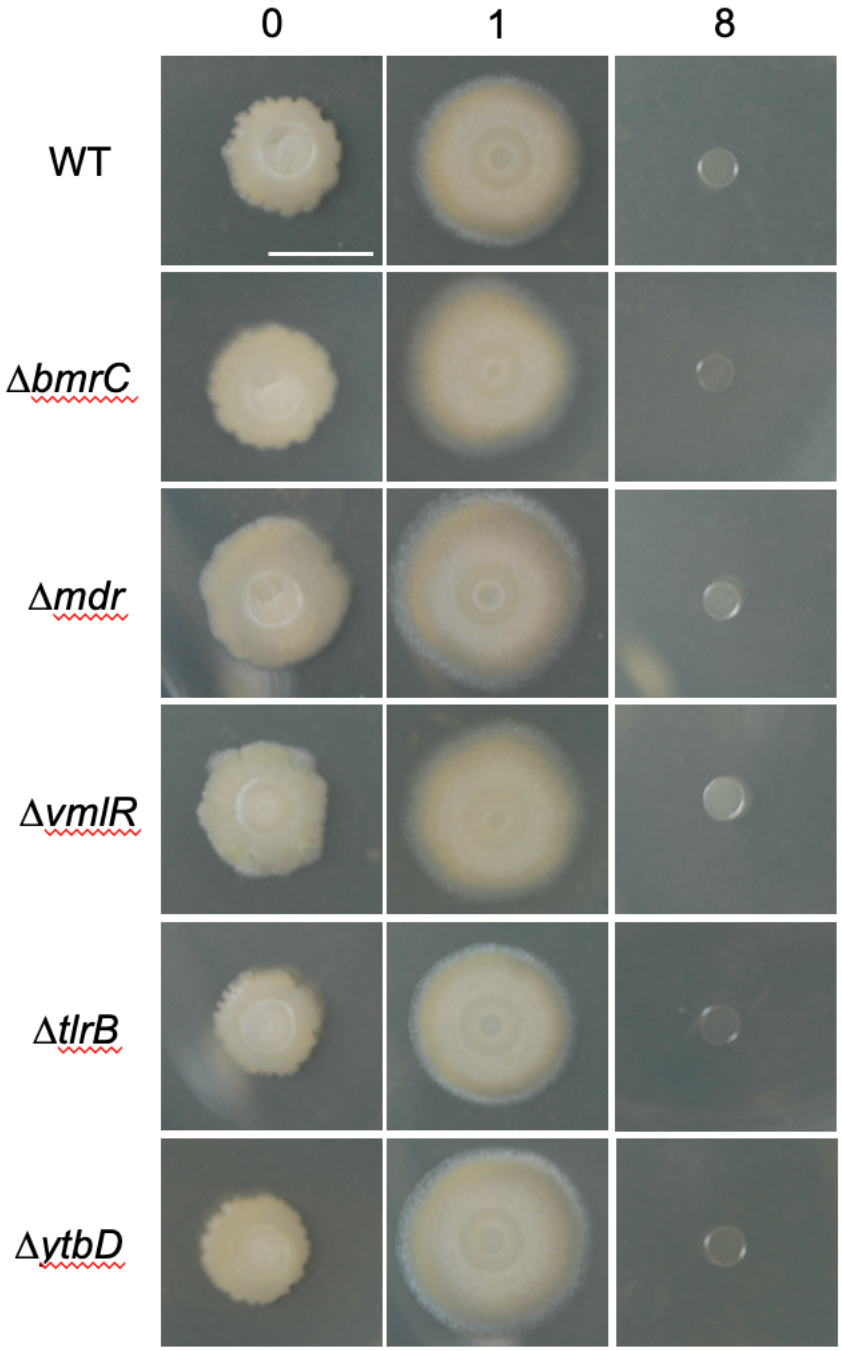


Figure S1. Five resistance elements (*bmrCD*, *vmlR*, *tlrB*, *mdr* and *ytbDE*) that respond to chloramphenicol (Cm) at 6 h were deleted. Wild type (WT) and each deletion strain were plated on the GYM7 plate with a range of subinhibitory concentrations of chloramphenicol (0-8 μM). Pictures were taken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

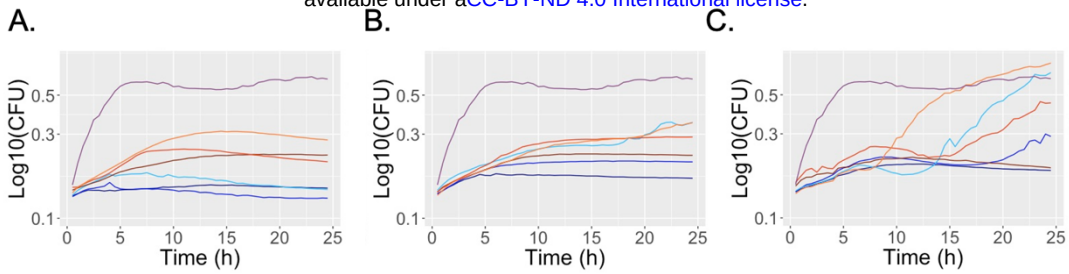


Figure S2. Preexposure to chloramphenicol provides slight increased resistance to antibiotics.

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

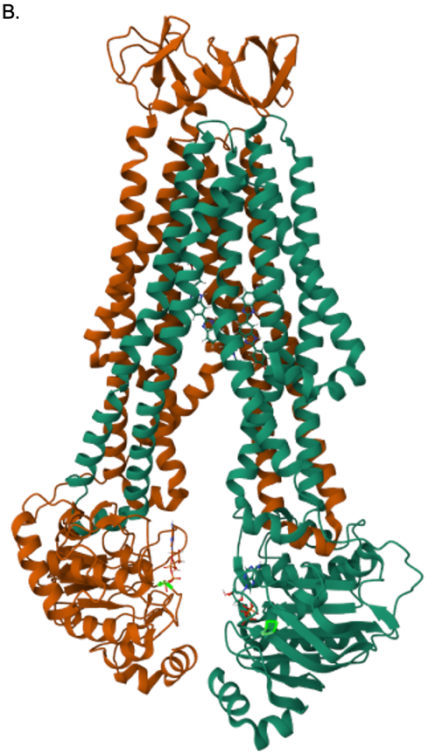
VSAGRVFELL EEKNTEEAGE PAKERALGRV EFRDVSFAYQ EGEEVLKHIS FTAQKGETVA LVGHTGSGKS SILNLLFRFY DAQKGDVLID GKSIYNMSRQ

ELRSHMGIVL QDPYLFSGTI GSNVSLDDER MTEEEIKNAL RQVGAEPLLK KLPKGINEPV IEKGSTLSSG ERQLISFARA LAFDPAILIL DEATAHIDTE

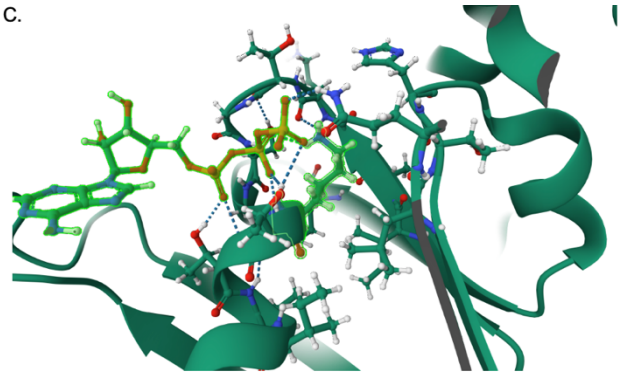
TEAVIQKALD VVKQGRTTFV IAHRLSTIRN ADQILVLDKG EIVERGNHEE LMALEGQYYQ MYELQKGQKH SIA

Walker A
*
Walker B

B.



C.



D.

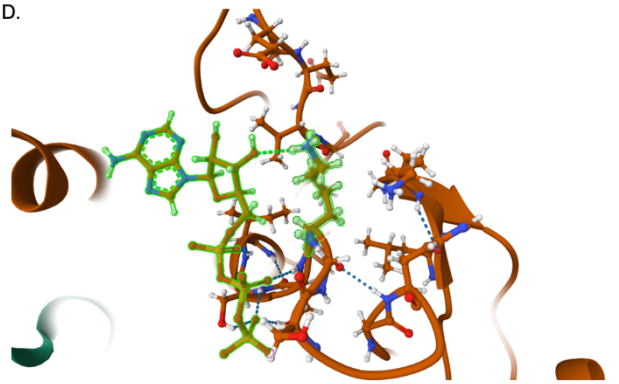


Figure S3. Walker A and B motifs of BmrCD

(**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**).

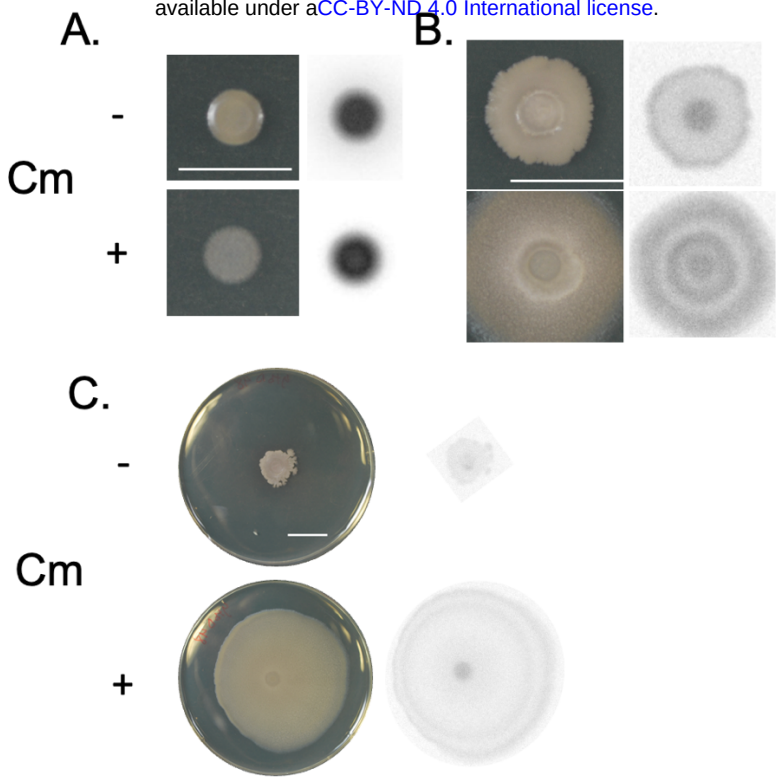


Figure S4. Gene expression of *ytbD* upon exposure to subinhibitory chloramphenicol using luciferase reporters.

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