IDENTIFICATION OF METABOLIC MECHANISMS FOR ANTIBIOTIC-INDUCED *BACILLUS SUBTILIS* MOBILIZATION

A Dissertation

by

YONGJIN LIU

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Chair of Committee,	Jean-Philippe Pellois
Committee Members,	David Russell
	Paul Straight
	Ryland Young
Head of Department,	Joshua Wand

August 2020

Major Subject: Biochemistry

ABSTRACT

Microbial communities have a widespread distribution on earth, ranging from soil to human gut. Each microbial community often contains various bacterial species. Interactions between species and their activities within microbial communities shape the environment where they live. To survive in such an environment, bacteria develop diverse strategies to interact with each other. To study these strategies, we developed a genetically tractable two-species model system composed of *Bacillus subtilis* and *Streptomyces venezuelae*. The interaction between the two species activates a mobile response in *B. subtilis*.

To uncover the molecular basis underlying this interspecies interaction, I first characterized the mobile response as sliding motility and identified the motility inducer as a ribosome-targeting antibiotics, chloramphenicol (Cm) and its brominated derivative at subinhibitory concentrations. Moreover, I determined that the sliding response is tied to the protein synthesis stress. To understand the connection between the subsequent responses after protein synthesis stress and the underlying genetic determinants, I used a combination of transcriptomics and metabolomics approaches to identify the key factors that contribute to the initiation of sliding motility. Results from these experiments suggest that a regulatory network leading to metabolic reprogramming governs the sliding response in response to antibiotic insults. Finally, I examined how *B. subtilis* survives in the presence of antibiotic-producing streptomycetes and found that the

intrinsic antibiotic resistance coupled with sliding motility enables *B. subtilis* to fit into their shared habitat. Overall, these findings indicate that bacteria can sense the invasion from competitors via detecting low-dose antibiotics and respond by adjusting their metabolic activities governed by complex gene regulation systems to control their physiological adaptations.

DEDICATION

To my wife, who always supports me in the last ten years. To my grandma and my parents, for raising me and educating me. To my sisters, who always believe in me.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Paul Straight, whose door is always open to me, for giving me the freedom to explore bacterial interactions in the beginning, guiding me with great patience along the way, and offering me an opportunity to observe microbial communities from different angles. Thank you for all the discussion on science, art and life.

I would also like to thank my committee members for their academic support, specifically, Dr. Jean-Philippe Pellois for teaching me not only science, but also responsibility, Dr. David Russell for help with mass spectrometry and his suggestion on my project, and Dr. Ryland Young for teaching me the critical thinking in the critical analysis class, bacteria and phage supergroup meeting and journal club over the years. I would like to acknowledge Dr. Jim Hu, who served on my committee for more than five years, for teaching me genomics and showing me his passion for science and his dedication to a better education during his life.

I would like to thank our collaborators, Dr. Carol LaFayette and Courtney Brake from the Department of Visualization, TAMU, for the training and help on acquiring nice movies and the discussion on art, and Dr. Amy Caudy from Stony Brook University, for running metabolomics samples.

I would like to thank Dr. Jennifer Herman for her advice and comments on my project, sharing bacterial strains and allowing me to use the fluorescence microscope, Dr. Larry Dangott for training on HPLC-MS/MS and being a great friend for many years, Dr. Jane Pishko, Dr. Megan Reynolds and Dr. Nicola Ayres for sharing teaching experience.

I would like to acknowledge Straight Lab members, current and former, especially Chris Hoefler and Reed Stubbendieck for all the training, suggestion and discussion on my project, and three great undergraduates who worked with me, Steven Kyle, Morgan Powers and Duha Eldow for assistance on my project.

Finally, I would like to thank my friends in the Biochemistry Program, Jesse Cahill, Kailu Yang and Tingting Zhao for their support through the years.

I am very grateful to all the nice people in the Department of Biochemistry and Biophysics. Their kindness and those sweet moments brought by them will always be a part of my treasured memory.

CONTRIBUTORS AND FUNDING SOURCES

This work was supervised by a dissertation committee consisting of Professors Paul Straight [advisor], Jean-Philippe Pellois, and Ryland Young of the Department of Biochemistry & Biophysics, and Professor David Russell of the Department of Chemistry at Texas A&M University. Professor Jim Hu of the Biochemistry & Biophysics at Texas A&M University also served as a member of the dissertation committee. All work for the dissertation was completed independently by Yongjin Liu.

Graduate study was supported by the National Science Foundation (http://nsf.gov/) (NSF-CAREER Award MCB-1253215) to Paul Straight, and the Robert A. Welch Foundation (http://www.welch1.org/) (Grant #A-1796) to Paul Straight. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of this dissertation. The contents of this dissertation are solely the responsibility of the authors and do not necessarily represent the official views of the National Science Foundation or the Robert A. Welch Foundation.

NOMENCLATURE

ABC	ATP-binding cassette
Cm	Chloramphenicol
EPS	Extracellular polysaccharide
HPLC	High performance liquid chromatography
Lm	Lincomycin
SKF	Spore-killing factor
SDP	Sporulation delay peptide
SPE	Solid phase extraction
T6SS	Type VI secretion system

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Bacterial interspecies interactions

Microbial communities exist almost everywhere on earth, ranging from soil to human gut[1–3]. Most of these microbial communities harbor diverse bacterial species of various taxa. Bacterial species is affected by the surroundings shaped by activities of other microbes[3]. It is well recognized that bacteria in microbial systems often exhibit behavior and metabolism different from those of bacteria cultured in isolation[3]. Competition for resources and space between different species is the driving force for bacteria to constantly adjust their physiological and metabolic state to adapt to local niches[3,4]. Moreover, it is increasingly evident that bacteria often exchange chemical signals with other species in the same community and guide their decision-making using this information[4]. In contrast, traditional studies of microbes in isolation often ignore these factors derived from other microbes. Therefore, studying how bacteria engage in interspecies competition is critical for understanding how bacteria coexist in the same habitat and how dynamic the whole community is. However, due to the vast complexity and large proportion of uncultured microbes within natural microbial communities[4], directly studying bacterial interspecies interaction in the context of natural settings is extremely challenging. Thus, developing a culture-based system that enables us to investigate detailed mechanisms underlying diverse bacterial interspecies interactions is required and necessary.

A mechanistic understanding of interspecies interactions has been expanded by many two-species interaction models, including fungi-bacteria interactions and bacteria-bacteria interactions. For example, in a *Pseudomonas* aeruginosa-*Candida albicans* model, *P. aeruginosa* kills *C. albicans* filaments by secreting virulence factors and forming biofilm on *C. albicans* filaments[5]. In a *Saccharomyces cerevisiae*-streptomycetes model, *Streptomyces venezuelae* colony expansion was triggered by *S. cerevisiae* via modification of medium pH and *S. venezuelae* produced volatile compounds to communicate with other streptomycetes[6]. For bacterial interactions, Muller *et al.* reported that *Bacillus subtilis* NICB3610 produced bacillaene to protect it from the predation by *Myxococcus xanthus*[7]. Also, it has been demonstrated that *Vibrio cholera* coexists with *Aeromonas hydrophila* in a microbial community by T6SS-mediated reciprocal antagonistic interactions[8]. These findings illustrate diverse mechanisms of interaction that we may expect to find by studying pairwise models. Although several multispecies interaction systems, such as the cheese model and THOR model, have been reported[9–11], they often do not resolve mechanisms underlying bacterial interactions.

Studies of a two-species interaction model system composed of soil Gram-positive bacteria, *B. subtilis* NICB3610 and *Streptomyces* species in our lab provide fascinating mechanistic insights into how bacteria interact with each other. For example, by culturing *Streptomyces sp.* Mg1 with *B. subtilis*, *S.* Mg1 caused the lysis of *B. subtilis*. The lysis agent was identified as a class of linearmycins[12,13]. It was later found

that linearmycin released from *S.* Mg1 could be delivered as a vesicle to mediate the lysis of *B. subtilis*[13]. To avoid killing by *S.* Mg1, *B. subtilis* formed mutants to survive in the shared habitat, via enhanced motility and elevated ability to export linearmycin[12]. Besides linearmycin, it was also shown that *S.* Mg1 secrets surfactin hydrolase to prevent the balding phenotype induced by surfactin produced from *B. subtilis*[14]. Results from previous studies suggest bacteria interact with their competitors directly or indirectly by sensing the environmental factors shaped by themselves.

Environmental factors for interspecies interactions

Bacteria sense environmental signals and cues to control their responses. Recent work provides insights on what such environmental factors can be and how they affect bacterial interactions[4]. These environmental signals often are specialized metabolites, secreted enzymes and extracellular vesicles[4]. Because most reported contact-dependent mechanisms mediate intraspecies interactions in Gram-negative bacteria, here the focus will be on secreted factors from bacteria.

Specialized metabolites

Unlike primary metabolism that is common to many biological taxa, secondary metabolism is unique within narrowly defined taxonomic groups. Specialized metabolites linked to secondary metabolism are different from those metabolites associated with the primary metabolism[15]. Specialized metabolites are usually produced at late stages of bacterial growth and not essential for bacterial growth under laboratory

conditions[15]. In few cases, specialized metabolites such as streptomycin and phenazines are required for bacterial growth[4,16,17]. The biological activities of specialized metabolites vary in different scenarios.

Antibiotics

Antibiotics are a common form of specialized metabolite. Antibiotics are the cornerstone of modern medicine. Hormesis, a dose-dependent phenomenon, is a significant feature of antibiotics[18]. Antibiotics exhibit antibiosis at higher concentrations, while at subinhibitory concentrations, antibiotics have profound effects on cellular transcription and metabolism[19]. Their different biological activities raise questions about their roles in nature. The view of antibiotics as signaling molecules is mainly due to the observation that antibiotic inhibitory concentrations are difficult to reach in nature. Several reports argued that antibiotics function as signaling molecules to induce bacterial physiological changes in bacteria instead of chemical weapons[20–22]. A recent study showed that instead of acting as cooperative signals, antibiotics at sub-lethal concentrations enable bacteria to detect and respond to competing species[23].

Antibiotics have been shown to induce diverse changes in bacterial gene expression and activities. Linares *et al.* reported that ciprofloxacin, tetracycline and tobramycin induce biofilm formation and tobramycin increases swimming and swarming motility in *P. aeruginosa*[22]. Using a library of promoter-*lux* reporter constructs, Goh *et al.* found that 5%-10% of genes are modulated by antibiotics with different structures

and modes of action in *Salmonella typhimurium*[24,25]. It is shown that several protein synthesis inhibitors induce global transcriptional response in *B. subtilis*[26]. Changes in gene expression and other activities such as virulence, motility and biofilm formation were also observed in other bacteria such as *E. coli*, *P. aeruginosa* and *S. pneunoniae*[4].

More importantly, several recent studies directly showed how antibiotics produced from one species can benefit bacterial competitive fitness during interspecies interaction in microbial communities. For instance, Bacillaene produced from *B. subtilis* has been shown to inhibit the production of another antibiotic prodigiosin in streptomycetes[27,28]and protects *B. subtilis* from predation by *Myxococcus xanthus*[7]. In human hosts, *Bacillus* bacteria produce fengycins to interfere with the quorum sensing in *S. aureus*, reducing the colonization of *S. aureus* in human[29]. The production of antibiotics from one species can also be beneficial to another species in a community. Meirelles *et al.* showed that *P. aeruginosa* secretes phenazines to increase ciprofloxacin tolerance in *Burkholderia cenocepacia* and *Burkholderia multivorans*[30].

Other types of specialized metabolites

In addition to antibiotics, several other specialized metabolites have been reported to confer benefits to bacterial survival and development. Siderophores are a class of iron chelators. The production of siderophores is upregulated under iron-limited conditions. Competing for limited iron source may escalate the competition between species. Surprisingly, it has been shown that *S. aureus* produces more siderophores in

the presence of *P. aeruginosa*, which promotes selection for *P. aeruginosa* cheaters[31]. The production of siderophore in this case can benefit both species. In another study of ion chelator, pulcherrimin has been reported to regulate *B. subtilis* biofilm formation in response to iron levels in the environment. In response to iron limitation, it is believed that pulcherriminic acid is synthesized and released into the extracellular environment, blocking biofilm expansion[32]. Numerous examples emphasize the role of iron-chelators in the interspecies interactions[33–35]. Quorum sensing signals have been shown to mediate not only intraspecies interaction but also interspecies interaction. For example, in AI-2 quorum sensing system, AI-2 has been reported to mediate interspecies interaction among diverse Gram-negative and Gram-positive bacteria and promote multispecies biofilm formation[4,36,37]. Because many gene clusters are only expressed under certain conditions[38], we can expect that bacteria may produce specialized metabolites that mediate diverse activities in bacterial communities.

Other types of secreted biological factors

Like specialized metabolites, most bacteria also secret enzymes and produce extracellular vesicles to enhance their competitive fitness during interspecies interactions. Secreted enzymes are believed to mainly act on the region close to cell surface[4]. It is not clear whether they can be transported into the inside of competitor cells. The functions of some secreted enzymes have been demonstrated in recent competition assays. For example, Hoefler *et al.* found that surfactin hydrolase released from *S.* Mg1 preserved the ability of *S.* Mg1 to sporulate by modifying surfactin

produced from *B. subtilis*[14]. Chen *et al.* reported that Esp protease secreted from *Staphylococcus epidermidis* blocked *S. aureus* biofilm formation by interfering with the murein hydrolase function and DNA release from *S. aureus*[39].

Due to different membrane structure between Gram-negative and Gram-positive bacteria, the extracellular vesicle biogenesis process is different. For Gram-negative bacteria, extracellular vesicles are generated by budding following pinching the outer membrane[4,40]. Another vesicle formation mechanism in Gram-negative bacteria is linked to prophage genes-induced lysis[41]. For Gram-positive bacteria, the detailed mechanism of how extracellular vesicles are produced remains unknown[4,42]. However, extracellular vesicles have been observed in several species such as *B. substilis*[43], *S. aureus*[44], *S. coelicolor*[45], and *S.* Mg1[13]. The role of vesicles may vary in different species, depending on the cargo delivered by vesicles, which can be lipids, proteins, nucleotides or signaling molecules. It has been reported that vesicles produced by *S. aureus* carry β-lactamases, thus preventing the enzyme from degradation[44]. Those vesicles also carry specialized metabolites such as actinorhodin in *S. coelicolor*[45] and linearmycin in *S.* Mg1[13]. Bacteria may employ specialized metabolites, secreted enzymes and extracellular vesicles individually or a combination of them when they interact with other competitors.

Bacteria respond to external stimuli by complex regulatory systems

Interspecies interactions often lead to observable physiological changes, such as motility, biofilm formation, sporulation and competence. In many cases, such

physiological changes are controlled by complex regulatory systems in bacteria. Numerous examples have been reported in diverse species[3,46]. Because *B. subtilis* is used in our two-species interaction model and the response of *B. subtilis* is studied in this dissertation, a few examples in *B. subtilis* are reviewed in this section.

Bacteria respond to external stimuli by modulating their transcriptomes via complex regulatory systems. Nicolas *et al.* measured the *B. subtilis* transcriptome in 104 conditions and found that around 66% variances in transcriptional activity could trace back to RNA polymerase sigma factors, suggesting flexibility and dynamics of gene regulation in response to a wide array of conditions[47]. In *B. subtilis*, changes in gene expression and the subsequent physiological changes are mainly governed by different regulators in a complex way. Extracellular signals affect *B. subtilis* cell fate through three master regulators, Spo0A, ComA and DegU[48] (Figure I-1). Regulation of responses include transformations of cells and populations that are observable. These include, motile populations, biofilms, sporulation and DNA uptake for transformation (competence)[49]. Each of these has a different regulatory system, although there is overlap in systems. In this section I will review the principal components of each system and how they are regulated and where they may overlap with other systems.

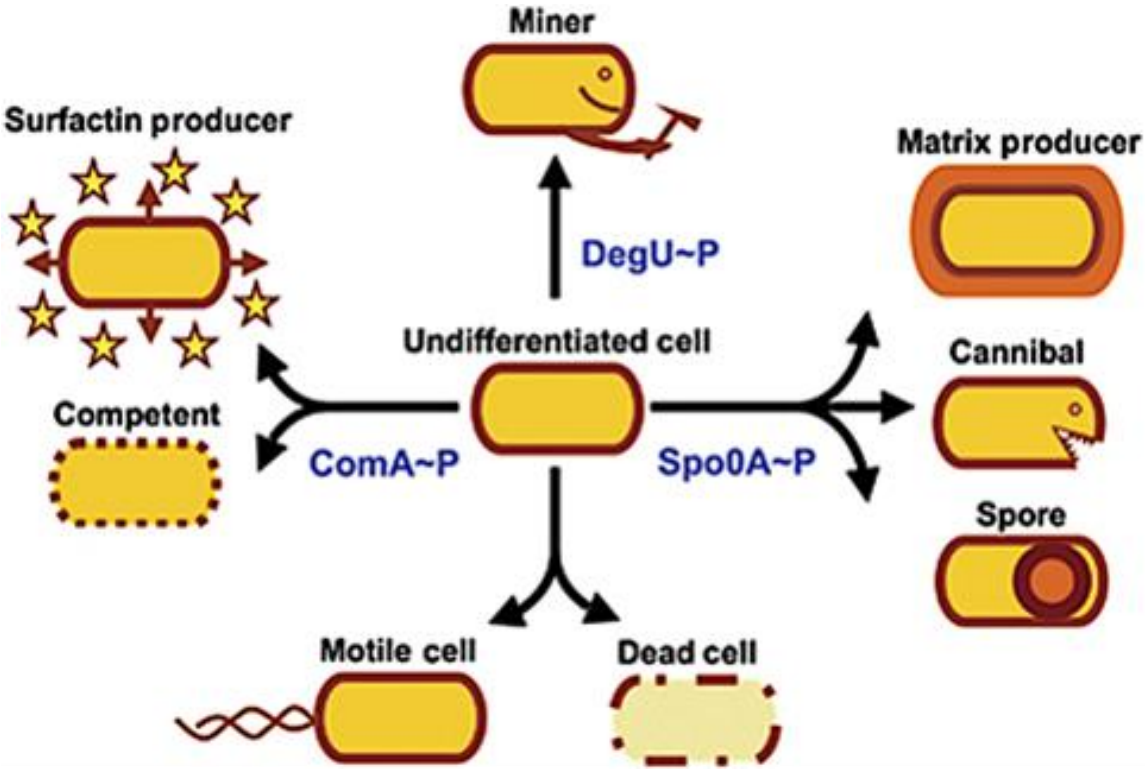


Figure I-1 *Bacillus subtilis* cell differentiation is governed by master regulators[48]. Three master regulators (in blue) Spo0A, ComA and DegU can be phosphorylated by different upstream kinases activated by extracellular signals and thus control cell fate by different degrees of phosphorylation levels. Arrows indicate the differentiation process. The motile cell in this figure is only linked to swimming and swarming motility. Figure I-1 was reprinted with permission from reference[48].

Motility-related regulation

In response to environmental stresses, motility enables bacteria to escape from hostile environments[12], occupy more resources and increase resistance to antibiotics[50,51]. *Bacillus subtilis* has three different types of motility: swimming, swarming and sliding[52]. Both swimming and swarming motility are flagellum-dependent. Swarming motility requires surfactin additionally, a surfactant to reduce surface tension. Sliding motility

depends on the presence of surfactin and extracellular polysaccharide(EPS)[53–56]. Neither swarming nor sliding is found in domesticated *B. subtilis* strains such as 168 and PY79, due to the loss of *sfp* gene encoding for surfactin synthesis enzymes[52]. Compared with swimming and swarming, the mechanism of sliding motility is not well understood. It was shown that sliding motility relies on the presence of potassium in the medium[55]. Because many mechanistic details are known, the focus will be on the regulation of swimming and swarming motility in this section.

In *B. subtilis*, *fla*/*che* operon comprises 31 genes responsible for the expression of flagella, sigma factor SigD and chemotaxis proteins[49,57]. It is induced when swimming and swarming are activated. One important regulator Spo0A controls the expression of those genes in *fla*/*che* operon. The levels of phosphorylated Spo0A (Spo0A-P) determine the expression levels of genes in *fla*/*che* operon. Spo0A phosphorylation is mediated by a phosphorelay system consisting of Spo0F and Spo0B. Phosphorylation of Spo0A is controlled by five kinases, KinA, KinB, KinC, KinD and KinE[58]. It was reported that at least two kinases KinA and KinB are involved in this system. It was shown that KinA and KinB have differential kinase activity on Spo0A in vitro[58,59], which results in different degrees of Spo0A activity. KinA and KinB transfer phosphoryl group to Spo0F, which can further transfer the phosphoryl group to Spo0B. Eventually, the phosphoryl group is transferred to Spo0A from Spo0B. The operon is inhibited by high levels of Spo0A-P, but activated by low levels of Spo0A-P[49] (Figur I-2).

One key regulator for the *fla*/*che* operon, SigD, controls the expression of *hag*, encoding the flagellar filament, flagellin. In addition, *motA* and *motB*, encoding flagellar rotation-required proteins, along with *lytA*, *lytD* and *lytF* genes functional for cell separation, are also activated by SigD[60]. *sigD* expression is controlled by another master regulator DegU. The phosphorylation state of DegU defines its activity on *sigD* expression level, with high activity for *sigD* expression at the unphosphorylated form[49,61]. Furthermore, SigD levels are also regulated by another transcription factor CodY[57].

The flagellum-dependent motility can be repressed by another regulator SinR given the costly process of expression of many genes. The activity of SinR is modulated by SlrR and SinI. By forming a complex with SlrR and SlrA, SinR regulates the levels of *sigD* expression[62], independent of $P_{fla/che}$ promoter. SinI can directly interact with SinR, antagonizing SinR activity[63](1993-Bai), while *sinI* expression is activated by Spo0A-P. Moreover, the SinR-SlrR complex can bind to the promoter of *lytABC* and *lytF*, regulating their expression[64]. These reports suggest motility is controlled by different layers of regulation in *B. subtilis*.

Biofilm-related regulation

Contrary to the mobile population, bacteria can form an immobilized structure consisting of single species or multispecies embedded in self-produced extracellular matrix, biofilms[65]. It has been shown biofilms promote bacterial survival when

environmental conditions are not favorable for bacteria. In *B. subtilis*, two major operons *epsA-O* and *tapA-sipW-tasA* are required for biofilm formation[49]. The expression of these two operons generates exopolysaccharides and amyloid TasA fibers, respectively. They hold cells together inside biofilms and are the major components of biofilms. Their expression are controlled by several regulators in *B. subtilis*. Spo0A is one of the most important regulators to regulate entry to biofilm formation. As discussed in the motility section, Spo0A activity is controlled by the phosphorylation state. When Spo0A-P reaches the low threshold levels, it regulates biofilm formation through two parallel pathways involved with SinR and AbrB, respectively[66]. In the pathway associated with SinR, SinR can directly repress the expression of both *eps* and *tapA* operons in the absence of its antagonist, SinI[63]. Spo0A-P activates the expression of *sinI*. When SinI interacts with SinR, it inactivates SinR, leading to the derepression of *eps* and *tapA* operons. Therefore, Spo0A regulates biofilm formation by affecting the expression of *sinI* in this pathway. In addition to SinI, another SinR anti-repressor SlrA has been reported, but *slrA* expression is activated by YwcC, instead of Spo0A-P[67,68]. Additionally, RemA and RemB were required for the activation of both matrix biosynthesis operons in SinR pathway[69]. It was shown that RemA serves as a transcriptional activator by directly binding to the *eps* promoter[70] (Figur I-2).

In another pathway related to AbrB, Spo0A-P controls AbrB in two different ways. First, Spo0A-P can repress the expression *abrB*. Second, Spo0A-P controls AbrB activity by enhancing the expression of AbrB anti-repressor AbbA. In both ways, AbrB-

mediated repression of *eps* and *tapA* operons will be relieved once Spo0A-P reaches the low threshold level[66]. Furthermore, it has been shown that positive feedback is generated through the relief of AbrB-mediated repression[71]. Because AbrB represses the expression of *sigH* and SigH activates several genes required for phosphorylation of Spo0A, the derepression of SigH leads to high levels of phosphorylation on Spo0A, thus ensuring the stimulation process can last for a period of time[65,66]. Overall, these reports imply regulation of biofilm formation is complex and mediated by multiple regulators, and several pathways present in the system enable *B. subtilis* to adapt efficiently to new niches. However, even in biofilms, there are some motile cells at the outer rim of biofilm, suggesting complex regulation mechanisms involved in biofilm formation[72].

Sporulation-related regulation

The non-motile cells in the center of mature biofilm often sporulate in response to nutrient starvation. Sporulation is a process that enables bacteria to adapt to hostile environments and preserve their DNA in spores. During this process, cells undergo dramatic morphological, physiological and biochemical changes. Unlike those genes controlling motility and biofilm formation in few operons, sporulation-associated genes are in distinct loci[73]. There are two major categories of sporulation genes, *spo* genes and *ger* genes. *spo* genes were originally designated based on whether mutations on these loci affect spore formation, while *ger* genes were originally named based on whether mutations on these loci affect spore germination. However, when spores germinate, those genes are still in the spore stage. Therefore, the distinction between these two

categories is not clear. There are more than 50 *spo* loci and more sporulation genes were reported based on a multitude of genetic studies. Several sigma factors such as SigH, SigF, SigE, SigG, and SigK have been reported to regulate the expression of sporulation genes at different stages[49,73]. Phosphorylation of major regulator Spo0A is critical to the initiation of sporulation, thus the role of Spo0A and its regulation at the initiation stage will be reviewed here.

The levels of Spo0A-P is gradually increased. When the low threshold levels of Spo0A-P are reached, Spo0A-P triggers the expression of two operons: eight-gene *skf* operon related to the expression of sporulation killing factor, and three-gene *sdp* operon related to the expression of sporulation delaying peptide[49,74]. These two factors kill the sibling cells and delay the factor-producing cells to initiate sporulation. Specifically, the sibling cells can be lysed by the action of SKF, releasing nutrients from some sporulating cells not completely committed to sporulation. The product from *sdp* operon is proposed to increase ATP production and lipid oxidation. Together, they delay the sporulation of SKF and SDP-producing cells in a *B. subtilis* population. When the high threshold levels of Spo0A-P are achieved, Spo0A-P activates the sporulation genes through a cascade of sigma factors[49,73] (Figure I-2).

Competence-related regulation

The regulatory system of competence in *B. subtilis* has some overlaps with motility, biofilm and sporulation regulation systems. Bacterial competence is a

physiological status in which bacteria take up foreign DNA. *Bacillus subtilis* can develop natural competence. Several regulators such as Spo0A, SigH, AbrB, ComK and DegU, have been identified in mutation studies of competence in *B. subtilis*[75]. ComK is the major regulator for competence. The expression of *comK* is activated by the ComXPA quorum-sensing pathway. ComX is a pheromone, which activates kinase ComP. Active ComP further phosphorylates ComA. Phosphorylated ComA activates the expression of ComK[76]. Moreover, the expression of *comK* is subject to a positive feedback loop[49]. ComK was also reported to inhibit the expression of *spo0A*, thus enabling *B. subtilis* to control the cell fate at the competent state[49]. More studies about how ComK activity is regulated by different regulators have been reported recently[49] (Figur I-2).

The crosstalk between these different regulatory networks in *B. subtilis* suggests that the regulation in response to most stimuli in the environment is not unitary but a multilayered and orchestrated process. Such complex regulation also highlights the importance of linking environmental stimuli to subsequent responses. Many two-component systems, SigB-mediated general stress response, stringent response and posttranscriptional regulation under diverse stressed conditions were also reported[77,78], which further complicates the regulatory networks in *B. subtilis*.

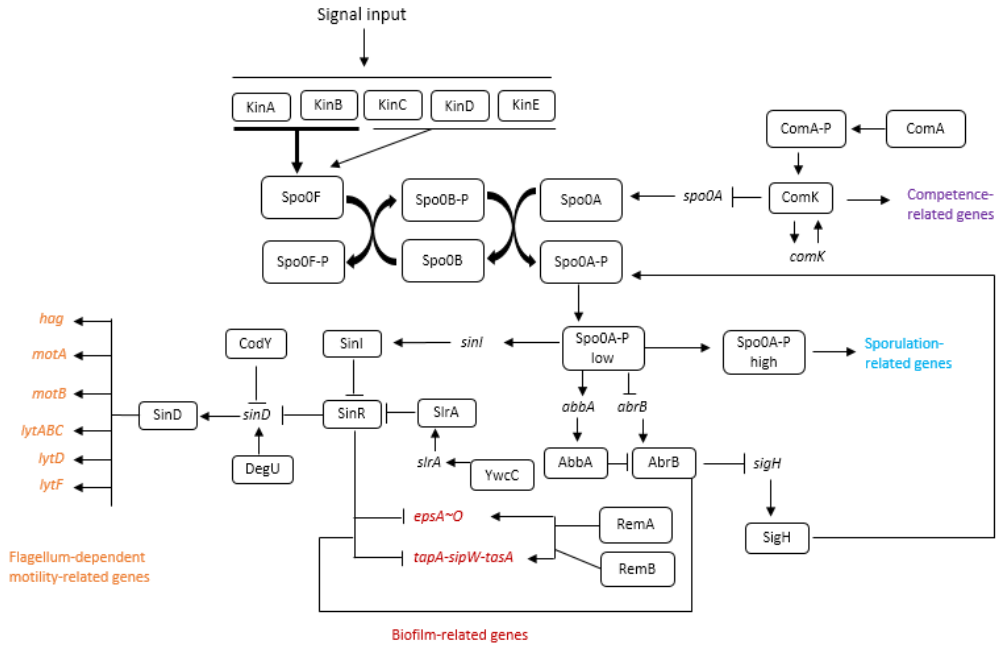


Figure I-2 Regulatory networks that control different responses in *B. subtilis.* Arrows indicate activation. T-bars indicate repression. Rounded rectangle boxes indicate proteins. Genes are italic texts. Genes related to different responses are marked with different colors.

In summary, *B. subtlis* senses diverse environmental conditions and responds by regulating gene expression through multiple and overlapped regulatory systems. However, such transcriptional changes are not sufficient to explain how bacteria adapt to new environments[79,80–82]. Other approaches will be needed to analyze how the adaption systems work.

Metabolic reprogramming regulates bacterial fitness

Metabolomics approach provides a complimentary avenue for transcriptional profiling to explain physiological adaptations. As the final products of macromolecule

interactions at different levels, metabolites play a crucial role in environmental adaptation in bacteria. Metabolites have diverse biological activities by interacting with nucleotides and proteins in vivo. For instance, guanine can directly bind to one riboswitch that controls *xpt-pbuX* operon to regulate gene expression in the purine metabolism pathways in *B. subtilis*[77]. cAMP binds CRP to increase *lac* operon expression in response to low levels of glucose in *E. coli*[83]. Therefore, the activity of interconnected metabolic pathways is governed by regulatory gene functions that coordinate an adaptive network.

The systematic study of metabolites, termed metabolomics, has been performed extensively to search for distinct metabolite patterns in response to diverse conditions. In particular, recent research in bacteria mainly focuses on how the altered metabolism changes the resistance or sensitivity to certain antibiotics[84]. It was reported that the addition of carbon sources such as glucose and fructose enables *E. coli* and *S. aureus* persisters to become sensitive to aminoglycoside antibiotic treatment. The killing mechanism is related to increased proton motive force (PMF) via respiration, which enhances the uptake of aminoglycoside antibiotics[85]. Using a GC-MS-based metabolomics approach, Peng *et al.* and Want *et al.* found that several metabolites alanine, glucose or fructose were decrease in multidrug resistance *Edwardsiella tarda* cells. Importantly, sensitivity to kanamycin was restored upon the addition of these metabolites. The mechanism is also linked to increased NADH and PMF[86,87]. Recently, Yang *et al.* developed a “white-box” approach to combine biochemical screening,

network modeling with machine learning to investigate the killing mechanism of aminoglycoside antibiotics. They found that antibiotic treatment decreased adenine abundance, resulting in increased ATP demand through purine biosynthesis pathway[88]. The killing action was mediated by the enhanced central carbon metabolism activities and oxygen consumption due to changes in these metabolites. These findings suggest that reprogramming the metabolome enables bacteria to modulate their fitness. Therefore, the ability of bacteria rewiring metabolism transforms the way they respond to environmental stresses.

Unlike genomics, transcriptomics, and proteomics studies, existing approaches to probe metabolomics are limited by dynamic metabolic fluxes and technical challenges of metabolite labeling and detection[89]. The view that transcript abundance determines metabolic flux has been challenged by a recent study which indicates that the levels of metabolites are not proportional to corresponding enzymes in many metabolic pathways[80]. Thus, the integration of different omics approaches would be required to unveil the mechanisms underlying the response of bacteria to environmental factors.

Importance of studying interspecies interactions in microbial communities

Most microbial communities are composed of diverse species instead of a single species. Examples can be extensively found in the environment such as soil, and in the host such as gut. Subtle changes in the community may lead to a chain of events. Studying interspecies interactions provides insights into how interactions between

species may stabilize the whole community. Knowledge from such work could apply to the agriculture and medical field. For example, *Bacillus* strains have been used as biocontrol agents for some plants due to their ability to secret some specialized metabolites to control the growth of other competitor species[90]. Importantly, studying drug metabolism in the context of the whole microbial community would help us better understand the drug activity. For instance, prontosil, which was used to treat *Streptococcus pyogenes* infection, is converted to the active form by other gut bacteria[91]. The activity of prontosil might not be found using modern standard in vitro screening assays. Interestingly, Zimmermann *et al.* investigated the metabolism of 271 drugs with 76 bacterial strains derived from human gut microbiota in mice and found 176 drugs could be metabolized. This finding further emphasizes the importance to study bacterial communities[92]. Moreover, it was shown in a recent study that the interaction between *P. aeruginosa* and *Burkholderia* pathogen promotes the resistance of *Burkholderia* strain to ciprofloxacin. Therefore, understanding how bacteria in microbial communities interact with each other is important for developing strategies to fight against pathogens[30].

Despite decades of study in interspecies interactions, our knowledge of bacterial interactions is still rudimentary. In particular, in terms of target inhibition and subsequent responses, the mechanisms underlying those changes observed in the majority of interspecies interactions remain largely elusive, which dramatically limits our ability to understand microbial communities. Thus, in this dissertation, I employ a model system composed of *Bacillus subtilis* and *Streptomyces venezuelae* to ask how

they compete with each other and how such interaction affects their survival and development.

CHAPTER II ANTIBIOTIC STIMULATION OF A BACILLUS SUBTILIS MIRGRAGTORY RESPONSE[1]

Summary

Competitive interactions between bacteria reveal physiological adaptations that benefit fitness. *Bacillus subtilis* is a Gram-positive species with several adaptive mechanisms for competition and environmental stress. Biofilm formation, sporulation, and motility are the outcomes of widespread changes in a population of *B. subtilis*. These changes emerge from complex, regulated pathways for adapting to external stresses, including competition from other species. To identify competition-specific functions, we cultured *B. subtilis* with multiple species of *Streptomyces*, and observed altered patterns of growth for each organism. In particular, when plated on agar medium near *S. venezuelae*, *B. subtilis* initiates a robust and reproducible mobile response. To investigate the mechanistic basis for the interaction, we determined the type of motility used by *B. subtilis* and isolated inducing metabolites produced by *S. venezuelae. Bacillus subtilis* has three defined forms of motility, swimming, swarming, and sliding. *Streptomyces venezuelae* induced sliding motility specifically in our experiments. The inducing agents produced by *S. venezuelae* were identified as chloramphenicol and a

---

[1]Reprinted from Liu Y, Kyle S and Straight PD. Antibiotic stimulation of a *Bacillus subtilis* migratory response. mSphere 3(1): e00586-17. doi: 10.1128/mSphere.00586-17 under the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided original work is cited. Copyright ©2018 Liu *et al.*

brominated derivative at subinhibitory concentrations. Upon further characterization of the mobile response, our results demonstrated that subinhibitory concentrations of chloramphenicol, erythromycin, tetracycline, and spectinomycin all activate a sliding motility response by *B. subtilis*. Our data are consistent with sliding motility initiating under conditions of protein translation stress. This study underscores the importance of hormesis as an early warning system for potential bacterial competitors and antibiotic exposure.

Introduction

Bacteria have varied mechanisms to maintain fitness under competitive stress. Examples of competitive fitness mechanisms include type VI secretion systems or contact-dependent inhibition [93–95], and chemical mechanisms as exemplified by antibiotics and other specialized metabolites [23,96–99]. Resistance to a specific challenge also promotes competitive fitness through chemical or genetic modifications to a target or a toxin [10,14,100]. Additionally, adaptations to the physiology of cells within a population or community may alter susceptibility to varied competitive stresses. For instance, bacteria may induce biofilm formation [23], enter a persister state[101], or activate specialized metabolism in response to competitors [102–105]. One adaptive mechanism available to many species is motility, which provides bacteria the ability to physically relocate in the event of a competitive challenge [6,12,106–108]. In some cases, the response may be chemotactic, avoiding a toxic substance through receptor activation of motility controls. Other sensing or stress mechanisms that activate mobility are not well defined.

In one example, swimming and swarming motility are enhanced when *P. aeruginosa* is exposed to the antibiotic tobramycin, but the underlying mechanism is unknown [22]. How bacteria sense and respond to antibiotic stress is of particular interest for understanding the development of antibiotic resistance. Indeed a connection between motility and antibiotic resistance has been found, wherein resistance is elevated in some motile populations of bacteria [51,109].

Bacillus subtilis serves as a model for motility of Gram-positive bacteria. *B. subtilis* has three described mechanisms of motility: swimming, swarming, and sliding [50,52,55,110,111]. Swimming and swarming motility are driven by the action of flagella, which provide propulsion to the bacteria. Swimming *B. subtilis* use multiple, peritrichous flagella to move as single cells through aqueous media. When the surrounding medium is sufficiently viscous, *B. subtilis* cells join into rafts that use swarming motility to migrate across surfaces under the power of flagella from multiple cells [50]. The third type of movement, sliding, is flagella-independent motility driven by growth. Sliding is currently understood to depend upon multiple factors, including potassium, production of the lipopeptide surfactin, exopolysaccharides (EPS), and extracellular proteins BslA and TasA [54–56]. At the vanguard of a sliding population, combinations of surfactin-producing cells and EPS-producing cells cooperate to generate 'van Gogh' bundles characteristic of sliding on specialized media [54]. The coordinate activities of cell subpopulations within a colony indicate orchestration of multiple events to promote cooperative sliding. Some regulatory functions that contribute to sliding

mobility have been described, but the overall process is less clearly understood than either swimming or swarming motilities [56]. Additionally, other competitive functions may be coordinately controlled with mobilization of cells. In combination with resistance functions, a mobilized bacterial population potentially possesses multiple advantages for competitive fitness.

Here we describe a competitive interaction between *Streptomyces venezuelae* and *B. subtilis*. We observed that, when cultured with *S. venezuelae*, *B. subtilis* activates a motile response. First, we identified the type of motility as sliding. Second, we extracted an inducer of sliding motility from agar plates of *S. venezuelae* and to our surprise identified the inducer as the antibiotic chloramphenicol. At subinhibitory concentrations, many antibiotics possess stimulatory activity, triggering a response in exposed bacteria. This phenomenon, known as hormesis, has been studied for many species and antibiotics [19,21]. Prior studies have shown that subinhibitory concentrations of antibiotics induce responses in exposed bacteria, including changes in transcription, biofilm formation, persistence, and altered virulence [19,22,26,97,112–114]. While tobramycin was previously seen to enhance motility of *P. aeruginosa*, induction of motility from an otherwise non-motile population has rarely been reported [12,22,28,112]. In addition to chloramphenicol, we found other antibiotics that target the ribosome also induce motility. Targeted analysis of genes associated with translation stress and antibiotic resistance suggested that the sliding response occurs when ribosome function is perturbed. Based on these observations, we

suggest that *B. subtilis* engages a programmed motile response to competitive stress from subinhibitory antibiotic interference with protein translation.

To identify patterns of interaction between *B. subtilis* NCIB 3610 and *Streptomyces* species, we plated pairs of two species on a rich agar media in a cross-wise pattern. The spotting pattern enables assessment of differential interactions determined by the proximity of competing species. *Streptomyces venezuelae* reproducibly induced proximal spots of *B. subtilis* to initiate a migration across the agar surface (Figure II -1A). By contrast, other species (e.g. *Streptomyces lividans* and *Streptomyces coelicolor*) also induce mobilization, but do so with delayed timing and to a lesser extent relative to *S. venezuelae* [28]. In some cases (e.g. *Streptomyces aizunensis* and *Streptomyces* sp. strain Mg1), mobilization is not observed, either due to lack of induction, or because lysis observed upon co-culture disrupts mobilization [12] (Figure II -1A). Based on the pattern and robust reproducibility of *B. subtilis* mobility induced by *S. venezuelae*, this interaction was investigated further.

Results

Competitive interaction with Streptomyces venezuelae induces mobilization of Bacillus subtilis NCIB 3610

Two features evident in the observed pattern indicated a complex interspecies interaction. First, the initial migration of *B. subtilis* across the agar surface is oriented

toward the competitor *S. venezuelae* (**Figure II -1B**, **Movie II-1**). The surface characteristics change for the *B. subtilis* mobile population, which acquires a rough appearance in contrast to the parent spot. The difference in colony texture indicates a major transition in cellular organization, reminiscent of swarming motility or biofilms [50,115]. Second, as the mobilized population progresses outward toward adjacent *S. venezuelae* patches, it appears to be repelled (**Figure II -1A, Movie II-1**). The observed patterns of migration towards *S. venezuelae* suggested that *B. subtilis* responds to the presence of diffusible substances produced by *S. venezuelae*. Based on the observed interaction pattern, we sought first to define the type of motility used by *B. subtilis*, and second to identify inducing substances produced by *S. venezuelae.*

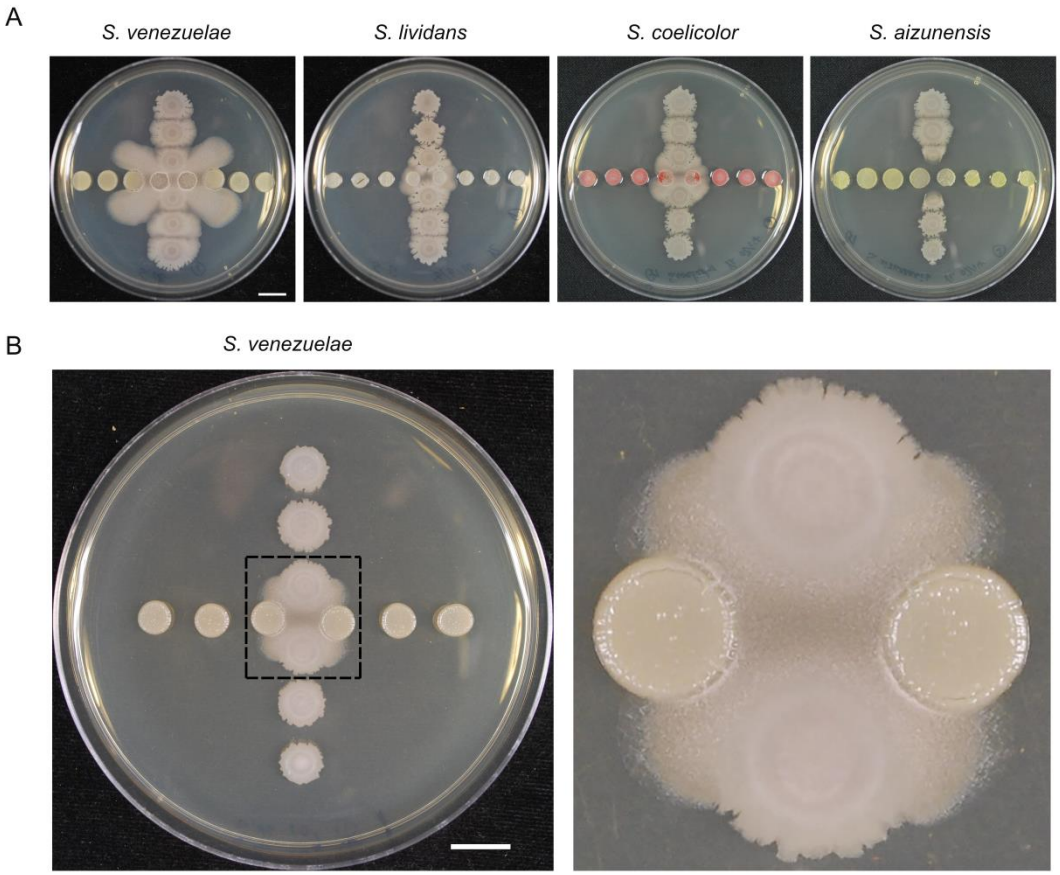


Figure II-1 *S. venezuelae* induces *B. subtilis* mobilization.
(A) Different species of *Streptomyces* were cultured with *B. subtilis* to identify patterns of interaction. *Streptomyces* species were spotted in the horizontal line and *B. subtilis* was in the vertical line. Pictures were taken at 40 h. (B) *S. venezuelae* (horizontal spots) induced proximal *B. subtilis* (vertical spots) to migrate across the agar surface in another assay, while this migration was not observed in the distal spots. The right panel is the enlarged view highlighting the mobile region inside the dashed box. The picture was taken at 18 h. Scale bars, 1 cm.

S. venezuelae induces flagella-independent sliding motility in B. subtilis

To understand the molecular basis for migration of *B. subtilis*, multiple approaches were used to identify the type of motility induced by *S. venezulae*. *Bacillus subtilis* migration depends upon the viscosity of the surrounding medium. For instance,

increasing agar concentrations limit the type of motility available. Agar concentrations above 0.3% and 1% prevent swimming and swarming motilities, respectively [52]. To characterize the motility of *B. subtilis* in response to *S. venezuelae*, we spotted both species to solid media of different agar concentrations. The motile response to *S. venezuelae* persisted up to an agar concentration of 2%, limiting the possibility of swimming or swarming as a basis for motility (Figure II -2A). A third type of motility, sliding, has been demonstrated on specialized media with agar or agarose concentrations typically less than 1% [54–56,116]. However, because the induced migration of *B. subtilis* was observed up to 2% agar, which has not been tested in sliding motility experiments, additional experiments were sought to determine the type of motility.

To identify genetic requirements for motility, we tested mutant strains that are defective for different types of motility. We first used a strain deficient in the flagellin protein (Δ*hag*), which is incapable of producing flagella [117,118]. Although the Δ*hag* mutation displays defects in colony morphology and motility, possibly due to overproduction of surfactin, the induction of migration by *S. venezuelae* was clearly observed using this strain [50](Figure II -2B). Therefore, the motility observed includes a flagella-independent component. Sliding motility depends on extracellular polysaccharides (EPS) and surfactin [54,55,111]. Mutant strains that are unable to produce a poly-N-acetylglucosamine component of EPS (*epsH*) or surfactin (*srfAA*) were unable to migrate in response to *S. venezuelae* [119](Figure II -2B). Because EPS and surfactin are both extracellular products, the single mutant strains, were combined to test for

extracellular complementation [54]. When the *B. subtilis epsH* and *srfAA* mutant strains are mixed and competed with *S. venezuelae*, the migration is restored. Consistent with these results, we identified disruptions in *eps* and *srf* genes in a transposon-mutagenesis screen for *B. subtilis* strains that failed to exhibit migration (Table 1). Together, these results strongly suggest that *B. subtilis* sliding motility is induced by *S. venezuelae*.

Table 1 Transposon mutagenesis results.

	Number	Frequency
Surfactin or EPS mutants	6	0.0575%
sigH	1	0.0096%
Unrecovered	2	0.0192%
Total number	10430	

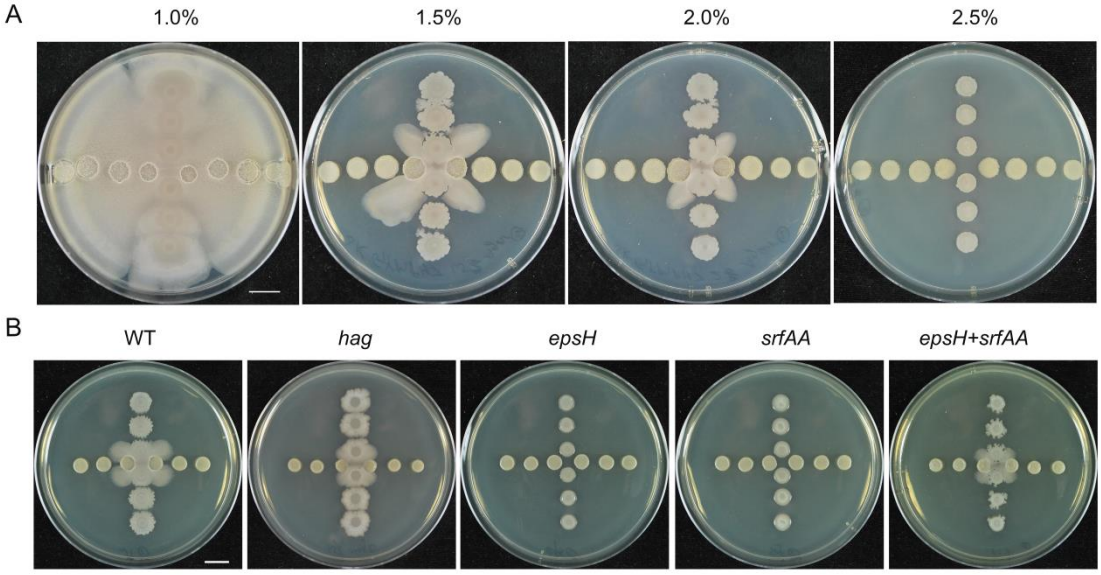


Figure II-2 Identification of *S. venezuelae*-induced mobility as sliding.
S. venezuelae* was spotted in the horizontal line in both (A) and (B). Pictures were taken at 48 h. (A) The mobilization induced by *S. venezuelae* was observed up to 2% agar. (B) Different *B. subtilis* mutants were cultured with *S. venezuelae*. The mobility of hag mutant was induced, but not observed in both *epsH* and *srfAA

Figure II-2 continued. mutants. However, when *epsH* and *srfAA* were mixed, the mixture was able to mobilize upon challenge with S. venezuelae. Pictures were taken at 24 h. Scale bars, 1 cm.

Identification of an inducing metabolite produced by S. venezuelae

The observed patterns of migration suggested that *S. venezuelae* produces a substance or substances that induce sliding by *B. subtilis*. One hypothesis is that a metabolite or enzyme secreted by *S. venezuelae* activates a specific response in *B. subtilis* cells, leading to the sliding motility observed. To identify an inducer substance, we extracted agar media after culturing *S. venezuelae* in isolation. Concentrated crude extracts were then added to wells adjacent to *B. subtilis* colonies to determine if inducing activity was present (Figure II -3A). Comparison to a medium-only control revealed robust inducing activity in the crude extract, which was subsequently fractionated using first solid-phase extraction followed by high-performance liquid chromatography (HPLC)(Figure II -S1). We then collected time-based HPLC fractions and screened for activity on agar plates. The inducing activity was abundant in a single fraction (Figure II -3A). The active fraction was analyzed by LC-MS/MS to identify candidate inducer metabolites (Figure II -S2A and II -S2B). An abundant signal identified by MS1 and MS2 analysis was consistent with that of monobromamphenicol, a variant of chloramphenicol where one chlorine atom is replaced by a bromine atom [120](Figure II -3B).

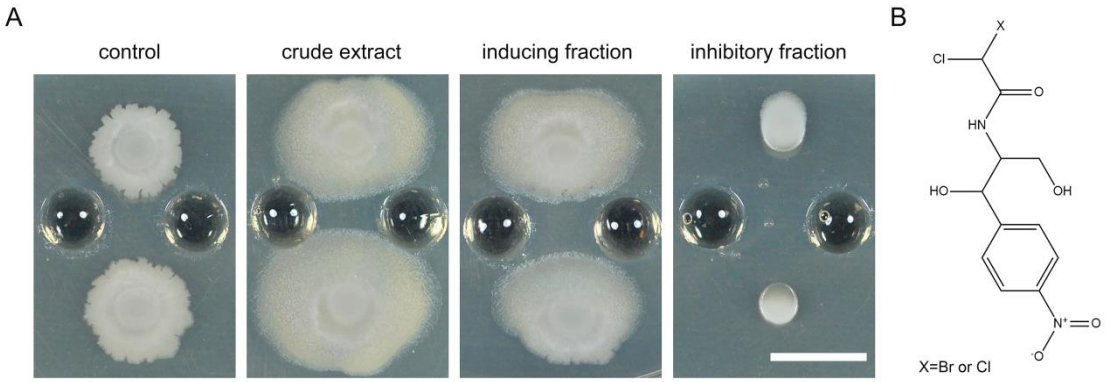


Figure II-3 Identification of monobromamphenicol as a sliding inducer. (A) Compared with the medium-only control, crude extract from *S. venezuelae* agar plates was loaded onto the wells near *B. subtilis* and induced robust sliding motility. All time-based HPLC fractions were collected and tested for activity. One fraction has the sliding inducing activity and one fraction has the growth inhibitory activity. Pictures were taken at 24 h. (B) The inducing fraction was brominated chloramphenicol (X=Br, as known as monobromamphenicol). The inhibitory fraction was chloramphenicol (X=Cl). Scale bar, 1 cm.

Streptomyces venezulae is well known as a producer of chloramphenicol, from which the antibiotic was originally identified [121,122]. Brominated and iodinated derivatives have been synthesized, but have not been described as natural products of *S. venezuelae* biosynthesis [120,123]. A possible explanation for the observed activity is that monobromamphenicol is a minor biosynthetic product of *S. venezuelae*, and that chloramphenicol is present at greater abundance in a separate fraction. We identified an inhibitory fraction among the HPLC fractions collected. The inhibitory fraction contained chloramphenicol as detected by LC-MS/MS (Figure II -S2C and II -S2D). We considered that chloramphenicol is primarily responsible for inducing *B. subtilis* sliding motility, but that upon concentrating the crude extract, the more abundant

chloramphenicol achieved inhibitory concentration and monobromamphenicol a stimulatory concentration. To determine whether chloramphenicol induces sliding mobility, the chloramphenicol-containing fraction was serially diluted, and each dilution tested for activity with *B. subtilis* (Figure II -4A). At a concentration approximately 8-fold lower than the parent fraction, chloramphenicol induced a sliding response by *B. subtilis* that was similar to challenge with *S. venezuelae* (Figure II -1B). Concentration-dependent differences in activity are described as hormesis, a phenomenon typically characterized by stimulatory effects of an agent at low doses and inhibitory or toxic effects of the same agent at higher concentrations [19,21]. To determine the corresponding concentration of chloramphenicol that is active for sliding induction, a commercially available source of pure chloramphenicol was serially diluted and added directly into the agar media. At 0.3 µg/ml chloramphenicol, which corresponds to an approximate concentration of 1 µM, we observed the maximal sliding response by *B. subtilis* (Figure II -4B and Movie II -2). These results demonstrate that subinhibitory amounts of chloramphenicol induce a widespread change in a population of *B. subtilis*, leading to mobilization of the colony.

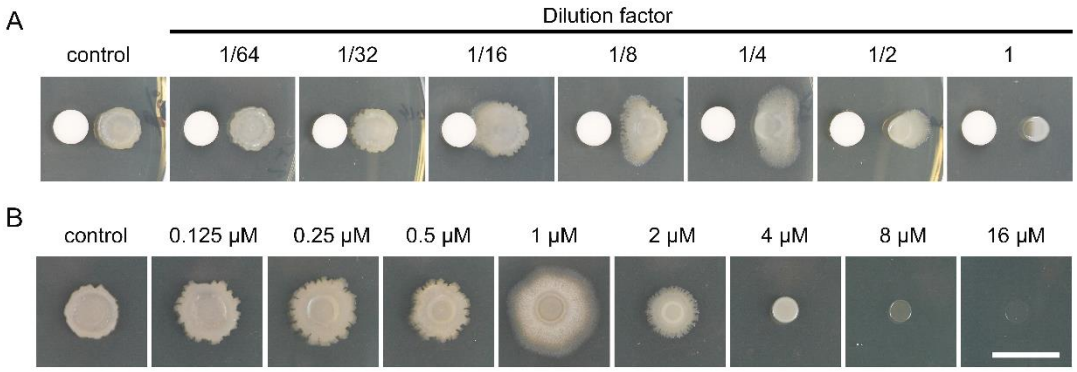


Figure II-4 Chloramphenicol induced *B. subtilis* sliding at subinhibitory concentrations.
(A) Chloramphenicol fraction was two-fold serially diluted and 10 μL of each dilution was applied onto a filter paper disc 0.6 cm away from *B. subtilis.* The control is the 40% methanol solvent. (B) Pure chloramphenicol was serially diluted and added to the agar plate. At 1 μM, the maximal sliding response was induced. The control is the plate without chloramphenicol. Pictures were taken at 24 h. Filter disc diameter, 0.6 cm; Scale bar, 1 cm.

Antibiotics that block translation induce B. subtilis sliding motility

To determine whether the sliding response was specific to chloramphenicol, we selected fourteen antibiotics to test for induction based primarily on their differing mechanisms of action. Serial dilutions of each antibiotic were spotted on filter discs placed adjacent to *B. subtilis*. In addition to chloramphenicol, three other antibiotics induced sliding mobility. The inducing antibiotics were tetracycline, erythromycin, and spectinomycin, which all target the ribosome and block protein translation (Table 2)[124–127]. Interestingly, no aminoglycoside antibiotic tested resulted in sliding mobility by *B.* subtilis, indicating that errors in translation do not trigger the sliding response. These

results led us to conclude that *B. subtilis* responds to some types of translation inhibitors at subinhibitory concentrations by activating sliding motility.

Table 2 Four of 14 tested antibiotics induced sliding.

Antibiotic	Motility Inducer	Target
Chloramphenicol	Yes	50S
Spectinomycin	Yes	30S
Erythromycin	Yes	50S
Tetracycline	Yes	30S
Apramycin	No	30S
Kanamycin	No	30S
Gentamycin	No	30S
Lincomycin	No	50S
Hygromycin	No	30S
Streptomycin	No	30S
Phleomycin	No	DNA
Novobiocin	No	DNA gyrase
Ampicillin	No	Transpeptidase
Rifamycin	No	RNA polymerase

In each case, the tested concentrations ranged from inhibitory levels to levels having no detectable effect

To determine whether the sliding response is dependent upon interaction of the antibiotics with the ribosome, as opposed to an unidentified cellular target, we investigated the effect of antibiotic resistance on sliding. First, a chloramphenicol resistant (Cm^R) *B. subtilis* strain, which expressed chloramphenicol acetyltransferase, was used to determine whether chemical modification of the antibiotic disrupted sliding. Acetylation of chloramphenicol interferes with binding of the drug to the peptidyl-transferase site on the ribosome [128–130]. The Cm^R strain did not induce sliding when challenged with chloramphenicol (Figure II-5A). Correspondingly, when wild-type *B.*

subtilis was treated with chloramphenicol acetate at an equivalent concentration where chloramphenicol induces sliding, there was no response (Figure II-S3). However, at elevated amounts (4-8× greater) chloramphenicol acetate was active for sliding induction, indicating that the resistance is overcome with greater amounts of the modified antibiotic. Second, to determine whether direct modification of the ribosome prevented sliding mobility, an erythromycin-resistant (Erm^R) *B. subtilis* strain was treated with inducing concentrations of erythromycin. The Erm^R strain expressed a methyltransferase that specifically methylates the 23S rRNA, which blocks erythromycin binding [131,132]. In comparison to wild type, the Erm^R *B. subtilis* did not induce sliding in response to erythromycin (Figure II-5B). Collectively, these results suggest that when present at subinhibitory concentrations, the sliding-inducer antibiotics are targeting the ribosome and presumably causing protein translation stress.

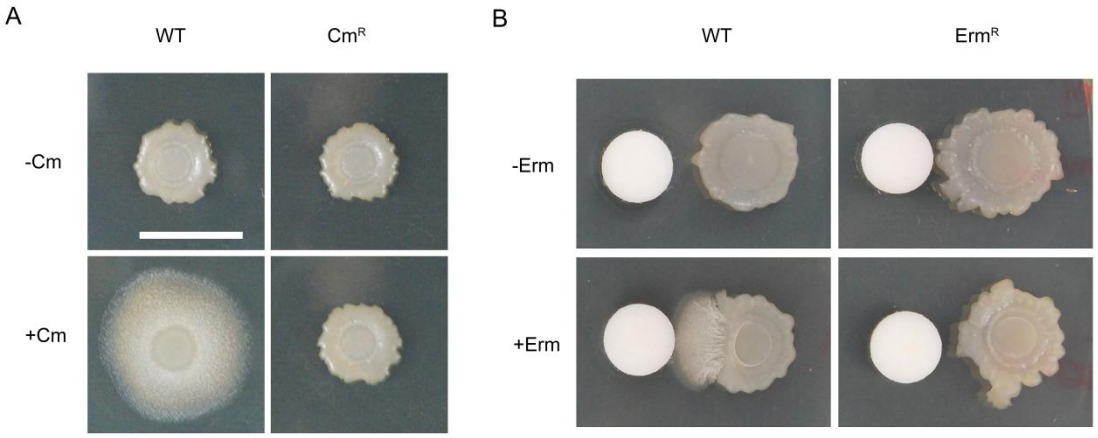


Figure II-5 The ribosome plays a key role in antibiotic-induced sliding.
(A) Wild type (WT) NCIB 3610 strain and chloramphenicol (Cm)-resistant strain CmR were spotted on the agar plate in the absence (-) or presence (+) of Cm. (B) Wild type (WT) NCIB 3610 strain and erythromycin (Erm)-resistant strain ErmR

Figure II-5 continued. were spotted on the agar plate in the absence or presence of Erm. Pictures were taken at 24 h. Filter disc diameter, 0.6 cm; Scale bar, 1 mm.

Induction of bmrCD by sub-inhibitory concentration of chloramphenicol is consistent with translation stress

Treatment of *B. subtilis* with chloramphenicol and other translation inhibitors at sub-inhibitory concentrations has been shown to affect gene expression [26,133,134]. Expression of several genes changed due to chloramphenicol exposure [26]. The *bmrCD* genes, which encode a multidrug efflux transporter, were subsequently shown to be dependent upon an upstream open-reading frame named *bmrB* [133]. The mechanism of expression control couples efficient translation of BmrB to transcription of downstream *bmrCD,* wherein disruption of translation by inhibitory antibiotics causes enhanced production of BmrCD. However, these prior studies investigated the laboratory strains *B. subtilis* 168 and 1A757 strains in liquid cultures where sliding would not be observed. To determine if undomesticated *B. subtilis* NCIB 3610 activates *bmrCD* expression in our sliding assays, the transcript abundance of *bmrCD* was monitored using quantitative reverse transcription PCR (qRT-PCR). Transcripts of *bmrCD* were elevated to a maximum of 12-fold over the untreated control abundance during the initial 12 hours of the experiment (Figure II-6A). The peak abundance of *bmrCD* transcript occurred between 6-12 hours. However, after 24 hours, when sliding motility is clearly observed, the *bmrCD* transcript abundance was restored to near wild-type levels. This pattern of *bmrCD* expression is consistent with the transient expression pattern observed

previously [133]. The elevated expression of *bmrCD* indicated that chloramphenicol at sub-inhibitory concentration was stressing protein translation, in accordance with the coupled transcription-translation of *bmrBCD*.

The induced transcription of *bmrCD* is not limited to chloramphenicol. Multiple antibiotics, all targeting the ribosome, were shown to also lead to elevated *bmrCD* expression when used at sub-inhibitory concentration [133]. Intriguingly, lincomycin was previously shown to induce *bmrCD* expression strongly at subinhibitory concentration but did not induce sliding at any concentration tested in our assays (Figure II-S4). This observation indicated independence between sliding motility and the effects of translation stress on expression of the BmrCD multidrug efflux pump. To determine whether *bmrCD* induction is required for sliding motility, *bmrC, bmrD*, and *bmrCD* mutant strains were challenged with sub-inhibitory chloramphenicol. Despite the absence of BmrCD, the sliding response was intact for the mutant strain (Figure II-6B and Figure II-S5). Therefore, the *bmrCD* genes are not required for sliding motility. To determine whether disrupting regulation of *bmrCD* would perturb the chloramphenicol-induced sliding, we generated a markerless deletion of the *bmrB* ORF, placing the *bmrCD* genes directly under transcriptional control of the *bmrB* promoter. When exposed to subinhibitory chloramphenicol, the *bmrB* mutant strain maintained the sliding response, further supporting the conclusion that *bmrCD* are not involved in sliding motility (Figure II-S5). Additionally, the mutant strains were not hypersensitive to chloramphenicol, either for sliding or growth (Figure II-6B and Figure II-S6). These

observations suggest that, while elevated *bmrCD* expression indicates translation stress, as yet unidentified events are the drivers of antibiotic-induced sliding motility.

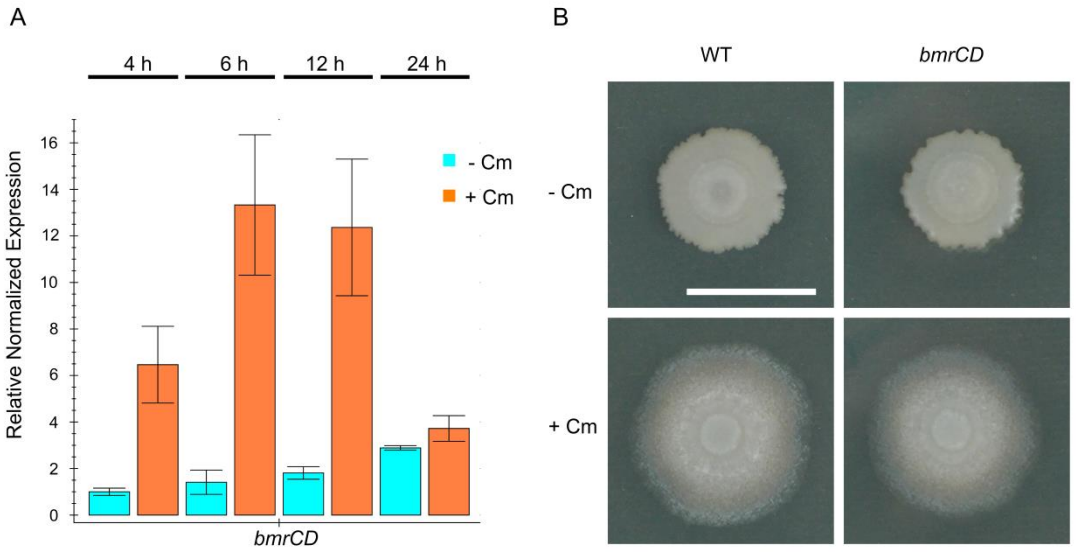


Figure II-6 *bmrCD* is related to translation stress but not required for sliding. (A) Quantitative RT-PCR of *bmrCD* transcript of wild type (WT) strain in the absence (-) and presence of chloramphenicol (Cm) at the indicated time points 4 h, 6 h 12 h and 24 h. Cq values were normalized to Cq for *gyrB*. Fold expression values are reported relative to the value for 4 h sample in the absence of Cm. (B) WT NCIB 3610 strain and a *bmrCD* deletion strain were spotted on the agar plate in the absence or presence of Cm. Pictures were taken at 24 h. Scale bar, 1 cm.

Discussion

Through tracking changes in colony morphology and mobility during competition between two species of bacteria, we observed that *S. venezuelae* induces sliding motility in *B. subtilis*. We found that exposure to low doses of monobromamphenicol and chloramphenicol induced mobilization of the *B. subtilis*

population. Subsequently, we found that multiple translation-inhibiting antibiotics induced *B. subtilis* sliding. The observed pattern of interaction is indicative of antibiotic hormesis. In this instance, exposure to low doses of translation inhibitory molecules triggers a mobilization of a *B. subtilis* population. The activation of sliding motility may provide a substantial competitive advantage to *B. subtilis*, enabling the cells to relocate rapidly and avoid inhibitory doses of antibiotics. Streptomycetes produce many translation-inhibiting antibiotics, consistent with our observation that sliding is frequently observed using pairs of *Streptomyces spp.* with *B. subtilis* NCIB 3610 (Figure II-1).

The perception of low doses of toxic or growth inhibitory substances provides an opportunity for bacteria to activate protective responses. For instance, biofilms provide a specialized niche for inhabitant bacteria, which alter their physiology and expression of resistance functions, and persisters are protected due to their paused growth and metabolism. Two described examples of antibiotic-protective responses are the formation of biofilms and persister cells, which lend adaptive resistance to the target organism [23,101,112,135,136]. In both cases, the outcomes are cells that become recalcitrant to antibiotic exposure. Upon exposure to subinhibitory translation stress, the outcome for *B. subtilis* is strikingly different. The cells engage a growth-dependent type of mobility, which provides a means to physically relocate a subpopulation. Thus, instead of preventing growth to avert antibiotic stress, *B. subtilis* is activating a growth-dependent mobilization. Induced motility has rarely been described in response to antibiotics.

Tobramycin was shown previously to enhance swarming motility of *Pseudomonas aeruginosa* [22]. In contrast, exposure to several antibiotics was found to diminish motility in multidrug-resistant *Salmonella enterica* serovar Typhimurium [97]. The observed effects of antibiotics suggest that enhanced motility plays an important role in physiological adaptations of bacteria to antibiotic exposure.

Bacillus subtilis displays a counterintuitive directionality for its reaction to chloramphenicol in our assays, which may have additional benefits suggested by the migration pattern relative to *S. venezuelae*. As observed in still images and video of the interaction, the initial response of *B. subtilis* is movement toward the colony of *S. venezuelae*. One speculative idea is that the apparent directionality is a product of the assay format, where cells on the proximal side of a patch are first to respond and expand outward. The outward expansion leads to rapid colonization of the agar surface, including the original spot of *S. venezuelae* (Figure II-7). Although further evidence is required, the interaction pattern suggests an early expansion of *B. subtilis* results in suppression of continued growth of the streptomycete, thereby preventing further production of chloramphenicol. Following several more hours of culture, the *B. subtilis* outward migration extends toward more distant spots of *S. venezuelae*. However, the migratory population is repelled from the *S. venezuelae* spots (Figure II-7). One possible explanation is that additional growth of the streptomycetes results in production of growth-inhibitory amounts of chloramphenicol or other antibiotics. If indeed the patterns reflect responses to changing antibiotic concentrations, the competitive fitness advantage

to early activation of sliding mobility would be dually protective, providing an early opportunity to overtake the competitor and an escape mechanism if antibiotic concentrations reach inhibitory levels.

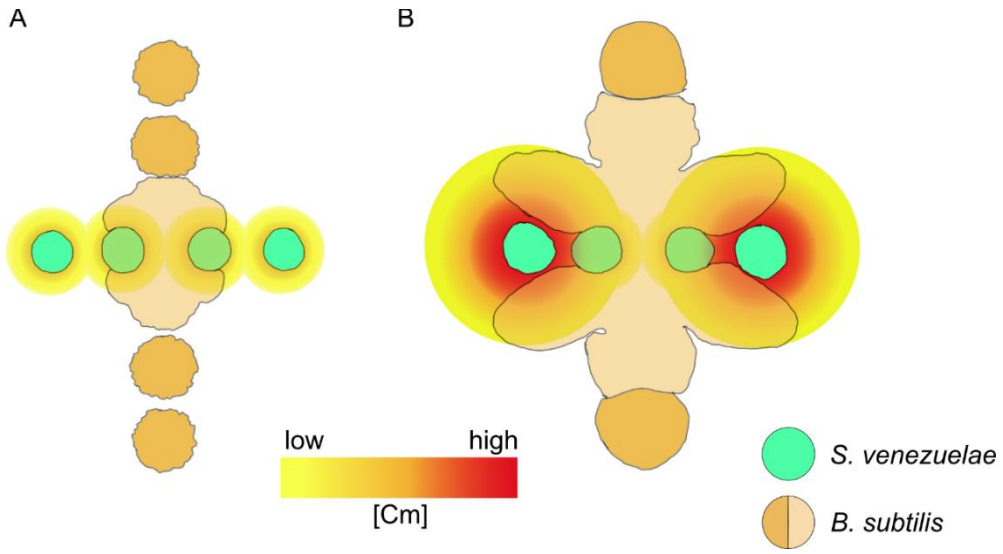


Figure II-7 Summary model for concentration-dependent effects of chloramphenicol on *B. subtilis*.
The competitive culture format for *S. venezuelae* and *B. subtilis* suggests a model for the temporal effects of population growth on production and diffusion of chloramphenicol in the agar medium. A. Early development (~24 hours) of the *S. venezuelae* (light green spots) results in low concentrations (yellow) of chloramphenicol in the medium, sufficient for stimulating sliding motility in the proximal *B. subtilis* (light tan shapes). B. Continued growth (~48 hours) and, presumably, chloramphenicol biosynthesis by the proximal *S. venezuelae* spot is impeded by the migratory population of *B. subtilis.* During this time, more distal spots of S. venezuelae grow to a greater extent and produce higher yields of chloramphenicol. The concentration of chloramphenicol (and possibly other, unidentified metabolites) becomes sufficient (red) to impede growth and progression of the sliding population of *B. subtilis*, which is therefore prevented from contacting the *S. venezuelae* population. The unaffected populations of *B. subtilis* (not mobilized by chloramphenicol exposure) are dark tan spots.

The mechanism by which subinhibitory antibiotics induce mobilization is likely linked to protein translation. The mechanism of action for each of the inducing antibiotics is to block translation. Intriguingly, the effect is not limited to a single site of action, such as the peptidyl-transfer site or the exit tunnel [130,137]. Instead, the mechanisms of activation converge on blockage to progression of translation, as opposed to mis-incorporation of amino acids or damage to other cellular structures [138–140]. This connection is illustrated by the transcriptional activation of *bmrCD* by subinhibitory concentrations of chloramphenicol and other antibiotics. Stalling in translation of BmrB permits the transcription of the *bmrCD* genes [133]. Because *bmrCD* are not required for mobilization, the induction of sliding must require other changes in *B. subtilis* exposed to inducing antibiotics. Further pursuit of changes in transcriptome, proteome, and metabolome will likely uncover key factors that lead from translation stress to sliding mobility for *B. subtilis*.

A growing body of evidence demonstrates the many mechanisms by which bacteria detect antibiotics in the environment and initiate protective responses. These responses include biofilm and persister formation, enhanced virulence, motility, and other physiological adaptations. The consequences of bacterial adaptation to low doses of antibiotics are likely to have substantial impacts on bacterial communities. Adaptive changes provide opportunities for bacteria to acquire specific resistance mechanisms to a given antibiotic or class of antibiotics [109,141,142]. In addition, adaptive changes that influence specialized metabolism, virulence, and mobility are likely to affect interactions

in ways that ripple outward to impact other species in a community and even plant and animal host organisms.

Materials and Methods

Strains, primers antibiotics and growth media

The strains of *Bacillus subtilis* used in this study are listed in Table S1. *Bacillus subtilis* mutant strains in 168 (originally from *Bacillus* Genetic Stock Center (BGSC)) or PY79 background were transduced into NCIB 3610 by SPP1 phage transduction using standard procedures [143]. The plasmid pDR244 was used to generate markerless deletions in the *mls* marked *B. subtilis* NCIB 3610 strains by looping out *loxP*-flanked MLS resistance cassette. To obtain a *bmrCD* double knockout strain with kanamycin resistance, long-flanking region homology (LFH) PCR was used. Primers *bmrC*-up1000-fwd and *bmrC*-up1000-rev were used to amplify the *bmrC* upstream 1 kb region and primers *bmrD*-down1000-fwd and *bmrD*-down1000-rev were used to amplify the *bmrD* downstream 1 kb region. Primers kan-fwd and kan-rev were used to amplify the kanamycin cassette. The primers are listed in Table S2. All antibiotics were purchased from Sigma. *B. subtilis* strains were cultured at 37°C in lysogeny broth (LB) and were inoculated onto GYM7 plates (0.4% D-glucose, 0.4% yeast extract, 1.0% malt extract, 1.5% agar, 100mM MOPS, 2.5mM KH_2PO_4, 2.5mM K_2HPO_4, pH7.0) when grown to OD_{600} = 1. *Streptomyces* spore stocks were maintained in water at 4°C.

Coculture assays and motility assays

Coculture assays were performed as previously described [12]. Briefly, 2.5 μL of a 10^7 spores/mL *Streptomyces* spores was spotted in the horizontal line and grown for 12 h at 30°C. 1.5 μL of *B. subtilis* was then spotted 6 mm from *Streptomyces sp.* in the vertical line. For motility assays, 1.5 μL of *B. subtilis* was spotted 6 mm from wells or filter discs on the agar plate.

Sliding inducer extraction and identification

Streptomyces venezuelae was cultured on the top layer of GYM7 plates separated from bottom layer by a sheet of cellophane. The top layer (5 mL) along with the cellophane was removed after 5 days of *S. venezuelae* growth. Metabolites were extracted from the lower layer (20 mL) by freezing the agar and separating aqueous media by filtration through 60 mL syringe containing a layer of Miracloth (EMD Millipore). The squeezed extracts were pooled and then lyophilized. The crude extract was suspended in 1/5 the original volume in H_2O. The crude extracts were initially fractionated by SPE C_{18} column (Sigma). To extract the mobility inducer, 3 mL crude extract was applied to the 3 mL SPE C_{18} column (Supelco). The column was washed with 6 mL of 10% methanol, followed by elution with a 20% stepwise gradient of methanol/H_2O (from 20%-100%). Methanol in all fractions was removed using a rotary evaporator. The concentrated fractions were suspended in 200 μL H_2O. All fractions were tested for mobility inducing activity by spotting 10 μL on a well or a filter disc 6mm away from *B. subtilis* colonies and the mobility induction was observed after 24 h. The 40% methanol fraction was

active and multiple 40% extracts were pooled for further analysis. The 40% methanol fraction was further fractionated by HPLC (Agilent 1200) using a semi-preparative C18 column (10 × 250 mm, 5 μm particles, Phenomenex). An isocratic method was used (30% solvent A, 70% solvent B. 20 min in total.) with a flow rate of 4 mL/min. Solvent A is acetonitrile. Solvent B is 0.1% formic acid (in H_2O). For each injection, 100 μL pooled active fraction was applied. Time-based fractions from HPLC were collected and tested for mobility inducing activity. Those active fractions were analyzed by LC-MS/MS. Specifically, LC-MS/MS was performed with an Agilent 1260 HPLC system coupled with a binary pump and a 1200 series diode array detector UV-Vis detector (compounds were detected at 254 nm, 340 nm and 420 nm) followed by a MicroTOF-Q Ⅱ mass spectrometer (Bruker Daltonics) using an ESI source. Separation was performed with a Supelcosil LC-18 column (15 cm X 3 mm, 3 μm particles, Supelco). LC conditions: t=0 min, 100% A; t=2 min, 100% A; t=12 min, 30% A; t=20 min, 30% A; t=25 min, 100% A; t=35 min, 100% A; t=40 min, 100% A. The flow rate was 400 μL/min. Solvent A is 5 mM ammonium acetate buffer, pH 6.6. Solvent B was 75% methanol and 25% H_2O. Mass spectrometer was calibrated with a diluted sodium acetate solution and six m/z values (158.9641, 362.9263, 498.9012, 566.8886, 634.8760 and 770.8509) were used for the calibration. The mass spectrometer was operated in positive mode in a mass range from 50 to 1500. The ion source temperature was maintained at 200°C with 8 eV ionization energy and 4,500 V capillary voltage. Helium was used as the collision gas.

RNA extraction

Wild-type *B. subtilis* NCIB 3610 was grown to early stationary phase (OD_{600} = 1) and was inoculated on GYM7 plates with or without 1 μM chloramphenicol, followed by incubation at 30°C. *B. subtilis* colonies at 4 h, 6 h and 12 h and the outer region of colonies at 24 h were scraped after treating with 3 mL of stabilization mixture (2 mL RNAprotect Bacteria Reagent (Qiagen) with 1 mL TBS buffer) on each plate. Bacterial suspension was transferred to 15 mL conical tube and mixed for 5s by vortexing and incubated at room temperature for 5 min. 500 μL aliquots were transferred to each 2 mL Eppendorf tube. Cell pellets were collected by centrifuge at 17,900 g for 10 min. RNA was isolated as previously described [12]. Briefly, cells were lysed with lysis buffer (15 mg/mL lysozyme, 5 mg/mL proteinase K in 100 mM Tris HCl -50 mM EDTA buffer, pH8.0) and vortexed vigorously for 45 min at ambient temperature. 1 mL Trizol reagent (Sigma) was added to each sample. RNA was precipitated using standard procedures. RNA samples were cleaned with a Turbo DNA-free kit (Applied Biosystems).

Quantitative RT-PCR(qRT-PCR)

qRT-PCR was performed as described previously [28]. Briefly, 50 ng of total RNA was used as the template for cDNA synthesis with a High-Capacity RNA-to-cDNA Kit (ThermoFisher Scientific). A SsoAdvanced Universal SYBR Green Supermix Kit (Bio-Rad) and CFX96 Touch real-time PCR thermocycler (Bio-Rad) were used to perform quantitative PCR as previously described [28]. *gyrB* was used as the reference gene. Target

abundance was normalized to *gyrB* and the fold change was calculated by comparing to the untreated sample at 4 h.

CHAPTER III

ANTIBIOTIC EXPOSURE TRANSIENTLY REPROGRAMS METABOLISM TO MOBILIZE A BACILLUS SUBTILIS BIOFILM

Summary

Bacteria often sense external stimuli from diverse competitors and respond by modulating their physiology to adapt efficiently to new environments. However, how physiological adaptations are regulated at the molecular level remains largely unknown. Previously, using a competition system composed of *Bacillus subtilis* and *Streptomyces venezuelae*, we found chloramphencol (Cm) secreted from *S. venezuelae* induced colony expansion in *B. subtlis*, sliding motility. To link the sliding response to underlying genetic determinants, we first determined that the sliding response is a regulatory response instead of mutations arising upon exposure to Cm. To understand how sliding motility is driven by Cm exposure, we sampled *B. subtilis* samples in the presence and absence of Cm at two stages, before and after the transition to colony expansion, followed by gene expression measurement across *B. subtilis* genome using RNA-Seq. Transcriptional profiling suggests that many genes are involved in the transition. To reduce the complexity of this regulation, we constructed a regulatory network based on the differential expressed genes to identify the key regulators that control the response for each stage. Regulator networks suggest changes in metabolic pathways are an important feature for the sliding response. Further metabolic profiling also highlights the role of metabolic changes. In addition, promoter-*lux* reporter strains used to monitor

changes in metabolic pathways revealed at least two major metabolic states in the sliding population. These results emphasize the importance of metabolic coordination and metabolic reprogramming in bacterial physiological adaptions in response to environmental stimuli.

Introduction

The natural history of many bacteria includes exposure to antibiotic metabolites produced by neighboring species[17,19,46,144]. Given sufficient concentration, antibiotics inhibit growth or kill the exposed bacteria. However, the concentrations of antibiotics in natural environments are a challenge to measure and therefore limit understanding of antibiotic-mediated interactions in nature[17,144]. Therefore, model systems are instrumental in determining the consequences of antibiotic exchanges between populations of bacteria and other microorganisms. Studies of sub-inhibitory antibiotic concentrations on bacteria have revealed a wide range of responses, which include changes in gene expression, motility, and virulence in the affected organisms[22,25,145]. These sub-inhibitory activities suggest that antibiotics may have substantial impacts on the organization of natural microbial communities. In the laboratory, studies of two species, cultured together, demonstrate the transformative activities that some metabolites exert on the dynamics of interactions[146].

The outcomes of a species-interaction study depend on many factors, including both the strains used and the culture conditions. Examples of different bacterial

responses to interacting species vary from measured changes in gene expression to dramatic changes in growth and metabolism. *Pseudomonas aeruginosa* protects *Staphylococcus aureus* from antibiotics by slowing growth[112,147,148]. *P. aeruginosa* also transforms the growth of *C. albicans*[5,149]. Other examples exhibit changes in bacterial development[12,104], biofilm formation[150], and motility[6].

Antibiotics from bacteria, by nature of their growth inhibitory activities, are a form of interference competition often characterized as chemical warfare. In this respect, the common response to exposure is to activate measures to avoid growth inhibition or cell death. These measures are a form of adaptive resistance to antibiotics[151]. An effective protective response to an antibiotic should be activated before the antibiotic concentration reaches an inhibitory threshold. Protective early-warning-systems have been described as competition sensing, wherein cellular damage caused by a competitor activates protective responses[23,96]. The responses identified include activation of antibiotic production or other countermeasures as a means of defense. In addition to a multiplicity of bioactive metabolites, the apparent activities of individual metabolites may change with concentration. This property of hormesis is best illustrated with antibiotics, which may be stimulatory or inhibitory with increasing concentration[19,152]. In particular, we found that exposure of *B. subtilis* to chloramphenicol and other translation inhibitors leads to a massive reorganization of growth. A *B. subtilis* colony exposed to chloramphenicol transitions to expansive growth known as sliding motility.

In some cases, discrete mechanisms are stimulated and the mechanisms controlling them are defined. In other cases, the responses may involve transformative changes in the growth of the organisms and may be more challenging to dissect into specific mechanistic details. The ribosome is a biosynthetic and regulatory centerpiece for growth control in all organisms[24,46,153,154]. Because translation is fundamental to cell function and is energy-intensive, cells are attuned to changes in translation efficiency. Examples include cis- and trans- functions that connect the efficiency of translation to growth control for the organism[154,156]. Typically, changes in nutrient availability or exposure to translation-disrupting agents may compromise the efficiency of translation, an insult to which the organism typically responds by reducing its rate of growth[154,157,158]. Mechanisms that connect translation and cell growth for bacteria include changes in metabolism. For example, the stringent response in bacteria occurs when, due to the depletion of amino acid pools, GTP is converted to (p)ppGpp[159]. The (p)ppGpp signal leads to a cascade of regulated effects that slow cell growth[160]. Recently, it was shown that (p)ppGpp could target several GTPases to affect ribosome assembly in Gram-positive bacteria[161,162]. In light of these connections, the anticipated outcome of reduced protein synthesis from antibiotic exposure is moderation of growth rate or activation of dormancy functions. However, our prior study revealed that for *B. subtilis*, a growth-dependent mode of surface motility, called sliding, emerges with exposure to translation inhibitors in nutrient-replete conditions[53]. This paradoxical result motivated the present study to identify changes in the *B. subtilis* population that correlate with active growth under antibiotic stress.

Here we show that colony expansion driven by sliding motility is defined by a substantial change in metabolism for *B. subtilis*. We have observed a change in expression, tied to translation efficiency and drug exposure. That is also linked to a massive change in population dynamics observed by colony expansion and biofilm-like colony morphology. We identify a network of regulatory functions that control the colony expansion. The expanding population comprises at least two major metabolic states, which we define by their emphatic differences in carbon and nitrogen metabolic functions. The results reveal a metabolic coordination across a population of cells, which fits a model of cooperative resource utilization for enhanced fitness in competition with antibiotic-producing species.

Results

Exposure to chloramphenicol causes a transient colony expansion of B. subtilis

Previously, we found *S. venezuelae* induces *B. subtilis* colony expansion by sliding motility and identified a subinhibitory concentration of chloramphenicol (Cm) that triggers the sliding response (**Figure III-1A and 3-1B, Movie III-1 and III-2**)[53]. This observation presents an opportunity to determine how *B. subtilis* regulates the activation of sliding motility. Because sliding motility is growth-dependent[163,164], we initially sought to determine whether it is an outcome of regulated changes in growth or due to mutations arising in the presence of Cm. If the response is transient, we predicted that the sliding population would revert to normal growth in the absence of Cm. We induced sliding motility using subinhibitory Cm and subsequently transplanted cells

from the sliding population to agar plates containing different concentrations of Cm (0, 1, and 16 µM). When transplanted to media without Cm, *B. subtilis* ceased colony expansion. In contrast, media containing the stimulatory concentration of Cm (1 µM) perpetuated the sliding motility. Overall growth was inhibited at the minimal inhibitory concentration (16 µM), indicating that the population had not acquired resistance to Cm (Figure III-1C). These results suggest that colony expansion is a transient characteristic stimulated by Cm exposure.

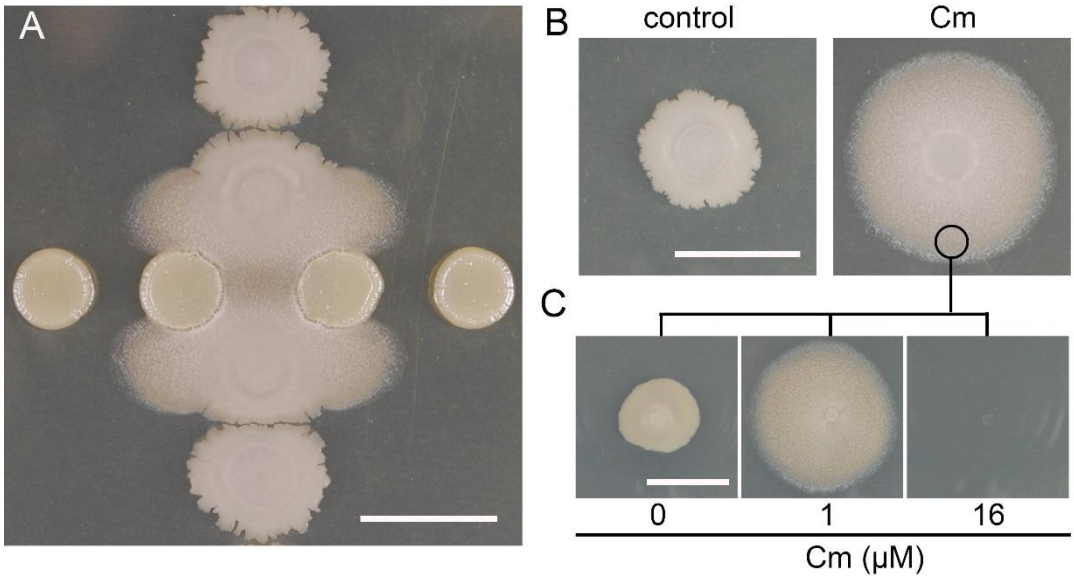


Figure III-1 Exposure to chloramphenicol enables *B. subtilis* to transition to a sliding mode.
A. Sliding motility is induced in proximal *B. subtilis* spots (vertical) when cocultured with *S. venezuelae* (horizontal spots) at 24 h due to the release of Cm from *S. venezuelae*. B. Direct plating of *B. subtilis* on an agar plate with a sub-inhibitory concentration of Cm (1 µM) leads to sliding, while sliding motility is not observed in the absence of Cm. C. Transplantation of *B. subtilis* cells in the sliding population to different agar plates with different concentrations of Cm: 0 µM, 1 µM (sub-MIC) and 16 µM (MIC). Bars, 1 cm.

In addition to chloramphenicol, other translation inhibitors trigger colony expansion by *B. subtilis*[53], suggesting that a reduction in protein synthesis underlies the transition to sliding motility. Alternative explanations include chemotaxis in response to the stimulatory compounds. For instance, attractant compounds can act as cues to promote chemotaxis in nutrient-replete conditions, well before nutrients are exhausted[165,166]. However, a deletion of the *cheV* and *cheW* genes, which completely blocks chemotaxis by *B. subtilis*[167], failed to prevent Cm-induced colony expansion (Figure. 3-S1). Therefore, we concluded that chemotaxis is not required for the antibiotic-induced response. Instead, we speculated that, most likely, the cause is directly related to the antibiotic effect of the drug on protein synthesis. Our previous study showed that strains resistant to chlorampenicol and erythromycin did not induce sliding upon exposure to subinhibitory Cm, suggesting indirectly that the mechanism requires the interaction of the drug with the ribosome[53]. As a direct test, we measured the rate of protein synthesis in the presence of 1 μM Cm, comparing Cm-treated and untreated cells using a Click-iT assay[168,169]. We observed a 60% reduction in the protein synthesis rate using 16 μM Cm (1-fold MIC) and a 7% reduction in protein synthesis rate with 1 μM Cm treatment. The small reduction suggests that protein synthesis activity is moderately affected by 1 μM Cm, which is consistent with motility arising as a cellular response to disrupted translation (Figure III-S2). Our observations support a model where low-level stress from antibiotic exposure leads to an adaptive physiological reprogramming of typical colony growth to colony expansion under sliding motility. We next sought to

determine a molecular basis for the antibiotic-induced transition to a mobilized population.

Patterns of gene expression suggest regulated changes in metabolism within the mobilized population

The application of a uniform concentration of antibiotic to agar media provides a straightforward approach to compare transcriptional states between Cm-treated and -untreated populations of bacteria. To identify changes in gene expression related to the mobile response, we performed transcriptional analyses of the whole *B. subtilis* genome in the absence and presence of Cm. In particular, we sought to identify differences in gene expression during the transition to colony expansion. For instance, the reported expression pattern for *bmrCD* in response to Cm exposure is transient, increasing from 4-6 hours, and subsequently decreasing from 12-24 hours[53,170]. The transient *bmrCD* expression pattern suggests gene expression states between early Cm exposure and later initiation of colony expansion may differ. We chose two times to sample the cells based on the following criteria: 1) expression of the *bmrCD* operon as an indicator of early (6 hours) Cm exposure, and 2) a time point (24 hours) when colony expansion is visible and the cells can be effectively isolated for RNA extraction. Thus, the selection of two times for sampling could provide a snapshot of the relevant transcriptional states during the transition from standard growth to colony expansion.

459 and 317 genes differentially expressed at 6h and 24h respectively (|fold change| ≥ 2 and adjusted p value ≤ 0.05) (Figure III-S3). To visualize the overall gene expression profile across all four conditions, we generated heatmaps using K-mean clustering (K=5) to group the data into 5 categories based on expression patterns. The heatmaps illustrate the complexity of the transcriptional changes under the conditions used, revealing nearly 100 genes with ≥ 2-fold change in expression for each category (Figure III-S3).

Functional analyses of those differentially expressed genes were performed based on Subtiwiki Database (REF). The results suggest that metabolic changes are predominant (Figure III-2). Moreover, a pattern of expression reversal occurs for some gene functions. A prominent example is purine metabolism. Simply sorting all changes by fold-change magnitude, the greatest degree of elevation is in purine synthesis at 6 hours and purine catabolism at 24 hours. This is reflective but does not capture the full complexity of the transition to expanding population. For instance, genes in the ribosomal biogenesis and translation category were mostly upregulated with Cm treatment at 6 hours. Interestingly, most genes in the stress response category at 6 hours were downregulated in the presence of Cm, suggesting suppression of the stressosome under the motility-inducing condition[171].

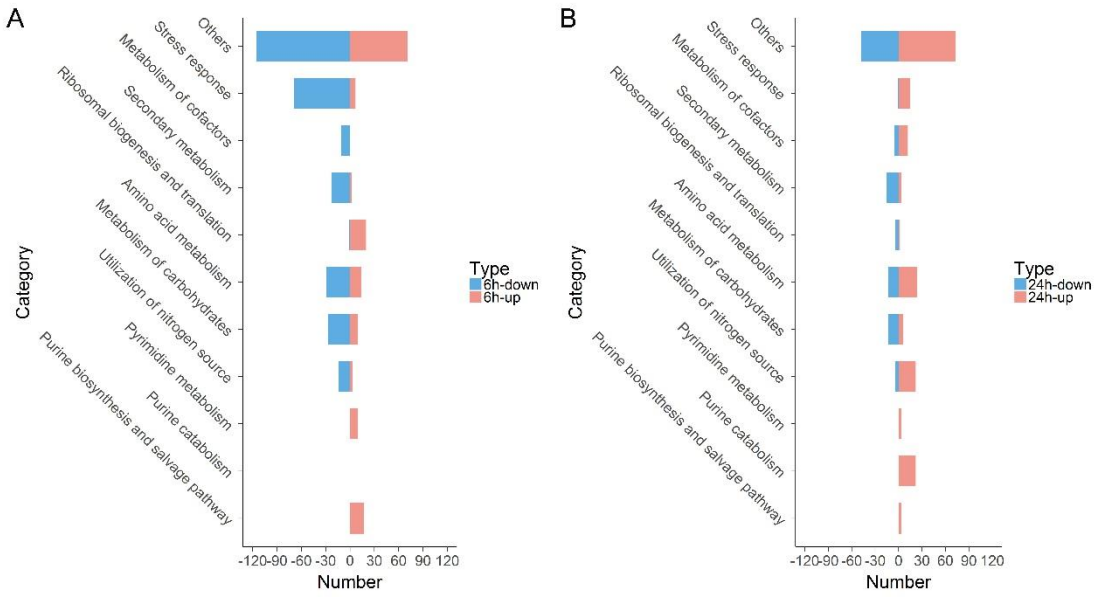


Figure III-2 Patterns of gene expression reflect regulated changes in metabolism within the mobilized population.
A. Functional classification of genes that change ≥two-fold with adjusted p value<0.05 in response to Cm at 6 h. B. Functional classification of genes that change ≥two-fold with adjusted p value<0.05 in response to Cm at 24 h. Blue: downregulation; Red: upregulation.

A regulatory network view of Cm-induced colony surface expansion

Transient use of sliding motility for colony expansion indicates that a regulated process controls a major transition, which is a common feature of *B. subtilis* and many other bacteria. An advantage of *B. subtilis* as a model for this study is the in-depth knowledge of gene regulatory functions encoded in the genome. Many genes are assigned to regulons involved in major changes in growth, metabolism, and stress responses for *B. subtilis*. We speculated that the engagement of different regulons under

Cm exposure may reveal key features controlling the transition to and perpetuation of colony expansion.

To visualize regulated changes in regulon activity upon chloramphenicol exposure, we downloaded the known regulons from Subtiwiki and paired them with our transcriptomic data. We organized the output into regulatory networks that emerge at 6 and 24 hours based on the number of genes within a given regulon that change expression by ≥ two-fold. (Figure III-3A and III-3B). To highlight the roles of major regulators in response to Cm, we applied a stringent cutoff of 40% of genes (the percentage of differentially expressed genes) to each regulon. In the network view, each node represents a regulator, connected by the shared genes (edge) with the network. The blue and red colors indicate downregulation and upregulation in each regulon, respectively. In some cases, the entire regulon is expressed or repressed according to Subtiwiki assignments (e.g. PurR). In other cases, only a fraction of the regulon appears to be engaged in the response (e.g. CodY). The regulator network output highlights multiple regulons that represent major changes under Cm-induced mobilization, which is reflective of the transcriptomic profile.

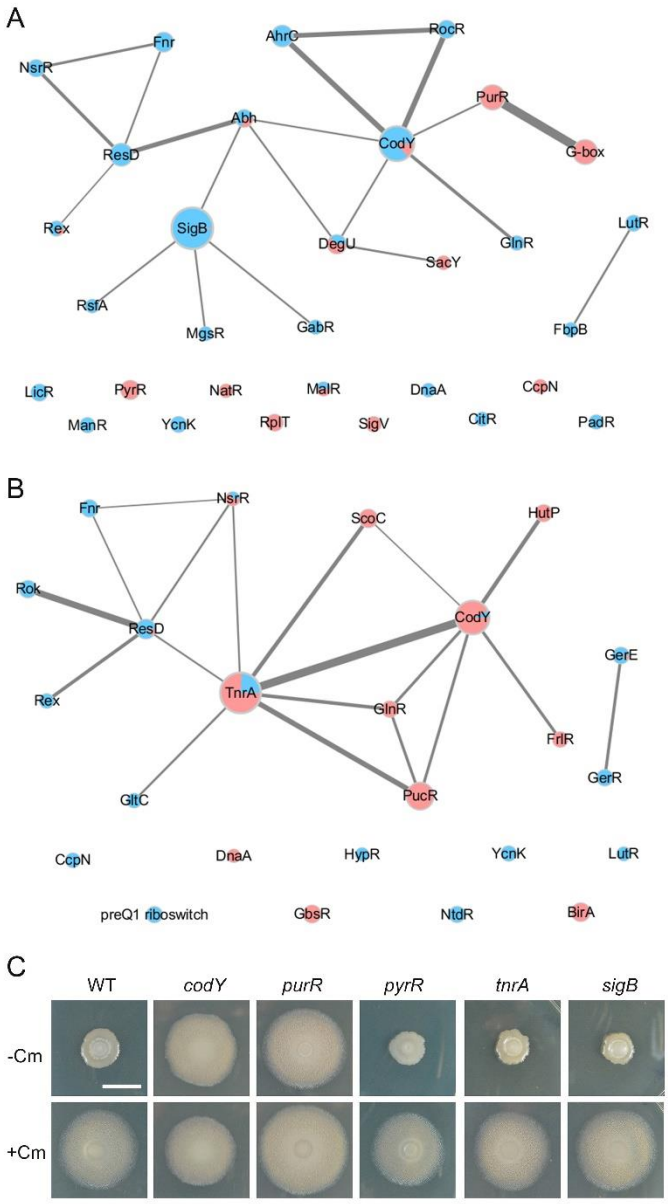


Figure III-3 A network view of regulation highlights pathways of control for adaptive colony surface expansion.
A. A cytoscape network of regulators recruited by genes that change ≥two-fold with adjusted p value<0.05 in response to Cm at 6 h. B. A cytoscape network of regulons recruited by genes that change ≥two-fold with adjusted p value<0.05 in response to Cm at 24 h. In both A and B, the size of each node represents weighted gene

Figure III-3 continued. number in each regulon (weighted by the percentage of differentially expressed genes in each regulon) and the edge width represents weighted overlapped gene number between two regulons (weighted by the percentage of differentially expressed genes among overlapped genes). Blue: downregulation; Red: upregulation. C. Genetic analysis of different regulator deletion in the absence (-Cm) or presence of Cm (+Cm). Pictures were taken at 24 h. Bar, 1 cm.

The network view focuses our attention on major functions that appear to control the transition to and perpetuation of the motile population. For instance, prominent among the regulators identified is CodY, which has been extensively studied for the regulation of target genes that changes depending upon nutrient conditions. Approximately 40% of the regulon is activated or deactivated under the conditions we used for these assays (CodY-2014-PNAS). Strikingly, a pattern emerges wherein at 6 hours, the majority of CodY-regulated genes are repressed (aka, CodY is active), and by contrast at 24 hours, the majority of the CodY-regulated genes are elevated in transcript abundance (CodY inactive). Because CodY is a global regulator of nutrient status and metabolism, the observed changes in CodY regulatory activity suggest a major transition in the metabolic state as cells transition from initial Cm exposure to colony expansion.

Based on the network node identities, we targeted regulatory genes for disruption to determine how the loss of their function affects the mobile response to Cm. For example, because the majority of CodY regulon is derepressed at 24 h, we predict that loss of CodY function could enhance colony expansion. Indeed, we observed that a Δ*cod*Y strain is constitutively expanding, even in the absence of Cm (**Figure III-3C**).

This result is consistent with CodY-repressed genes having active roles in perpetuating the mobile population. Whether the CodY regulation is direct or indirect through other regulators remains to be determined. Other prominent regulators in the network control more specific functions than the global regulator, CodY. Compared with wild type, a *purR* mutant strain exhibited sliding in the absence of Cm, highlighting the role of purine metabolism in sliding motility (**Figure III-3C**). In contrast, we observed no visible phenotype for *pyrR*, *tnrA*, and *sigB* deletion strains despite their presence in the 24-hour networks (**Figure III-3C**). These results suggest that disruption of genes within specified regulons will enable us to parse the functions into those essential for sliding and those with nuanced functions separable from sliding motility.

We chose to focus here on CodY regulated functions due to its prominent role in regulating sliding motility. Genetic disruption of CodY function leads to constitutive sliding and CodY controls 15% of genes that change expression in response to Cm. An indication of dynamic CodY regulation is observed in the pattern of changes within the regulon over time. We isolated the CodY-regulated genes and sorted them according to GO analysis (Figure III-S4A).There are 57 and 61 genes within the CodY regulon that change ≥2-fold in response to Cm at 6 hours and 24 hours, respectively. Among these genes, 25 are changing at both stages, and 24 out of 25 of them change in the opposite direction. For example, *guaC* regulates purine salvage, and its expression is upregulated at 6 hours and downregulated at 24 hours (**Figure III-S4A, III-S4B**). The opposing

change in transcript level indicates a requirement for CodY-activated purine salvage that is relieved during the transition to sliding motility. We considered genes whose products may be related to the mechanical functions that support sliding motility. Two genes encoding extracellular proteases, *aprE* and *vpr*, show repressed expression at 6 hours and increased expression at 24 hours, possibly implicating a requirement for protein degradation to facilitate colony expansion.

According to our functional assignments, genes involved in nitrogen metabolism are abundant among the regulated network. We observe gene expression within this category is predominantly induced at 6 hours and repressed at 24 hours. For those genes that are not overlapped at two stages, most of their functions are associated with the utilization of nitrogen source, amino acid metabolism (mainly BCAA, Arg, His and Asn), secondary metabolism (polyketide and surfactin) and unknown functions (**Figure III-S4A, III-S4B**). Specifically, the increase of genes responsible for surfactin biosynthesis at 6 hours indicates that cells are preparing the requirements for colony expansion before transition to sliding mode. Together, these changes suggest that cells are changing their metabolic states to promote colony expansion. Transcription data are only reflective of metabolic states. Combining insights from transcripts with direct measurements of metabolites could focus our attention on key pathways that drive colony expansion under antibiotic exposure.

Direct measurements of cellular metabolite pools indicate major changes in glycolysis and nucleotide metabolism during the transition to colony expansion

Because changes in gene expression of metabolic functions do not necessarily reflect underlying changes in metabolite pools, we next sought to measure directly differences in metabolite pools between treated and untreated samples. We measured metabolites using LCMS at 6 versus 24 hours for Cm treated and untreated samples. Because the transcriptional data emphasize changes in phosphate-bearing nucleotide pools, we used an ion-pairing approach for chromatographic separation of extracts to identify changes in metabolite abundances that result from Cm exposure (6 hours) and that characterize the expanding colony (24 hours). Figure III-4 illustrates the results using a heat map to visualize changes in metabolite abundance under the given conditions. It shows that central carbon metabolites such as pyruvate, phosphoenolpyruvate, and glutamine were elevated, while metabolites in purine metabolic pathways such as guanosine, inosine, and dAMP, and dihydroxyisovalerate, one metabolite in branched-chain amino acid pathway were reduced at 6 hours. However, we observed increased gene expression in purine biosynthesis pathway, implying the transcriptional data are not consistent with metabolomics data. How to clearly explain these changes in metabolic pools will need more experiments using mutants or metabolic labeling in these pathways. However, we would capture the most significant features in both transcriptomics and metabolomics in the beginning to facilitate our analysis. At 24 hours, the most significant change is allantoin, in the purine catabolism pathway, which is consistent with the transcriptional data. Therefore,

to understand these changes during transition to sliding motility, it would be better to integrate both datasets.

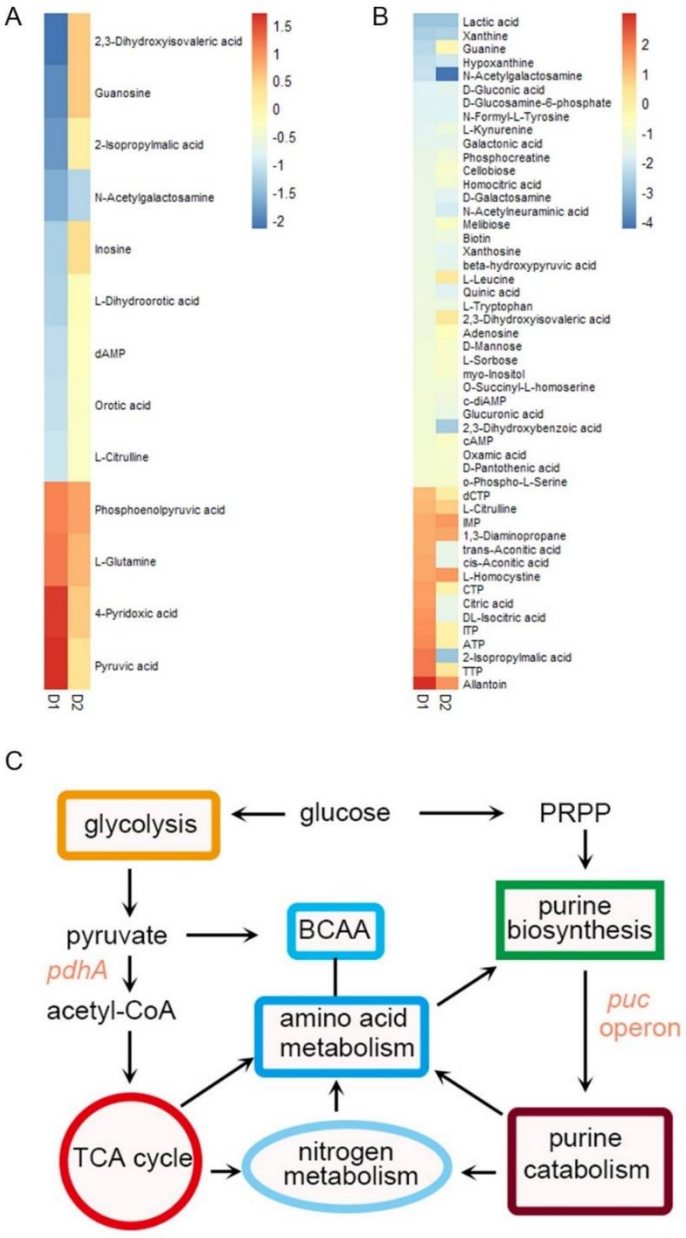


Figure III-4 Metabolomics analysis underscores the pattern of shifting metabolism

Figure III-4 continued. reflected by transcriptional analysis. Metabolomics profile of wild type and *codY* deletion strains at 6 h (A) and 24 h (B). Metabolites that change ≥two-fold in wild type are listed, while the corresponding metabolites in *codY* deletion are also listed on the side. (C). An overview of changes in different pathways revealed by both transcriptomics and metabolomics data analysis.

Because CodY appears to contribute significantly to colony expansion and the Δ*codY* phenotype is constitutive expansion, we also extracted metabolites from the Δ*codY* strain in parallel with the wild type. We hypothesize that the metabolite profiles would differ between conditions and thus the contrast would highlight Cm-specific events from other CodY-regulated functions. Figure 4 illustrates several differences in the metabolite pools upon comparison. Notably, glycolysis stands out as a major factor at 6 hours, where accumulated pyruvate is the dominant change(**Figure III-4A**). At 24 hours, nitrogen cycle functions appear dominant, e.g. the accumulation of allantoin(**Figure III-4B**). Combining these results with transcription profiles, we built a diagram of the major metabolic features indicated by transcriptional and metabolomics data. (**Figure III-4C**). The abundance of pyruvate at 6 hours suggests a high demand for glycolysis/energy early in the response. The abundance of allantoin and nucleotides, and the expression of the PucR regulon, suggests nitrogen cycle characterizes the latter population. Interestingly, N-acetylgalactosamine is reduced in both times and both strains. N-acetylgalactosamine is reported to participate in cell wall biosynthesis. It is possible that during colony expansion driven by sliding motility, cells need more N-acetylgalactosamine for cell wall biosynthesis (**Figure III-4A and III-4B**).

pdhA is critical for sliding motility

To understand the role of carbon and nitrogen metabolism in the development of colony expansion, we generated several mutants in glycolysis, purine biosynthesis, and purine catabolism pathways. The results indicate disruption of purine biosynthesis and purine catabolism pathways did not affect sliding motility upon exposure of Cm (**Figure III-5**). However, the deletion of *pdhA* abolished sliding motility, although Cm increased the size of *pdhA* colony compared with the untreated. To further investigate the effect of *codY* on the phenotype of Δ*pdhA* in Cm-induced colony expansion, we constructed *pdhA* and *codY* double deletion strain. Exposing Δ*pdhA* Δ*codY* strain to Cm did not induce the sliding response or increase the colony size, suggesting the additive effect of *pdhA* and *codY* on the colony expansion triggered by Cm.

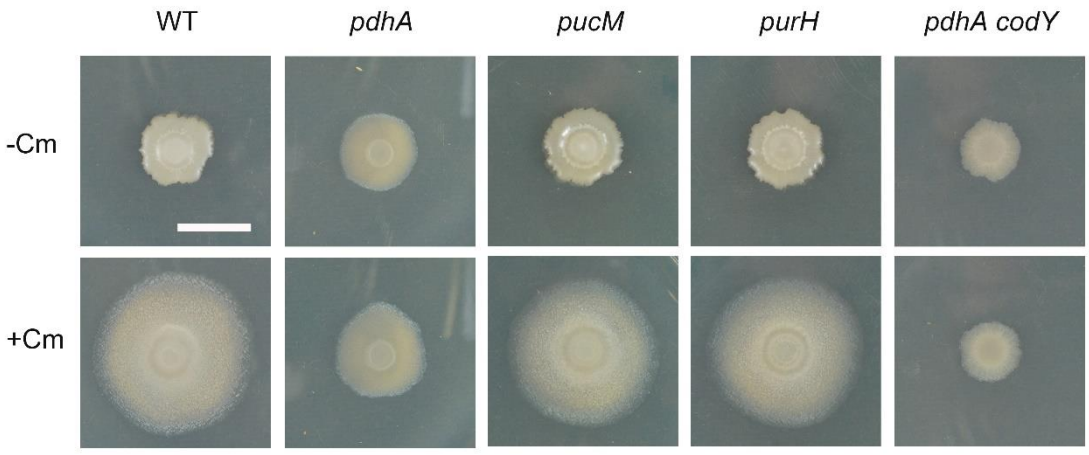


Figure III-5 *pdhA* is required for colony expansion on the Cm plate.
Wild type, *pdhA*, *pucM*, *purH* and *pdhA codY* double knockout strains were spotted on the agar plate in the absence (-Cm) or presence of Cm (+Cm). Pictures were taken at 24 h. Bar, 1 cm.

We next sought to study how carbon sources contribute to Cm-induced colony expansion by either modifying GYM7 medium component or generating mutants in the carbon overflow pathway. First, we removed glucose and malt extract from GYM7 medium (Y7) or replaced glucose with glycerol in GYM7 medium (YG7). We then tested the sliding motility with the modified media in the presence of Cm. Compared with the GYM7 medium, sliding motility was slightly reduced on YG7 plate and not observed on Y7 plate (**Figure III-S5A**), suggesting certain amount of carbon source is required for initiation of sliding motility. Second, we deleted *alsR* gene, which is required for the activation of *alsS* and *alsD* in the conversion of pyruvate to acetoin, to favor the flow of pyruvate to TCA cycle (not sure). *alsR* strain exhibited constitutive sliding motility on the GYM7 plate, suggesting more energy was produced to support colony expansion by shifting pyruvate to TCA cycle (**Figure III-S5B**)

However - the pattern observed in metabolic data also raised some questions - for example, why is the PucR regulon expressed (highly) while the PurR regulon expression is maintained - which appears as a futile cycling. The pattern suggested that sampling the entire population averaging transcripts and metabolites does not fully capture the dynamic population states represented during colony expansion. For example, surfactin and EPS are expressed in different population[172]. Therefore, we selected different reporters for metabolism based on the transcriptomic and metabolomic data. Using the reporters, we can monitor changes in metabolic states continuously to better understand

the coordination of populations. For this work, we chose to use the *luxABCDE* operon[173] as a reporter, realizing that standard fluorescence or beta-lactamase reporters would not be responsive to both up and down shifts in promoter activity.

Coordination of carbon and nitrogen metabolism drive colony expansion

Based on the changing pattern of expression and metabolite accumulation, we hypothesized dynamic changes occur as the population moves. For instance, at 6 hours we observe de novo purine biosynthesis transcripts are elevated. However, we do not observe major changes in purine pools at this time. By contrast at 24 hours, we observe striking elevation of purine catabolism genes in addition to expression of de novo purine biosynthesis genes, although at a reduced level compared to 6 hours. Our metabolomics data reveal elevated pools of phosphonucleosides at 24 hours. The patterns suggested to us a conflict in the need to produce and recycle nucleotides in populations under Cm-induced colony expansion.

To resolve the changing patterns of metabolic gene expression over time in a sliding population, we sought suitable reporters of gene expression activity in the population. Based on the accumulated transcriptional, regulatory and metabolomic data, we modeled the process based on carbon metabolism through glycolysis and nitrogen metabolism through nucleotide biosynthesis and catabolism. We selected reporters specifically based on the major changes observed, which led us to *pdhA* and *pucA*. *pdhA*,

linking glycolysis and TCA cycle, encodes pyruvate dehydrogenase, which converts pyruvate to acetyl-CoA. The product of *pucA* is xanthine dehydrogenase in the purine degradation pathway, catalyzing xanthine to urate. The results from reporter assays indicate that *pdhA* was upregulated at the early stage of colony development and mainly active in the periphery of the colony during the process of colony expansion (48 hours), while *pucA* was off at early phase and expressed at the later stage (48 hours) in the interior region when colony developed to a certain size (**Figure III-6, Movie III-3**). These patterns suggest that cells coordinate colony expansion with metabolic status at different spatial locations.

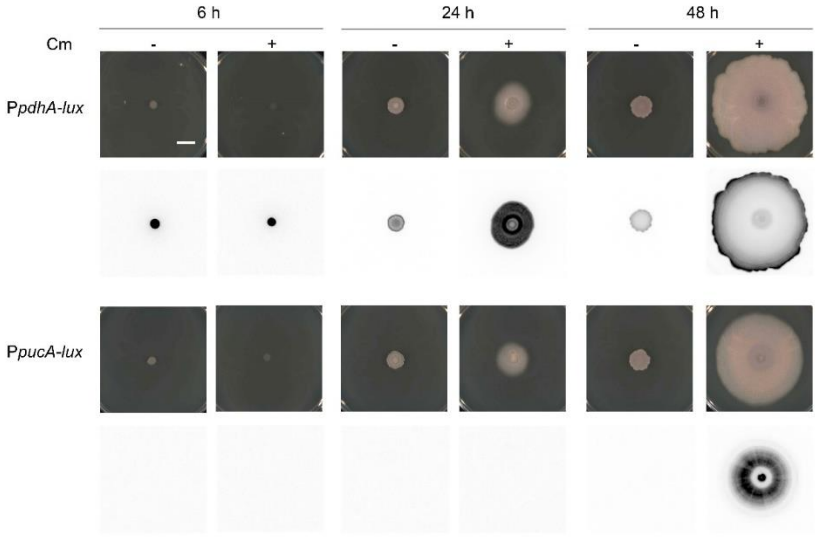


Figure III-6 Spatial metabolism in the sliding population supports colony migration.
Reporter strains with luciferase operon *luxABCDE* fused to *pdhA* or *pucA* promoter in the wild type *B. subtilis* background were spotted on the agar plate without (-) or with Cm (+). Pictures were taken with phase contrast (top) and chemiluminence (bottom) mode at different time points: 6 h, 24 h, and 48 h. Bar, 1 cm.

Given the constitutive mobile phenotype for *codY* deletion strain, we wonder whether disruption of CodY function would change the observed spatial metabolic distribution in the sliding population in the wild type background. Therefore, we constructed luciferase strains in Δ*codY* background. We found *pdhA* expression pattern is similar to that observed in wild type. It is active at the early stage of colony expansion (6 hours) and increases its expression at the periphery of the sliding population in the presence of Cm at 24 hours and 48 hours. Intriguingly, *pucA* expression differs from that seen in wild type background. *pucA* is on at 24 h and active at the rim of the sliding population for both Cm-treated and untreated groups, instead of the interior region, suggesting disruption of CodY function leads to the change of metabolic patterns (**Figure III-S6**).

Discussion

It has been debatable that antibiotics may act as signals versus weapons during interspecies interactions. The role of antibiotics in natural habitats is likely determined by their dose. Antibiotics have been reported to trigger tremendous effects at sublethal concentrations. However, their consequences on bacterial development are largely unknown. Here, we study the effect of chloramphenicol on *B. subtilis* over the course of colony expansion. A combination of transcriptomics, metabolomics, and genetic approaches were used to probe the different states over the course of Cm exposure. Our results indicate a network of regulators are involved in the transition from non-motile

state to motile state. Moreover, the colony expansion is delicately coordinated with spatial metabolism through carbon metabolism represented by glycolysis and nitrogen metabolism represented by purine metabolism.

Transcriptome analysis indicates that hundreds of genes change their expression in response to chloramphenicol at 6 hours and 24 hours, both elevated and reduced. The number and classification of genes differ from previous reports of Cm exposure on *B. subtilis*[26], likely due to the culture format, medium composition, strains used in the assay and the timing to collect samples. The organization of genes that respond to Cm into regulator networks allows us to focus on key determinants mediating the transition. One prominent example is CodY. CodY is a regulator known for regulating metabolism in response to nutrient conditions. The reversal of CodY regulon at two stages and the phenotypic change upon loss of *codY* suggest that CodY is a key regulator in the transition to sliding. Although CodY regulates many genes related to swarming motility[174], all of them did not change more than two-fold (most of them are downregulated below two-fold cutoff), further implying Cm-induced mobile response is sliding motility.

The transition to sliding motility upon loss of *purR* in the absence of Cm indicates the derepression of PurR-repressed genes enhances mobility. It has been identified that genes (*purD* and *purH*) in the purine de novo biosynthesis pathway are required for sliding due to growth on the minimal medium[54,55,111]. However, on the rich medium used

in this paper, deleting either of these two genes did not abolish the sliding motility (**Figure III-S7**). Increased mobility upon deletion of *purR* in the presence of Cm suggests the importance of PurR-repressed genes in the sliding motility. Most regulators recruited in the regulator networks are involved in cellular metabolism, suggesting the metabolic processes during transition to sliding are tightly controlled. Metabolomics data support the changes observed in transcriptomics, especially in glycolysis and nucleotide metabolism. The time-lapsed movie for colony expansion suggests *B. subtilis* sliding population allocates resources to promote the mobile response (**Movie III-3**).

Biofilm, a cooperative lifestyle allowing microbes to resist antibiotic attacks and allocate resources, is a multicellular community embedded in a self-produced matrix comprising extracellular polysaccharide (EPS), and other species-specific components[65]. Biofilm is triggered by diverse environmental cues and conditions, such as antibiotics, small molecules, and nutrient starvation[23,65,136]. The pathways controlling biofilm formation have been well studied in model organism *B. subtilis*[65]. Sliding motility shares one common property with biofilm, EPS. In *B. subtilis*, surfactin is reported to induce potassium leakage to control biofilm formation. This suggests that sliding motility and biofilm are very similar in terms of the requirements for both activities.

Colony expansion for a biofilm has been shown to require oscillations of glutamate and ammonia metabolism from exterior to interior[175]. Glutamate in the case of an MSgg biofilm is the primary source of N and glycerol for C. In our work, the cells are

grown on rich media with a wide variety of potential nutrients. However, glucose is likely to predominate as a carbon source until exhausted by rapidly growing cells. When *B. subtilis* senses danger in otherwise favorable nutrient conditions, it goes on the move to expand, fight and retain possession of its territory. We thought the process could be coordinated by not only a metabolic cooperation, but a regulated shift in metabolism to support the expanding population and its continued growth. To assess whether a division of labor exists, we looked for hallmarks of the relevant pathways that could be used to characterize colony metabolic subdivisions while growing continuously on the surface of an agar plate using luciferase reporter strains. Although we identified a few key metabolic changes such as pyruvate and allantoin that are correlated with transcriptional data, how other metabolites contribute to the initiation would await further investigation.

Materials and methods

Strains, primers and growth media

The strains of *Bacillus subtilis* used in this study are listed in Table 3-1. *Bacillus subtilis* mutant strains in 168 or PY79 background were transduced them to *B. subtilis* NCIB3610 by SPP1 phage transduction. The primers are listed in Table 3-2. *Bacillus subtilis* strains were cultured at 37℃ in lysogeny broth(LB) and were inoculated onto GYM7 plates (0.4% D-glucose, 0.4% yeast extract, 1.0% malt extract, pH7.0) when grown to OD600=1.0.

Sliding motility assay

B. subtilis cells grown in 5 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. For the luciferase reporter assays, images were captured with an Amersham imager 600. For the coculture assay, 2.5 μl of *Streptomyces venezuelae* spores and 1.5 μl of *B. subtilis* (OD600=1.0) cells were spotted in a cross pattern with a distance of 1 cm between spots for each species.

RNA extraction

Wild-type *B. subtilis* NCIB3610 was grown to early stationary phase (OD600=1.0) and was inoculated on GYM7 plates without or with 1 μM chloramphenicol, followed by incubation at 30 ℃ for 6 hours and 24 hours. *Bacillus subtilis* colonies at 6 hours and 24 hours were scraped from agar plates. RNA was extracted using TRI reagent (Sigma) with standard procedures[176]. Turbo DNA-free kit (Applied Biosystems) was used to remove DNA from RNA samples.

RNA-Seq and data analysis

50 bp single-end read libraries were constructed using a TruSeq Stranded mRNA kit and sequenced using the Illumina HiSeq2500. We generated raw counts by mapping reads to all the open reading frames (ORFs) in the *B. subtilis* 168 genome (Gene Bank: NC_000964.3) plus *sfp*, *swrA* and ORFs in the plasmid pSB32 with Kallisto

(V0.42.2.1)[177]. Raw counts were used as the input for DESeq2 (R/Bioconductor)[178] to generate normalized counts for differentially gene expression analysis. Transcript data were normalized to library size. ORFs with |fold change|≥ 2 and Padj≤ 0.05 are defined as differentially expressed genes in this study.

Heatmap

To organize differentially expressed genes into a meaningful structure, heatmap combined with K-means clustering was used. Normalized counts for these differentially expressed genes were extracted for calculation of Z-scores for further analysis. Z-scores were calculated based on the formula: (X-Y)/Z (X: normalized counts of the sample; Y: average normalized counts of all four samples; Z: standard deviation for all four samples). Z-scores were used as a matrix to generate heatmap with Heatmap.2 (gplots) package of R/Bioconductor. We used NbClust (R/Bioconductor) to determine the cluster number as K=5. Column clustering was hierarchical. Hierarchical clustering and K-means clustering were applied to cluster lines in the heatmap.

Cytoscape network

Identities of known regulons were downloaded from Subtiwiki and paired with our transcriptomic data. The output was organized as regulatory networks that emerge at 6 and 24 hours based on the number of genes within a given regulon that change expression by $\geq 2X$ and adjusted $p \leq 0.05$. Each node represents an individual regulator.

Each node represents a regulator. Node size represents the normalized number of genes that change expression relative to the number of overall genes in each regulon. The edge connecting nodes represents shared genes between two regulons. Edge width represents the normalized number of shared genes that change expression relative to the number of total number of shared genes. Blue and red inside each node represent the percentage of upregulation and downregulation in each regulon, respectively.

Metabolite extraction and HPLC-MS/MS

Metabolite extraction was adapted from the previous method[179]. Briefly, cells were collected with cell scrapers from agar plates and immediately transferred to the low-temperature organic solvent (methanol: acetonitrile: H2O=40:40:20). After three rounds of freeze-thaw between -20 °C and -80 °C, cells were spun down and saved for DNA content measurement by diphenylamine assay, and supernatants were collected and dried using nitrogen gas. Samples were subject to HPLC-MS/MS analysis in Dr. Amy Caudy's laboratory at the University of Toronto.

Click-iT assay

The method used in this study was adapted from Click-iT HPG Alexa Fluor protein synthesis assay with slight modifications. *B. subtilis* cells cultured in S7 (methionine-free) medium overnight were passaged to 500 µl S7 medium to an optical density of 0.08 at 600 nm (OD600=0.08) and grown to OD600=0.5 in a 30 °C shaker with a speed of 230 rpm in the dark. 5 µl of 100 µM Cm was added to 495 µl *B. subtilis*

culture at OD600=0.08 to a final concentration of 1 μM Cm. Then, 5 μl of 5 mM HPG was added to the culture to a final concentration of 50 μM and grown in the same condition. For, 16 μM Cm sample, 5 μl of 1.6 mM Cm was added to 490 μl *B. subtilis* culture with 5 μl of 5 mM HPG at OD600=0.5 to a final concentration of 16 μM Cm. After 5 min incubation, S7 medium containing HPG was removed (Eppendorf benchtop centrifuge, 15,000 rpm, 1 min) and cells were washed once with 500 μl PBS (15,000 rpm, 1 min). Then, cells were fixed in 500 μl 4% paraformaldehyde (PFA) (in 1X PBS) at room temperature for 7 min. Cells were washed once with 500 μl PBS and resuspended in 500 μl PBS followed by sonication for 20s to separate cells (1s/1s; 20% amplitude with Thermo FB-20). For permeabilization, cells were washed twice with 3% BSA (in 1X PBS), and then permeabilized in 500 μl 0.5% Triton X-100 (in 1X PBS) for 20 min at room temperature. To detect the HPG incorporation, we followed the Click-iT HPG Alexa Fluor protein synthesis assay kits user guide. Briefly, cells were washed twice with 3% BSA (in 1X PBS) to remove the permeabilization buffer and resuspended in 500 μl Click-iT reaction cocktail (prepared 15 min before the reaction) for a total of 30 min incubation in the dark. Cells were further washed with Click-iT reaction rinse buffer and resuspended in PBS before flow cytometry analysis (BD Accuri C6). The mean fluorescence intensity was calculated with 20,000 cells for each sample.

CHAPTER IV

ANTIBIOTIC RESISTANCE COUPLED WITH SLIDING MOTILITY PROMOTES B. SUBTILIS SURVIVAL DURING INTERSPECIES COMPETITION

Summary

Bacteria evolve diverse strategies to survive during competition with other species in microbial communities. To study these strategies, we previously developed a model system comprising *Bacillus subtilis* and *Streptomyces venezuelae* and found that *S. venezuelae* produces chloramphenicol (Cm) to induce sliding motility in *B. subtilis*. In the process to probe the driving force behind Cm-induced sliding motility in *B. subtilis* using RNA-Seq, we found several resistance genes in *B. subtilis* increase their expression upon early exposure to Cm and during the transition to a sliding population. However, the resistance to Cm does not change upon the loss of these resistance genes. A screening of resistance functions resulted in the identification of a phleomycin and bleomycin transporter, BmrCD. In addition, we identified colony expansion in *B. subtilis* as a common response during competition with antibiotic-producing streptomycetes. These data suggest *B. subtilis* may use a combination of antibiotic resistance and sliding motility to improve its fitness during interspecies competition. Importantly, antibiotic-induced colony expansion was also observed in other species, implying a widespread function of colony expansion as a response to antibiotic insults.

Introduction

Antibiotic resistance has increasingly become a global concern due to the emergence of diverse resistance mechanisms bacteria possess. In general, antibiotic resistance can be classified into two categories, intrinsic resistance and acquired resistance. Intrinsic resistance is a bacterial inherent trait, usually linked to particular cellular structure or function[180], while acquired resistance is the resistance acquired by mutations in the chromosome or exogenously through horizontal gene transfer[180]. Many genes have been identified to be associated with intrinsic resistance through high-throughput genetic screens[180], such as genes encoding outer membrane porin proteins[181], efflux pumps and antibiotic modification enzymes[180]. These gene products either reduce the concentration of toxic substances inside bacterial cells or prevent the access of toxin to cellular targets. Most identified intrinsic resistance mechanisms are related to efflux pumps[180]. Unlike acquired resistance, which is achieved through natural selection, intrinsic resistance can be regulated by environmental signals such as iron limitation[182] and antibiotics[133,183]. It has been shown that the expression of efflux pumps increases in the presence of antibiotics[180]. Both intrinsic resistance and acquired resistance enable bacteria to survive in the presence of antibiotic-producing competitor species in microbial communities.

Bacillus subtilis is a Gram-positive soil organism that has multiple adaptive functions to cope with environmental changes and competitor organisms. Many genes and corresponding developmental pathways have been well studied in *B. subtilis*, which

makes it an excellent model organism to study antibiotic resistance. It is shown that at least 78 ABC transporters have been annotated in *B. subtilis* genome[184]. The function of these transporters may vary in different contexts[185,186], but several reported transporters are related to intrinsic antibiotic resistance[183,187]. Antibiotics have been shown as chemical agents to mediate bacterial interactions in natural settings[188]. In response to antibiotics produced from other competitor species in microbial communities, *B. subtilis* can modulate its growth to adapt to new settings. An example is changing motility, which enables *B. subtilis* to avoid unfavorable conditions and move to safety. Three types of motility so far have been reported in *B. subtilis*, including swimming, swarming and sliding[52]. Unlike flagellum-dependent swimming and swarming, sliding relies on the production of surfactin and extracellular polysaccharide (EPS) and it is flagellum-independent[163,172]. It also has been reported that a mobile population may increase the resistance to antibiotics[51,109]. Thus, the intrinsic resistance in *B. subtilis* is likely affected by the presence of antibiotic-producing species in the same habitat due to the interspecies interaction. Although antibiotic resistance has been studied for decades, much of the effort was focused on the efficacy of one antibiotic in the treatment. How cellular responses resulted from one antibiotic change the activity of other antibiotics and benefit bacterial survival remains largely unknown.

Previously, using a competition model composed of *B. subtilis* and *S. venezuelae*, we identified Cm as a sliding inducer in *B. subtilis*[53]. We investigated how *B. subtilis* regulates gene expression to transition from a non-mobile phase to a mobile phase. In the

course of these studies, we found that Cm changes the expression of several known and predicted resistance genes during a transition from static colony growth to colony expansion by sliding. Furthermore, we demonstrated that Cm treatment results in cross-resistance to phleomycin and bleomycin in *B. subtilis*. As a direct outcome of competition, sliding presumably plays a crucial role in the survival of *B. subtilis* in response to competitors in the environment. Our data indicate that Cm is not the only sliding inducer secreted by *S. venezuelae* in our competition model and several other streptomycetes are able to activate the sliding response in *B. subtilis*. Importantly, the colony expansion induced by Cm is also observed in a model organism for *Mycolicibacterium tuberculosis, M. smegmatis*. Thus, we conclude that *B. subtilis* and possibly other bacterial species use a combination of intrinsic resistance and sliding motility to survive in response to diverse competitors in the environment. In addition, the effect of antibiotics on surface translocation in different bacteria implicates that it may be worth considering bacterial interactions when developing effective strategies to fight against pathogens.

Results

Antibiotic resistance genes change their expression in response to sub-MIC Cm

Among those genes (~400) changing expression after Cm exposure, we identified a specific subset of genes either known or predicted to be involved in antibiotic resistance. The genes are *vmlR, yxjB, bmrCD, mdr, ytbDE* and *liaIH. vmlR, yxjB* and *liaIH* have different antibiotic specificities(**Figure IV-1**), all of which are unrelated to

chloramphenicol. *vmlR* encodes an ABC transporter that binds to the ribosome and mediates the resistance to lincomycin and virginiamycin M[189,190]. *yxjB* encodes a 23S rRNA methyltransferase that confers resistance to tylosin[191]. *liaIH* encode proteins that mediate resistance to cell wall antibiotics such as daptomycin and bacitracin[192]. The function of *bmrCD* has been demonstrated as a heterodimer transporter for several fluorescent drugs in a vesicle assay[185]. Importantly, these genes are not all regulated under control of a single regulatory protein, so they do not fit into any of the known stress response pathways under sigma factor control. However, three of them share a terminator-antiterminator regulatory mechanism that is dependent upon the protein synthesis rate. It is not clear whether they are related to Cm resistance and what benefits their expression has on *B. subtilis* under Cm exposure.

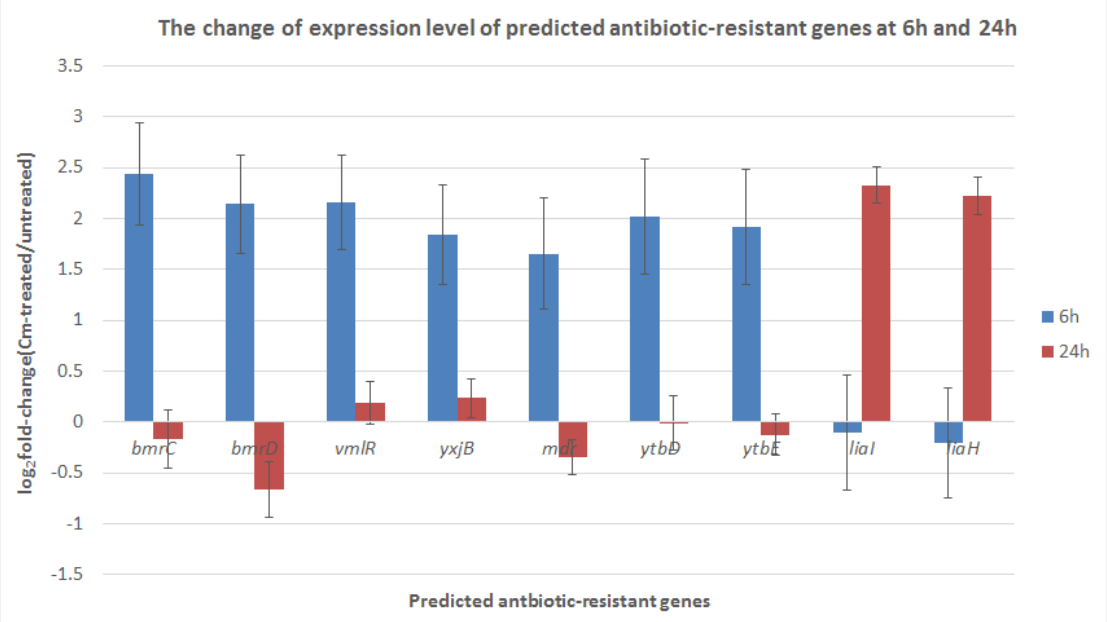


Figure IV-1 Resistance profile in response to Cm changes over time.

Figure IV-1 continued. In response to 1 μM Cm, *bmrCD, vmlR, yxjB, mdr, ytbDE* increase more than two-fold at 6 h, while *liaIH* increase more than two-fold at 24 h. However, the change of *bmrCD, vmlR, yxjB, mdr, ytbDE* at 6 h and *liaIH* at 24 h are not significant (< two-fold).

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

It has been reported that antibiotic resistance could be induced by the same antibiotic. Examples include induced chloramphenicol resistance through expression of *cat-86* by chloramphenicol[193], enhanced tetracycline resistance linked to *tetR* by tetracycline[194], and elevated erythromycin resistance associated with *ermC* by erythromycin[195]. Therefore, if resistance is critical for the survival of *B. subtilis* cells in response to sub-MIC Cm, then removal of resistance-associated genes, especially those genes that provide resistance at 6 hours should significantly enhance the sensitivity to Cm. To determine whether these predicted antibiotic resistance genes contribute to Cm resistance, we deleted all antibiotic resistance genes that are elevated at 6 hours. Since sliding motility induced by Cm is a dose-dependent phenomenon[53], subtle changes in the sensitivity may lead to dramatic differences in the sliding pattern. We performed the sliding assay at a range of sub-MIC Cm (0-8 μM). However, we found no difference was observed between *B. subtilis* NCIB 3610 wild type strain and Δ5 strain (**Figure IV-2**), suggesting that increased expression of these resistance genes does not confer resistance to Cm. This result implies that increased expression of these predicted antibiotic resistance genes does not affect Cm sensitivity and in the meantime raises a question of how they benefit the survival of *B. subtilis*.

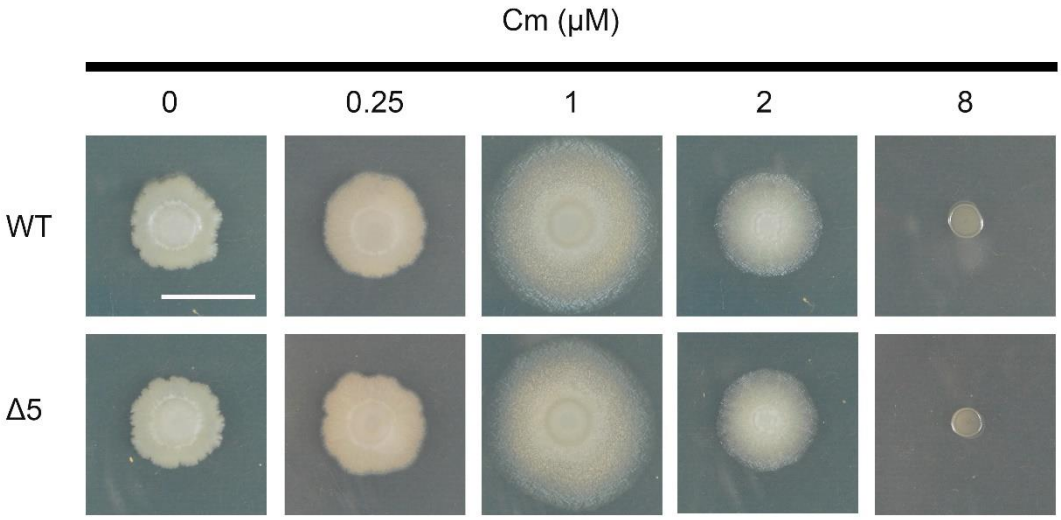


Figure IV-2 Deletion of resistance genes does not change the sensitivity to Cm. Five resistance elements (*bmrCD, vmlR, yxjB, mdr* and *ytbDE*) that respond to Cm at 6 h were deleted (refer to as Δ5 strain). Wild type (WT) and Δ5 strains were plated on the GYM7 plate with a range of sub-MIC Cm (0-8 μM). Pictures were taken at 24 h. Bar, 1 cm.

Cm treatment leads to increased phleomycin and bleomycin resistance via BmrCD

Previous studies show that *vmlR* and *yxjB* are related to lincomycin and tylosin resistance, respectively [189,191]. Thus, we expect that *B. subtilis* may actually increase the expression of these resistance genes to counteract any possible insults from the environment. To investigate this idea, we tested the sensitivity of Δ5 to a list of antibiotics in the presence and absence of Cm. Among those tested antibiotics (**Table 2**), in contrast to wild type strain, Δ5 is more sensitive to phleomycin (**Figure IV-3**), but not to other antibiotics tested in this study, indicating that at least one of these deleted genes should be responsible for phleomycin resistance.

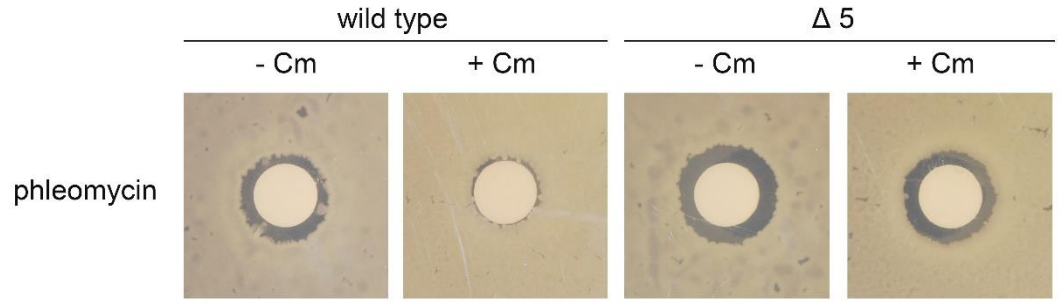


Figure IV-3 Cm treatment leads to increased phleomycin resistance.
Wild type and Δ5 strains (OD600=1.0, 100 µl) were spread on the GYM7 agar medium in the absence (-) and presence of 1 µM Cm. Then, 10 µl of 125 µg/ml phleomycin was added onto the paper disc. Cm treatment reduced the inhibition zone in wild type strain, but not in Δ5 strain. Pictures were taken at 24 h. Diameter of paper disc, 6 mm.

To further investigate which gene correlates with phleomycin resistance, we assayed each gene deletion strain and found *bmrCD* genes contribute to the resistance to phleomycin (**Figure IV-4A**). *bmrCD* genes encode a heterodimer BmrCD transporter, which is predicted as a multidrug ABC transporter. Although BmrCD has been shown to transport several fluorescent dyes in a vesicle assay, its transporter function has not been reported in vivo. To validate the function of *bmrCD* as a phleomycin transporter and uncouple phleomycin resistance from Cm induction in wild type strain, we designed a *bmrCD* overexpression strain under the control of an IPTG-inducible promoter (P*hyperspank*). Although there is some leaky expression for this overexpression system, the inhibition zone caused by phleomycin significantly reduced to the level observed in the Cm treatment group in the presence of IPTG (**Figure IV-4A**). Complementation of P*hy-bmrCD* in Δ*bmrCD* strain at the *amyE* locus restored the phleomycin resistance

phenotype. In the presence of IPTG, the inhibition zone returned to the level comparable to that of Cm-treated sample. To further verify this result, we introduced P*hy-bmrCD* with point mutations in the Walker A motif of BmrC and BmrD into both wild type and Δ*bmrCD* strains (REF). Consistent with the above results, loss of the function of binding nucleotide completely abolished BmrCD transporting ability for phleomycin (**Figure IV-4A**). The BmrCD exhibits crippled function to transport phleomycin in both strains, even in the presence of IPTG. Altogether, these results indicate that BmrCD is a phleomycin transporter in vivo.

Since phleomycin is in the class of glycopeptide antibiotics, we wonder whether BmrCD is able to transport other structurally similar antibiotics in this class. To test this idea, we chose two other antibiotics in the glycopeptide class, bleomycin and vancomycin. Although both of them resemble phleomycin structurally, bleomycin is more similar to phleomycin functionally. Using a modified Kirby-Bauer assay, we found BmrCD confers resistance to both phleomycin and bleomycin, but not vancomycin **(Figure IV-4B)**. Importantly, the antibiotic resistance is more obvious with bleomycin treatment. However, the difference between phleomycin and bleomycin may be due to the concentration used in the assay. We further confirmed this result using the P*hy-bmrCD* overexpression strain and *bmrCD* point mutant (**Figure IV-4B**). These results suggest that BmrCD may have a very constrained substrate specificity. Because both bleomycin and phleomycin target DNA, and other antibiotics that inhibit DNA

replication were transported by BmrCD in a previous study[185], it would be attractive to study whether BmrCD has a specific substrate determinant at the structural level.

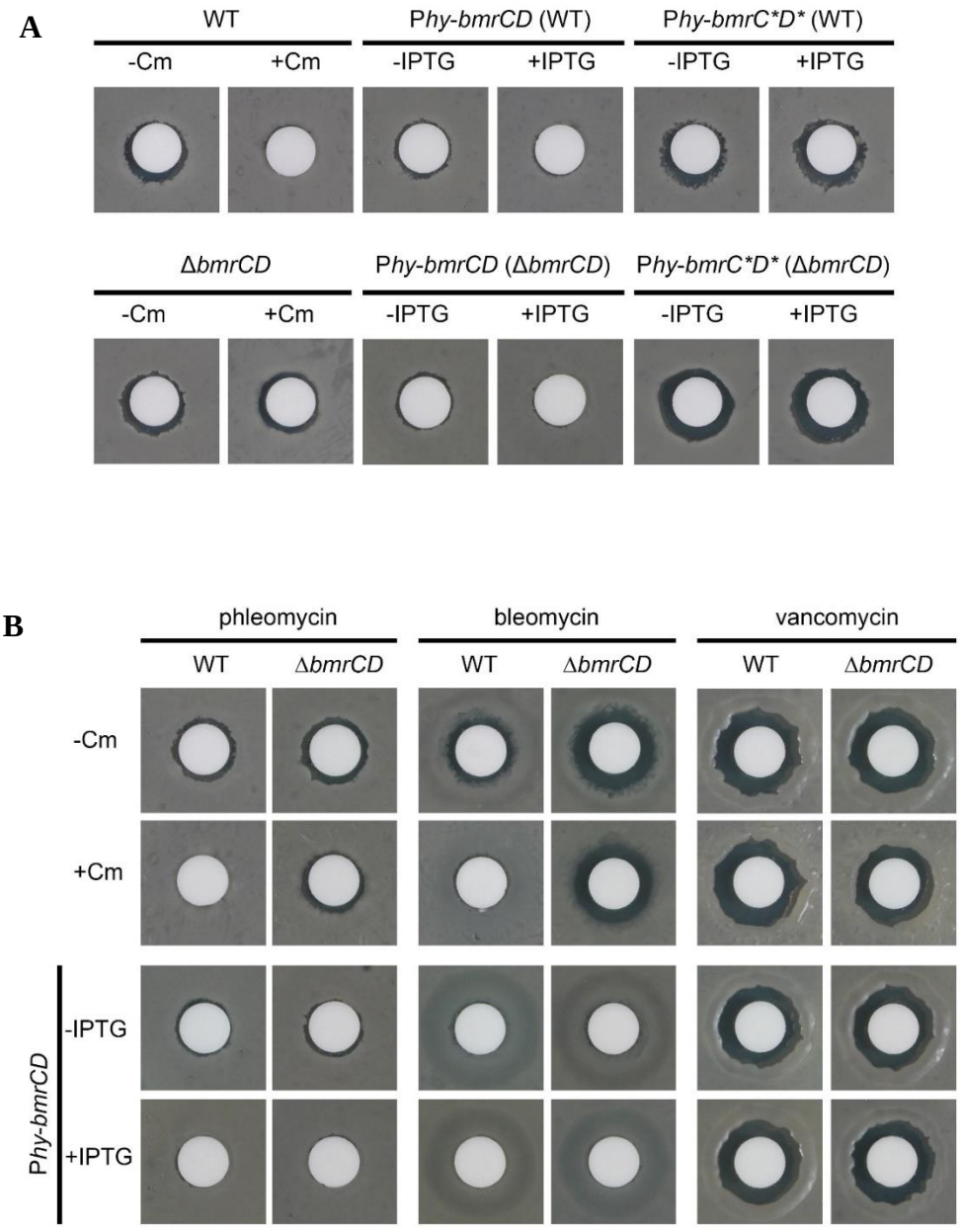


Figure IV-4 BmrCD is transporter for phleomycin and bleomycin.
(A). Left: WT and Δ*bmrCD* strains (OD600=1.0, 100 µl) were spread on the GYM7 agar medium in the absence (-) and presence of 1 µM Cm. Then, 10 µl of 125 µg/ml

Figure IV-4 continued. 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. There is some leaky expression for this promoter. Right: point mutations introduced to Walker A motif of BmrC and BmrD in both WT and Δ*bmrCD* strains abolished the BmrCD function as a phleomycin transporter, with an observation of inhibitory zone similar to the group without IPTG. Pictures were taken at 24 h. Diameter of paper disc, 6 mm. (B) 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.

Deletion of vmlR leads to induced sliding motility under lincomycin exposure

These five genes are not related to Cm resistance, suggesting that Cm-induced resistance gene expression is not antibiotic-specific, but it is a type of stress response related to protein synthesis. Since sliding motility is tied to protein synthesis stress and lincomycin does not induce sliding motility in wild type *B. subtilis* background, we wonder whether we could observe the sliding response in the absence of *vmlR*. To do so, we constructed *vmlR* deletion strain in NCIB 3610 background and performed sensitivity and sliding assay in a wide range of lincomycin concentrations. Compared with wild type strain, Δ*vmlR* is more susceptible to lincomycin, which inhibits Δ*vmlR* growth at a concentration of 1.56 µg/ml (**Figure IV-5**). Intriguingly, we found that sliding motility is observed in Δ*vmlR*, but not in wild type strain. The possible explanation may be linked to the binding efficiency of lincomycin to the ribosome. This result confirms the linkage

between the function of *vmlR* and lincomycin resistance, suggesting *B. subtilis* cells employ Cm as a warning cue to prepare themselves before threats emerge.

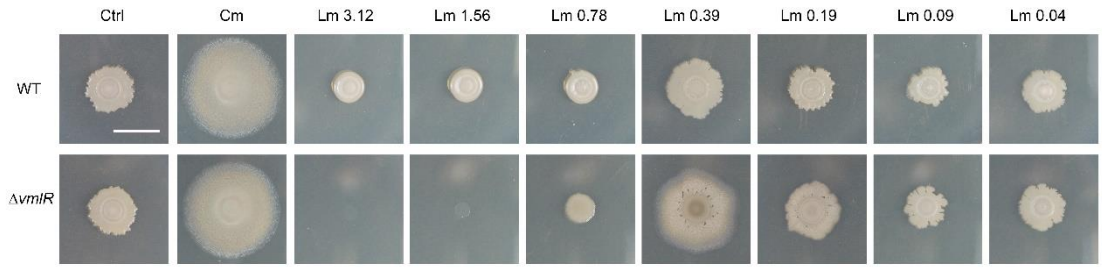


Figure IV-5 Deletion of *vmlR* leads to induced sliding motility under lincomycin exposure.
Wild type and Δ *vmlR* strains were plated on the GYM7 plate with 1 μM Cm (control) and a wide range of lincomycin(Lm) concentrations (0.04-3.12 μg/ml). Δ*vmlR* is more sensitive to lincomycin, not to Cm. The minimal inhibitory concentration Δ*vmlR* in this assay is 1.56 μg/ml. Pictures were taken at 24 h.

Cm is not the only sliding inducer in the competition model system

Besides intrinsic resistance conferred by those resistance genes, as an outcome of translation stress, sliding motility also promotes the survival of *B. subtilis* in the competition model. Since *S. venezuelae* harbors a variety of gene clusters responsible for production of diverse antibiotics including ribosome-targeting antibiotics, it would be intriguing to study whether Cm is the only sliding inducer secreted from *S. venezuelae*. To test this idea, we deleted *cmlP* (*sven0922*) involved in Cm biosynthetic pathway. Because *cmlP* encodes a key enzyme that carries adenylation, PCP and reductase domains in the Cm biosynthesis pathway, the deletion of *cmlP* should abolish Cm production. To confirm Δ*cmlP* no longer produces Cm, we extracted metabolites from

buffered GYM plates where we grew wild type and Δ*cmlP* separately under the same growth conditions, followed by HPLC-MS/MS analysis. The disappearance of peak 321.00 and corresponding isotope peak profile in Δ*cmlP* implies that Cm production is disabled or significantly repressed (**Figure IV-6A**). Thus, we cultured Δ*cmlP* with *B. subtilis* and found sliding motility was still observed. However, the pattern is completely different from the X-shaped form observed with wild type *S. venezuelae* (**Figure IV-6B**). We expect that the difference at 48 h and 72 h may be due to loss of accumulated Cm around the distant Δ*cmlP* patches and insufficient unknown sliding inducer released from the mutant. This result suggests that aside from Cm, *S. venezuelae* secretes another sliding inducer when it competes with *B. subtilis*.

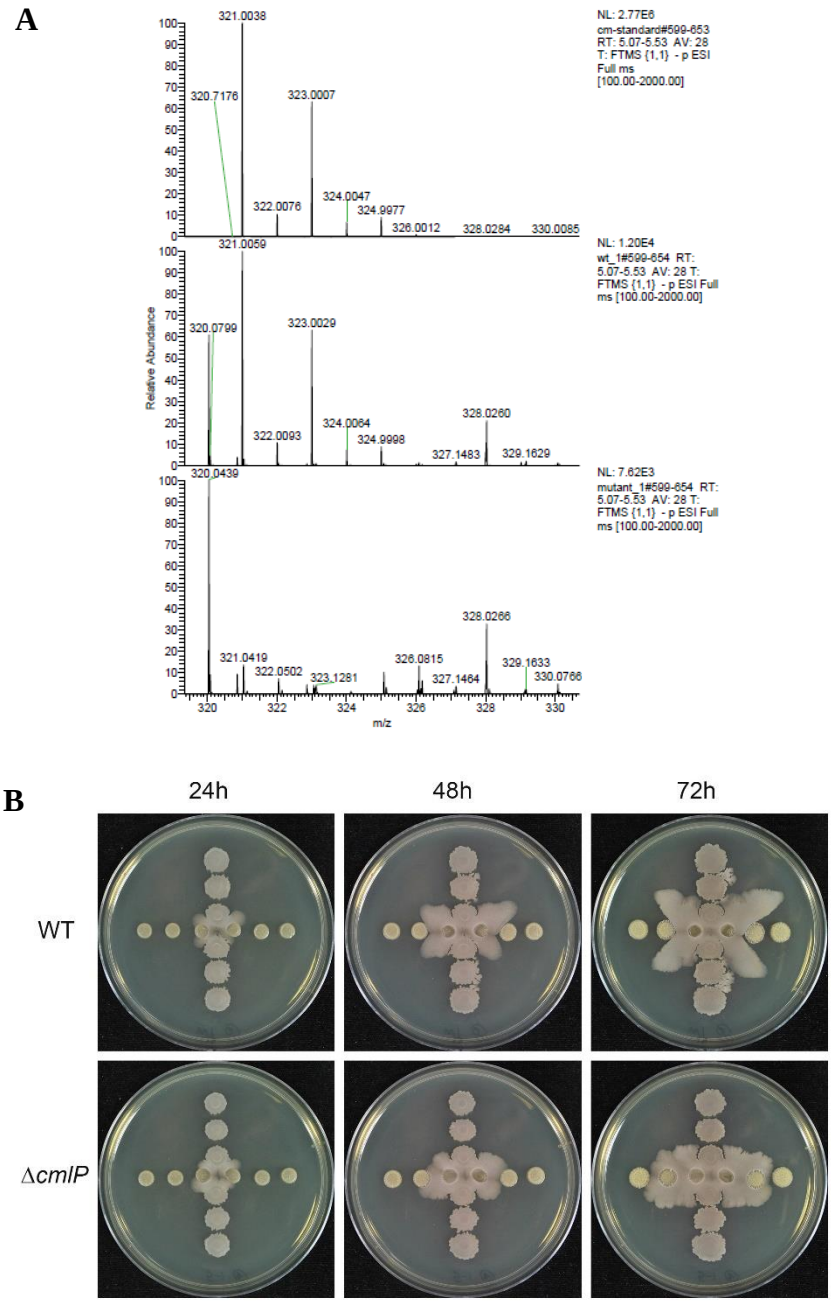


Figure IV-6 Cm is not the only sliding inducer secreted from *S. venezuelae*.
(A) The isotope peak profile of Cm standard (top), crude extract from GYM7 medium where wild type strain was grown (middle), crude extract from GYM7 medium where wild type strain was grown (bottom). (B) Wild type and Δ*cmlP* mutant (no production of Cm) strains were cultured with *B. subtilis* NCIB 3610. Sliding motility was induced in both strains but with different patterns. Pictures were taken at 24 h, 48 h, 72 h. Diameter of the petri dish, 8.4 cm.

Colony expansion is a common response to many streptomycetes in B. subtilis

Our data show that several bacteriostatic antibiotics induce sliding motility in *B. subtilis*. Because most antibiotics are produced by streptomycetes, we wonder if other species in *Streptomyces* genus stimulate colony expansion in *B. subtilis*. To explore this possibility, we expand the analysis to several streptomycetes with a sequenced genome. Pairwise competition assays revealed that seven out of nine species tested have different degrees of sliding induction ability (**Figure IV-7**). However, sliding motility is not activated by *S. griseoflavus* or *S.* sp. AA4 in this assay (**Figure IV-7**). We speculate that the concentration of potential sliding inducers from these streptomycetes may play a crucial role in colony expansion, as observed in Cm-induced sliding motility. Another possibility is that the sliding response may be masked by activities of other metabolites produced by these two streptomycetes. Therefore, further analysis of their sliding-inducing ability under different experimental conditions would be required. These results suggest that sliding-inducing property is not restricted to *S. venezuelae*, but is also possessed by many other streptomycetes.

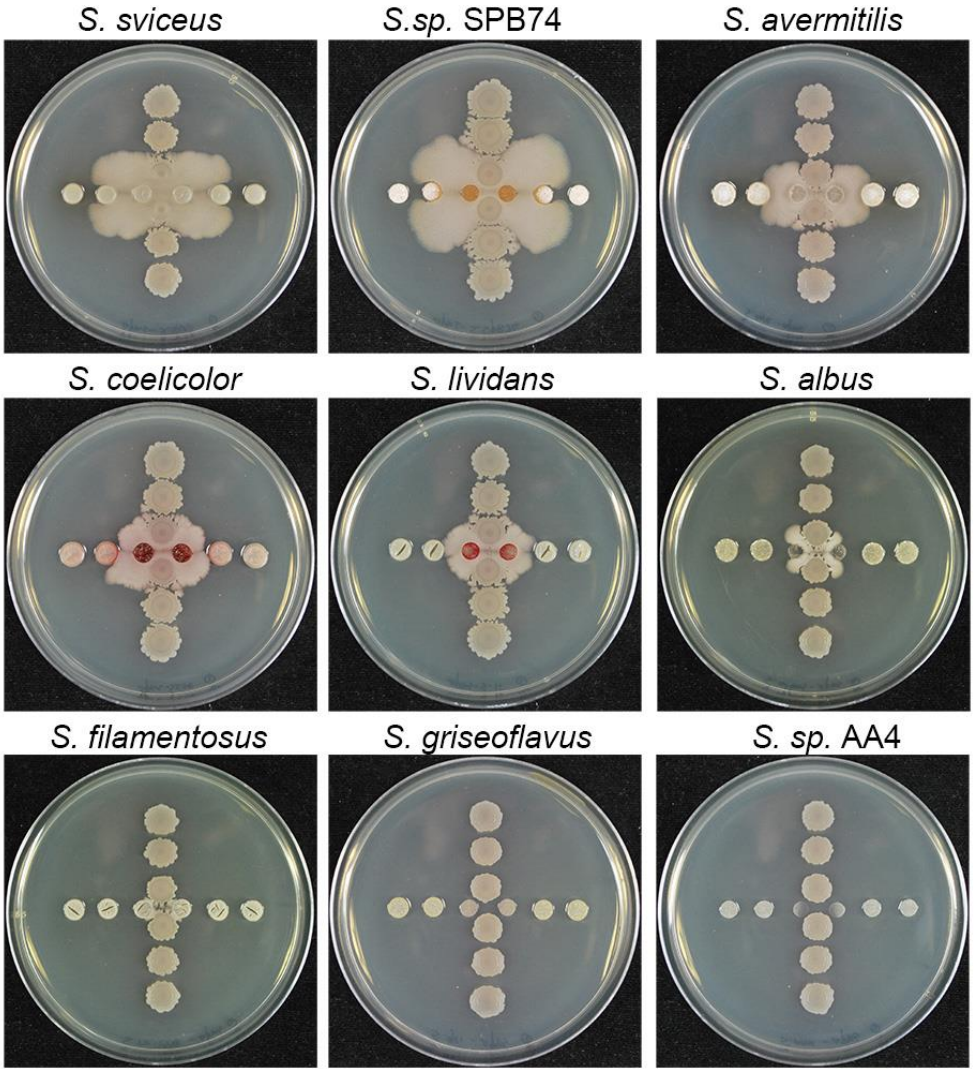


Figure IV-7 Multiple streptomycetes induce colony expansion in *B. subtilis*. Spores collected from each Streptomyces species were spotted (2.5 µl of 10^7 spores/ml) on the GYM7 and then cultured with *B. subtilis* (1.5 µl , OD600=1.0). Pictures were taken at 48 h. Diameter of petri dish, 8.4 cm.

Cm-induced colony expansion is observed in M. smegmatis

Because several other species have been shown to possess sliding ability, Cm-induced sliding motility in *B. subtilis* led us to explore whether Cm stimulates colony

spreading in other bacteria. Notably, *codY*-deficient *B. subtilis* strain is trapped in a sliding "on" mode in our study. Therefore, we tested this idea based on either of these two criteria, observed sliding motility or confirmed CodY function in the species. Thus, we selected *Mycolicibacterium smegmatis*[196], *Listeria innocua* and *Clostridium difficile* [197], and challenged them with sub-MIC Cm. Unfortunately, we did not observe the difference between Cm-challenged and control group in *L. innocua* and *C. difficile* (data not shown). We suspect that the ability to spread over the surface in response to sub-MIC Cm may be linked to the growth conditions and special requirements for surface translocation in various bacteria species. However, we found that compared with the untreated group, Cm is able to induce colony expansion in *M. smegmatis*, a model organism for the pathogenic *M. tuberculosis*, and the onset of Cm-induced expansion could be observed at 48 h (**Figure IV-8**). The difference in the initiation timing for sliding in *M. smegmatis* in comparison to *B. subtilis* may be due to the variant inherent growth rate under different growth conditions.

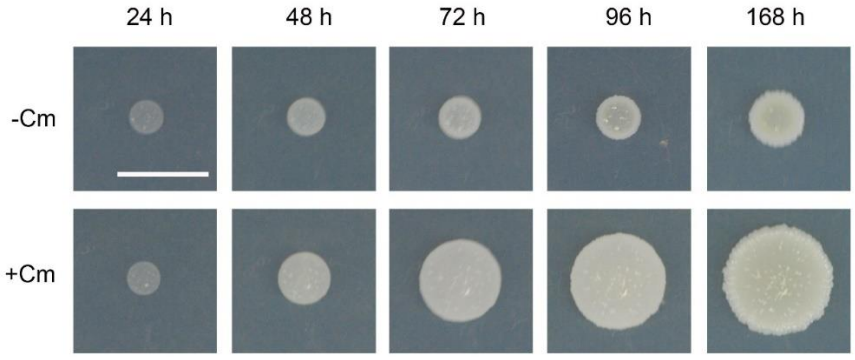


Figure IV-8 Cm induce colony expansion in *M. smegmatis*.
***M. smegmatis* was spotted on the 7H9 medium in the absence (-) and presence (+) of 1 µM Cm. Pictures were taken at 24 h, 48 h, 72 h, 96 h and 168 h. Bar, 1 cm.**

Discussion

Competition between *B. subtilis* and *S. venezuelae* activates defense mechanisms in *B. subtilis*. In response to sub-MIC Cm, *B. subtilis* cells protect themselves from external stresses by enhancing the expression of resistance genes. Furthermore, the resistance pattern induced by Cm varies over time. More importantly, such a response leads to cross-resistance to several other antibiotics. Sliding motility may provide an additional way for *B. subtilis* to defend competitors when the resistance profile changes over time. We found sliding motility in *B. subtilis* is not just activated by *S. venezuelae*, but also stimulated by many other streptomycetes, consistent with our previous observation that several antibiotics produced by streptomycetes could induce sliding motility in *B. subtilis*. Our data also suggest that Cm triggers colony expansion in *M. smegmatis*, highlighting the importance of studying bacterial interactions to facilitate antibacterial treatments given the widespread use of antibiotics in the environment as well as in the host.

The change of Cm-induced resistance profile over time reflects the complex regulation of antibiotic resistance genes. Upon early exposure to sub-MIC Cm, *B. subtilis* increases the expression of several genes that are known to be responsible for antibiotic resistance. These include *vmlR* for lincomycin resistance, *yxjB* for tylosin resistance, and *liaIH* for bacitracin[190–192]. It is likely that *mdr* and *ytbDE* at the early response stage are linked to other antibiotics. At 24 hours, when sliding motility is observed, the expression of these genes decreases, whereas *liaIH* increase. These results

suggest that the expression of these resistance genes is transient and *B. subtilis* has different layers of resistance modules to protect it from antibiotic stresses at different stages. The same sensitivity to Cm in *B. subtilis* after the deletion of five resistance elements at 6 h may further support this expectation. Notably, the resistance enhanced by Cm is not specific to certain types of antibiotics or cellular targets since both lincomycin and tylosin target the ribosome to prevent protein synthesis, while phleomycin and bleomycin inhibit the DNA activity.

Intrinsic resistance induced by one antibiotic is often related to the regulation of the same antibiotic. The mechanism underlying this phenomenon is likely linked to antibiotic-mediated transcription attenuation and/or translation attenuation mechanisms and various riboswitches. Examples can be found in *yxjB* and *vmlR* expression[183,191]. However, it is also shown that some antibiotics induced cross-resistance to other antibiotics. 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[195]. This type of cross-resistance is due to the modification of the same target. As a transporter, BmrCD has been reported to function in vitro to pump a few fluorescent drugs out of vesicles[185]. However, its role as a drug transporter in vivo has never been proved in previous studies. Here, we demonstrate that Cm increases the resistance to phleomycin and bleomycin in *B. subtilis* by upregulating an efflux pump BmrCD expression level. The substrate specificity of BmrCD is not well understood right now. In combination with one previous study from

Torres *et al.*, it seems that BmrCD prefers to pump DNA-targeting drugs out of the cell. However, this speculation needs to be proved by more experiments. 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[30]. These data suggest that antibiotic-induced cross-resistance may be a common phenomenon, which may promote biodiversity in the microbial communities composed of diverse species.

Physiological adaptions provide *B. subtilis* a complimentary way to compete with other microbes. In response to environmental cues, *B. subtilis* is able to differentiate into diverse cell types to adapt to local settings through a complex gene regulation network. In our bacterial competition model, *B. subtilis* cells initiate a sliding response to evade from *S. venezuelae*. The resistance profile change in a sliding population compared with that at the early Cm exposure stage. It is likely that a sliding population would show increased resistance to some other antibiotics because there are other upregulated genes with unknown functions based on our transcription analysis. Since *B. subtilis* sliding motility is surfactin and EPS-dependent[54], it resembles a mobile biofilm. Understanding how widespread the sliding response is present may provide clues to study bacterial interactions at different levels and for optimized bacterial infection treatments. Our data suggest that *B. subtilis* cells activate sliding motility in response to a wide array of streptomycetes. Because sliding motility is induced by several protein synthesis inhibitory antibiotics and streptomycetes are the major antibiotic-producers, further

identification of sliding inducers may lead to the discovery of new translation inhibitors. Importantly, we found that Cm-induced surface translocation is also detected in *M. smegmatis*. Although more bacterial species need to be examined in the survey of antibiotic effect on colony expansion, our results imply that bacteria seem to preserve the property to escape from hostile niches by sensing antibiotics via diverse mechanisms and their ability to do so may go beyond our imagination.

Materials and methods

Strains, primers and growth media

Bacillus subtilis strains and primers used in this study are listed in Table 4-1 and Table 4-2, respectively. Mutant strains in 168 or PY79 background were transduced into NCIB 3610 by SPP1 phage transduction with standard procedures. *B. subtilis* strains were cultured at 37°C in lysogeny broth (LB) and were inoculated onto GYM7 plates (0.4% D-glucose, 0.4% yeast extract, 1.0% malt extract, 1.5% agar, 100mM MOPS, 2.5mM KH_2PO_4, 2.5mM K_2HPO_4, pH7.0) when grown to OD_{600} = 1. All plate assays were performed at 30°C.

Modified Kirby-Bauer assay

GYM7 agar plates were prepared one day before the experiment. Agar plates were dried under the hood for 25 min before use. Overnight LB cultures were diluted to 0.08 OD_{600}, then grown to 1.0. 100 μL of different *B. subtilis* cultures ($0D_{600}$=1.0) were spread on the plate using glass beads and then dried for 5 min with the petri dish lid

open. Paper discs were then placed on the GYM7 plate, followed by adding 10 μL different antibiotics. Finally, plates were transferred to a 30°C incubator.

Overexpression and point mutation of bmrCD

hyperspank promoter, *lacI* and *spec* resistance cassette were amplified from pDR111, and *bmrCD* was amplified from *B. subtilis* NCIB3610 genomic DNA using the primers listed in Table IV-2. These four fragments were assembled to the *bmrCD* overexpression plamid using Gibson Assembly method. The resulting overexpression plasmid was transformed to *B. subtilis* PY79 using *B. subtilis* one-step transformation method, followed by transduction into *B. subtilis* NCIB3610 SPP1 phage transduction. Point mutations were introduced by designing overlap primers on *bmrC* and *bmrD* based on the *bmrCD* overexpression plasmid using primers listed in Table IV-2.

Sliding motility assay

Coculture assays were performed as previously described. Briefly, 2.5 μL of a 10^7 spores/mL *Streptomyces* spores was spotted in the horizontal line and grown for 12 h at 30°C. 1.5 μL of *B. subtilis* was then spotted 6 mm from *Streptomyces sp.* in the vertical line. For motility assays, 1.5 μL of *B. subtilis* was spotted on the GYM7 plate.

Construction of cmlP deletion strain

An in-frame deletion of *cmlP* (*sven_0922*) was generated with a cosmid 4P22 (a gift from Dr. Marie Elliot) using ReDirect technology (2013-Gust et al.). The *cmlP*

coding sequence was replaced by an apramycin resistance cassette. The *cmlP* deletion was verified by PCR with primers located upstream and downstream of *cmlP*.

Extraction of crude metabolites from S. venezuelae wild type and ΔcmlP strains

The extraction of crude metabolites from *S. venezuelae* wild type and Δ*cmlP* strains was performed as described previously. Briefly, each Streptomyces strain was grown on the top layer (5 ml) of GYM7 plate, separated from the bottom layer by a sheet of cellophane. After five days of growth, metabolites deposited at the bottom layer (20 ml) were extracted using the freezing-squeezing method, followed by lyophilization as described.

HPLC-MS/MS analysis

HPLC-MS/MS was performed with an Exactive mass spectrometer (Thermo) using an ESI source. Separation was performed with a Luna C18 column (15 cm X 3 mm, 3 µm particles, Phenomenex). LC conditions: t=0 min, 0% A; t=2 min, 50% A; t=10 min, 50% A; t=12 min, 95% A. The flow rate was 400 µL/min. Solvent A is 100% acetonitrile. Solvent B is 0.1% formic acid (in H_2O). The mass spectrometer was operated in the negative mode within a mass range from 100 to 2000 Da.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

CONCLUSIONS

Microbial communities are complex and dynamic entities, in which the interactions among various species shape the environment where bacteria reside. To survive, bacteria evolve diverse mechanisms to interact with other species. Two-species interaction models provide a platform for us to explore the underlying interaction mechanisms. To understand these mechanisms, we developed a model system composed of *B. subtilis* NCIB3610 and *S. venezuelae*, and found *S. venezuelae* activates a mobile response in *B. subtilis*. The mobile phenotype raises many questions concerning this pairwise interaction such as what type of motility it is, what agent induces the mobile response, how the mobile response is induced and what benefits it confers to both species. In this dissertation, I sought answers to several questions related to this mobile response from different perspectives.

Identification of protein synthesis inhibitors as sliding inducers

Based on the genetic determinants and environmental factors associated with different types of motility in *B. subtilis*, we first characterized the mobile response triggered by *S. venezuelae* as sliding motility. We then identified the inducing agents released from *S. venezuelae* as chloramphenicol and its derivative, monobromamphenicol at sub-lethal concentrations. Upon further exploration of motility

inducers in light of the chloramphenicol action, we found that motility induction ability is not restricted to chloramphenicol, but also is extended to other bacteriostatic antibiotics, erythromycin, tetracycline and spectinomycin. In addition, we demonstrated that blocking the binding of antibiotics to the ribosome abolished sliding motility, suggesting that inhibition of protein synthesis plays a crucial role in sliding induction in *B. subtilis*. These findings imply that exposure to low doses of antibiotics provides a warning signal to bacteria and enables bacteria to modulate their activities to adapt to new environments. We speculate that such competitive fitness developed through interspecies interactions to promote bacterial survival in complex and dynamic microbial communities.

Antibiotic-mediated metabolic reprograming contributes to the physiological adaptation

The physiological change in *B. subtilis* triggered by sub-MIC Cm opens the door to a more challenging question: how to link the sliding response to the underlying genetic determinants. To answer this question, we first tested whether the property of sliding motility is due to mutations in *B. subtilis*. We found that Cm-induced colony expansion is a transient character, indicating the sliding response is a genetically regulated response. Then, we sought to ask the next level of question regarding the translation efficiency in *B. subtilis* in the presence of sub-MIC Cm. Our data revealed that *B. subtilis* protein synthesis rate is reduced with early exposure of sub-MIC Cm, suggesting subsequent responses following protein synthesis stress result in sliding motility. To further explore the regulatory mechanism underlying Cm-induced sliding

motility, we performed RNA-Seq to measure transcript levels across the whole genome in the absence and presence of Cm at the early stage (6 h) and at the moment (24 h) when sliding motility can be observed. Transcriptional analyses show a change of expression in an array of genes, which pose a challenge to identify the key determinant for the sliding response. To define the key feature that governs the sliding response, we constructed a regulatory network comprising regulators that control the differentially expressed genes for each stage. Regulatory networks emphasize the role of metabolic shifts in the physiological transition to colony expansion. Further characterization of metabolome profiling reflects the substantial change in metabolome and provides additional clues to the function of metabolic changes. A combination of transcriptional data and metabolomics data highlight the role of carbon and nitrogen resource utilization in the sliding response. In light of these findings, we designed two promoter-*lux* reporters to monitor metabolic pathways for carbon and nitrogen resource utilization, respectively. Results from reporter assays suggest that the sliding population exhibits at least two major metabolic states. These findings imply that metabolic reprogramming and metabolic coordination in the clonal population enable the *B. subtilis* population to expand in response to exogenous insults.

Enhanced antibiotic resistance and sliding motility enable B. subtilis *to coexist with antibiotic-producing streptomycetes*

We further explore how *B. subtilis* integrates different responses to promote bacterial survival in the presence of antibiotic insults. Antibiotic resistance is becoming a

global concern. The coexistence of *B. subtilis* with antibiotic-producing streptomycetes provides a clue to understand antibiotic resistance. Our transcriptional data reveal a dynamic resistance profile over time in response to sub-MIC Cm. Particularly, several antibiotic resistance genes increase upon early exposure to sub-MIC Cm. Further experiments indicate that increased expression in these resistance genes does not confer resistance to Cm, but instead to a selection of unrelated antibiotics. A survey of resistance gene functions led to the identification of phleomycin and bleomycin transporter BmrCD. Moreover, our data suggest colony expansion may be a common response for *B. subtilis* to evade invasion by streptomycete competitors. Importantly, we show that antibiotic-induced colony expansion is not only limited to *B. subtilis*, but also observed in other species. These results suggest that *B. subtilis* employs a combination of increased resistance to antibiotics and sliding motility to enhance its fitness during interspecies competition.

Future Directions

Bridge the gap between subsequent responses to protein synthesis stress and sliding motility in B. subtilis

Our data indicate sliding motility is gradually induced by a cascade of events downstream of an initial insult to protein synthesis. Integration with other omics approaches may provide more clues about how the response is generated. Our transcriptomic analyses suggest that sub-MIC Cm induces substantial changes in *B. subtilis* at the transcriptional level and a resulting transcriptional regulatory network

coupled with metabolic changes defines the physiological change in the clonal population. However, gene expression is regulated at both the transcriptional level and translational level. Although transcription and translation are coupled in bacteria, changes at the transcriptional level may not really reflect those at the translational level. Moreover, the protein synthesis rate is reduced in the presence of sub-MIC Cm, which suggests translation stress is induced. Thus, directly probing the affected genes at the translational level when Cm binds to the ribosome would be critical for uncovering the mechanism by which Cm-induced translation stress leads to sliding motility. Therefore, it is tempting to perform ribosome profiling to examine which subset of genes alter their expression in response to sub-MIC Cm at a series of time points. A combination of different omics data will facilitate the identification of key factors that govern the sliding response.

Identify new sliding inducers

How bacteria sense and respond to external signals is of particular importance for us to understand bacterial physiology. Studying the nature of these external signals and subsequence responses induced by them will reveal mechanistic insights into bacterial interspecies interactions. Since several protein synthesis inhibitors stimulate the sliding response in *B. subtilis* and streptomycetes contain many specialized metabolite gene clusters, the observation that different *Streptomyces* species induce colony expansion in *B. subtilis* motivates us to explore the identity of sliding inducers. It is attractive to expect that identification of these sliding inducers may lead to the discovery of new

protein synthesis inhibitors and corresponding gene clusters in streptomycetes. Alternatively, new sliding mechanisms will be uncovered in *B. subtilis* if the inducer does not block protein synthesis.

Meanwhile, many questions remain in this dissertation. For instance, what other cellular targets besides the ribosome in the protein synthesis process are linked to sliding motility? How many metabolic states exist in the clonal sliding population? How do different subpopulations interact to promote colony expansion? Whether Cm can be metabolized at sub-Inhibitory concentrations? What is main factor that drives the sliding response? How does the interaction between these two soil bacteria affect the dynamics of the soil microbial community? However, with more methods such as CRISPR introduced into the interaction model system in this dissertation, more details about how bacteria interact will be unveiled.

We realize that probing the mechanism underlying pairwise interactions is just the initial step to understanding the complex and dynamic interaction network within microbial communities. As the saying goes, “Rome was not built in one day”, we believe that incorporation of individual pairwise interaction into the complex community network will provide significant insights about how exogenous stimuli lead to diverse responses in bacteria and how these responses in various species ensure the dynamics of the whole microbial community.

REFERENCES

1. Eckburg, P. B. *et al.* Microbiology: Diversity of the human intestinal microbial flora. *Science.* **308,** 1635–1638 (2005).
2. Kent, A. D. & Triplett, E. W. Microbial Communities and Their Interactions in Soil and Rhizosphere Ecosystems. *Annu. Rev. Microbiol.* **56,** 211–236 (2002).
3. Straight, P. D. & Kolter, R. Interspecies chemical communication in bacterial development. *Annu. Rev. Microbiol.* **63,** 99–118 (2009).
4. Stubbendieck, R. M. & Straight, P. D. Multifaceted interfaces of bacterial competition. *J. Bacteriol.* **198,** 2145–2155 (2016).
5. Hogan, D. A. & Kolter, R. Pseudomonas - Candida Interactions : An Ecological Role for Virulence Factors. *Science (80-.).* **296,** 2229–2233 (2002).
6. Jones, S. E. *et al.* Streptomyces exploration is triggered by fungal interactions and volatile signals. *Elife* **6,** e21738 (2017).
7. Müller, S. *et al.* Bacillaene and sporulation protect bacillus subtilis from predation by myxococcus xanthus. *Appl. Environ. Microbiol.* **80,** 5603–5610 (2014).
8. Wong, M. J. Q. *et al.* Microbial herd protection mediated by antagonistic interaction in polymicrobial communities. *Appl. Environ. Microbiol.* **82,** 6881–6888 (2016).
9. Wolfe, B. E., Button, J. E., Santarelli, M. & Dutton, R. J. Cheese rind communities provide tractable systems for in situ and in vitro studies of microbial diversity. *Cell* **158,** 422–433 (2014).
10. Kelsic, E. D., Zhao, J., Vetsigian, K. & Kishony, R. Counteraction of antibiotic production and degradation stabilizes microbial communities. *Nature* **521,** 516–519 (2015).
11. Lozano, G. L. *et al.* Introducing THOR, a model microbiome for genetic dissection of community behavior. *MBio* **10,** 1–14 (2019).
12. Stubbendieck, R. M. & Straight, P. D. Escape from Lethal Bacterial Competition through Coupled Activation of Antibiotic Resistance and a Mobilized Subpopulation. *PLoS Genet.* **11,** e1005722 (2015).
13. Hoefler, B. C. *et al.* A Link between Linearmycin Biosynthesis and Extracellular Vesicle Genesis Connects Specialized Metabolism and Bacterial Membrane Physiology. *Cell Chem. Biol.* **24,** 1238–1249.e7 (2017).
14. Hoefler, B. C. *et al.* Enzymatic resistance to the lipopeptide surfactin as identified through imaging mass spectrometry of bacterial competition. *Proc. Natl. Acad. Sci.* **109,** 13082–13087 (2012).
15. Davies, J. Specialized microbial metabolites: Functions and origins. *J. Antibiot. (Tokyo).* **66,** 361–364 (2013).
16. Price-Whelan, A., Dietrich, L. E. P. & Newman, D. K. Rethinking 'secondary' metabolism: Physiological roles for phenazine antibiotics. *Nat. Chem. Biol.* **2,** 71–78 (2006).
17. Davies, J. Are antibiotics naturally antibiotics? *J. Ind. Microbiol. Biotechnol.* **33,**

496–499 (2006).
18. Calabrese, E. J. & Blain, R. The occurrence of hormetic dose responses in the toxicological literature, the hormesis database: An overview. *Toxicol. Appl. Pharmacol.* **202,** 289–301 (2005).
19. Davies, J., Spiegelman, G. B. & Yim, G. The world of subinhibitory antibiotic concentrations. *Curr. Opin. Microbiol.* **9,** 445–453 (2006).
20. Romero, D., Traxler, M. F., López, D. & Kolter, R. Antibiotics as signal molecules. *Chem. Rev.* **111,** 5492–5505 (2011).
21. Yim, G., Wang, H. H. & Davies, J. Antibiotics as signalling molecules. *Philos. Trans. R. Soc. London Ser. B, Biol. Sci.* **362,** 1195–1200 (2007).
22. Linares, J. F., Gustafsson, I., Baquero, F. & Martinez, J. L. Antibiotics as intermicrobial signaling agents instead of weapons. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 19484–9 (2006).
23. Oliveria, N. M. *et al.* Biofilm Formation As a Response to Ecological Competition. *PLoS Biol.* **13,** e1002191 (2015).
24. Tsui, W. H. W. *et al.* Dual Effects of MLS Antibiotics : Transcriptional Modulation and Interactions on the Ribosome. *Chem. Biol.* **11,** 1307–1316 (2004).
25. Goh, E. B. *et al.* Transcriptional modulation of bacterial gene expression by subinhibitory concentrations of antibiotics. *Proc. Natl. Acad. Sci. U. S. A.* **99,** 17025–17030 (2002).
26. Lin, J. T., Connelly, M. B., Amolo, C., Otani, S. & Yaver, D. S. Global Transcriptional Response of Bacillus subtilis to Treatment with Subinhibitory Concentrations of Antibiotics That Inhibit Protein Synthesis. *Antimicrob. Agents Chemother.* **49,** 1915–1926 (2005).
27. Straight, P. D., Fischbach, M. A., Walsh, C. T., Rudner, D. Z. & Kolter, R. A singular enzymatic megacomplex from Bacillus subtilis. *Proc. Natl. Acad. Sci. U. S. A.* **104,** 305–310 (2007).
28. Vargas-Bautista, C., Rahlwes, K. & Straight, P. Bacterial competition reveals differential regulation of the pks genes by Bacillus subtilis. *J. Bacteriol.* **196,** 717–28 (2014).
29. Piewngam, P. *et al.* Pathogen elimination by probiotic Bacillus via signalling interference. *Nature* **562,** 532–537 (2018).
30. Lucas A. Meirelles, Elena K. Perry, Megan Bergkessel, D. K. N. *Bacterial defenses against a natural antibiotic promote collateral resilience to clinical antibiotics*. (2020). doi:https://doi.org/10.1101/2020.04.20.049437
31. Harrison, F., Paul, J., Massey, R. C. & Buckling, A. Interspecific competition and siderophore-mediated cooperation in Pseudomonas aeruginosa. *ISME J.* **2,** 49–55 (2008).
32. Arnaouteli, S. *et al.* Pulcherrimin formation controls growth arrest of the Bacillus subtilis biofilm. *PNAS* **116,** 13553–13562 (2019).
33. Stubbendieck, R. M., Vargas-Bautista, C. & Straight, P. D. Bacterial communities: Interactions to scale. *Front. Microbiol.* **7,** 1–19 (2016).
34. Galet, J. *et al.* Pseudomonas fluorescens pirates both ferrioxamine and ferricoelichelin Siderophores from Streptomyces ambofaciens. *Appl. Environ.*

Microbiol. **81,** 3132–3141 (2015).
35. Traxler, M. F., Seyedsayamdost, M. R., Clardy, J. & Kolter, R. Interspecies modulation of bacterial development through iron competition and siderophore piracy. *Mol. Microbiol.* **86,** 628–644 (2012).
36. McNab, R. *et al.* LuxS-Based Signaling in Sfilm Formation with Porphyromonas gingivalis. *J. Bacteriol.* **185,** 274–284 (2003).
37. Structural and chemical aspects of resistance to the antibiotic, fosfomycin, conferred by F. from B. cereus. 基因的改变NIH Public Access. *Bone* **23,** 1–7 (2011).
38. Imai, Y., Sato, S., Tanaka, Y., Ochi, K. & Hosaka, T. Lincomycin at Subinhibitory Concentrations Potentiates Secondary Metabolite Production by Streptomyces spp. *Appl. Environ. Microbiol.* **81,** 3869–79 (2015).
39. Chen, C. *et al.* Secreted proteases control autolysin-mediated biofilm growth of staphylococcus aureus. *J. Biol. Chem.* **288,** 29440–29452 (2013).
40. Schwechheimer, C., Sullivan, C. J. & Kuehn, M. J. Envelope control of outer membrane vesicle production in Gram-negative bacteria. *Biochemistry* **52,** 3031–3040 (2013).
41. Turnbull, L. *et al.* Explosive cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms. *Nat. Commun.* **7,** (2016).
42. Brown, L., Wolf, J. M., Prados-Rosales, R. & Casadevall, A. Through the wall: Extracellular vesicles in Gram-positive bacteria, mycobacteria and fungi. *Nat. Rev. Microbiol.* **13,** 620–630 (2015).
43. Brown, L., Kessler, A., Cabezas-Sanchez, P., Luque-Garcia, J. L. & Casadevall, A. Extracellular vesicles produced by the Gram-positive bacterium Bacillus subtilis are disrupted by the lipopeptide surfactin. *Mol. Microbiol.* **93,** 183–198 (2014).
44. Lee, J. *et al.* Staphylococcus aureus extracellular vesicles carry biologically active β-lactamase. *Antimicrob. Agents Chemother.* **57,** 2589–2595 (2013).
45. Schrempf, H., Koebsch, I., Walter, S., Engelhardt, H. & Meschke, H. Extracellular Streptomyces vesicles: Amphorae for survival and defence. *Microb. Biotechnol.* **4,** 286–299 (2011).
46. Fajardo, A. & Martínez, J. L. Antibiotics as signals that trigger specific bacterial responses. *Curr. Opin. Microbiol.* **11,** 161–167 (2008).
47. Nicolas, P. *et al.* Architecture in Bacillus subtilis. *Science (80-.).* **335,** 1103–1106 (2012).
48. López, D. & Kolter, R. Extracellular signals that define distinct and coexisting cell fates in Bacillus subtilis. *FEMS Microbiol. Rev.* **34,** 134–149 (2010).
49. Lopez, D., Vlamakis, H. & Kolter, R. Generation of multiple cell types in Bacillus subtilis. *FEMS Microbiol. Rev.* **33,** 152–163 (2009).
50. Kearns, D. B. & Losick, R. Swarming motility in undomesticated Bacillus subtilis. *Mol. Microbiol.* **49,** 581–590 (2004).
51. Butler, M. T., Wang, Q. & Harshey, R. M. Cell density and mobility protect swarming bacteria against antibiotics. *Proc. Natl. Acad. Sci. U. S. A.* **107,** 3776–81 (2010).

52. Kearns, D. B. A field guide to bacterial swarming motility. *Nat. Rev. Microbiol.* **8,** 634–644 (2010).
53. Liu, Y., Kyle, S. & Straight, P. D. Antibiotic Stimulation of a Bacillus subtilis Migratory Response. *mSphere* **3,** e00586-17 (2018).
54. van Gestel, J., Vlamakis, H. & Kolter, R. From Cell Differentiation to Cell Collectives: Bacillus subtilis Uses Division of Labor to Migrate. *PLoS Biol.* **13,** 1–29 (2015).
55. Kinsinger, R. F., Shirk, M. C. & Fall, R. Rapid surface motility in Bacillus subtilis is dependent on extracellular surfactin and potassium ion. *J. Bacteriol.* **185,** 5627–31 (2003).
56. Grau, R. R. *et al.* A Duo of Potassium-Responsive Histidine Kinases Govern the Multicellular Destiny of Bacillus subtilis. *MBio* **6,** e00581 (2015).
57. Ababneh, Q. O. & Herman, J. K. CodY regulates SigD levels and activity by binding to three sites in the fla/che operon. *J. Bacteriol.* **197,** 2999–3006 (2015).
58. Jiang, M., Shao, W., Perego, M. & Hoch, J. A. Multiple histidine kinases regulate entry into stationary phase and sporulation in bacillus subtilis. *Mol. Microbiol.* **38,** 535–542 (2000).
59. LeDeaux, J. R., Yu, N. & Grossman, A. D. Different roles for KinA, KinB, and KinC in the initiation of sporulation in Bacillus subtilis. *J. Bacteriol.* **177,** 861–863 (1995).
60. Marquez-Magana, L. M. & Chamberlin, M. J. Characterization of the sigD transcription unit of Bacillus subtilis. *J. Bacteriol.* **176,** 2427–2434 (1994).
61. Tsukahara, K. & Ogura, M. Promoter selectivity of the Bacillus subtilis response regulator DegU, a positive regulator of the fla/che operon and sacB. *BMC Microbiol.* **8,** 1–12 (2008).
62. Cozy, L. M. *et al.* SlrA/SinR/SlrR inhibits motility gene expression upstream of a hypersensitive and hysteretic switch at the level of σ D in Bacillus subtilis. *Mol. Microbiol.* **83,** 1210–1228 (2012).
63. Bai, U., Mandic-Mulec, I. & Smith, I. SinI modulates the activity of SinR, a developmental switch protein of Bacillus subtilis, by protein-protein interaction. *Genes Dev.* **7,** 139–148 (1993).
64. Chai, Y., Kolter, R. & Losick, R. Reversal of an epigenetic switch governing cell chaining in Bacillus subtilis by protein instability. *Mol. Microbiol.* **78,** 218–229 (2010).
65. Vlamakis, H., Chai, Y., Beauregard, P., Losick, R. & Kolter, R. Sticking together: Building a biofilm the Bacillus subtilis way. *Nat. Rev. Microbiol.* **11,** 157–168 (2013).
66. Cairns, L. S., Hobley, L. & Stanley-Wall, N. R. Biofilm formation by Bacillus subtilis: New insights into regulatory strategies and assembly mechanisms. *Mol. Microbiol.* **93,** 587–598 (2014).
67. Kobayashi, K. Gradual activation of the response regulator DegU controls serial expression of genes for flagellum formation and biofilm formation in Bacillus subtilis. *Mol. Microbiol.* **66,** 395–409 (2007).
68. Chai, Y., Kolter, R. & Losick, R. Paralogous antirepressors acting on the master

regulator for biofilm formation in Bacillus subtilis. *Mol. Microbiol.* **74,** 876–887 (2009).
69. Winkelman, J. T., Blair, K. M. & Kearns, D. B. RemA (YlzA) and RemB (YaaB) regulate extracellular matrix operon expression and biofilm formation in Bacillus subtilis. *J. Bacteriol.* **191,** 3981–3991 (2009).
70. Winkelman, J. T. *et al.* RemA is a DNA-binding protein that activates biofilm matrix gene expression in Bacillus subtilis. *Mol. Microbiol.* **88,** 984–997 (2013).
71. Chung, J. D., Stephanopoulos, G., Ireton, K. & Grossman, A. D. Gene expression in single cells of Bacillus subtilis: Evidence that a threshold mechanism controls the initiation of sporulation. *J. Bacteriol.* **176,** 1977–1984 (1994).
72. Gingichashvili, S. *et al.* Bacillus subtilis Biofilm development - a computerized study of morphology and kinetics. *Front. Microbiol.* **8,** 1–9 (2017).
73. Errington, J. Bacillus subtilis sporulation: Regulation of gene expression and control of morphogenesis. *Microbiol. Rev.* **57,** 1–33 (1993).
74. Stevick, P. T., Soule, M. & Ayala, F. J. Cannibalism by Sporulating Bacteria. *Science (80-.).* **301,** 510–513 (2003).
75. Properties, P. & Regulation, C. hy Study Competence? .....9.6.... 0..... 396 Properties. *Microbiol. Rev.* **55,** 395–424 (1991).
76. Magnuson, R., Solomon, J. & Grossman, A. D. Biochemical and genetic characterization of a competence pheromone from B. subtilis. *Cell* **77,** 207–216 (1994).
77. Mandal, M., Boese, B., Barrick, J. E., Winkler, W. C. & Breaker, R. R. Riboswitches control fundamental biochemical pathways in *Bacillus subtilis* and other bacteria. *Cell* **113,** 577–586 (2003).
78. Gundlach, J., Rath, H., Herzberg, C., Mäder, U. & Stülke, J. Second messenger signaling in Bacillus Subtilis: Accumulation of cyclic di-AMP inhibits biofilm formation. *Front. Microbiol.* **7,** 1–8 (2016).
79. Schilling, O. *et al.* Transcriptional and metabolic responses of Bacillus subtilis to the availability of organic acids: Transcription regulation is important but not sufficient to account for metabolic adaptation. *Appl. Environ. Microbiol.* **73,** 499–507 (2007).
80. Chubukov, V. *et al.* Transcriptional regulation is insufficient to explain substrate-induced flux changes in Bacillus subtilis. *Mol. Syst. Biol.* **9,** 1–13 (2013).
81. Chubukov, V., Gerosa, L., Kochanowski, K. & Sauer, U. Coordination of microbial metabolism. *Nat. Rev. Microbiol.* **12,** 327–340 (2014).
82. Kochanowski, K. *et al.* Few regulatory metabolites coordinate expression of central metabolic genes in Escherichia coli . *Mol. Syst. Biol.* **13,** 903 (2017).
83. Kimata, K., Takahashi, H., Inada, T., Postma, P. & Aiba, H. cAMP receptor protein-cAMP plays a crucial role in glucose-lactose diauxie by activating the major glucose transporter gene in Escherichia coli. *Proc. Natl. Acad. Sci. U. S. A.* **94,** 12914–12919 (1997).
84. Yang, J. H., Bening, S. C. & Collins, J. J. Antibiotic efficacy — context matters. *Curr. Opin. Microbiol.* **39,** 73–80 (2017).
85. Allison, K. R., Brynildsen, M. P. & Collins, J. J. Metabolite-enabled eradication

of bacterial persisters by aminoglycosides. *Nature* **473,** 216–220 (2011).
86. Su, Y. Bin, Peng, B., Han, Y., Li, H. & Peng, X. X. Fructose restores susceptibility of multidrug-resistant Edwardsiella tarda to kanamycin. *J. Proteome Res.* **14,** 1612–1620 (2015).
87. Peng, B. *et al.* Exogenous Alanine and/or Glucose plus Kanamycin Kills Antibiotic-Resistant Bacteria. *Cell Metab.* **21,** 249–262 (2015).
88. Yang, J. H. *et al.* A White-Box Machine Learning Approach for Revealing Antibiotic Mechanisms of Action. *Cell* **177,** 1649–1661.e9 (2019).
89. Takhaveev, V. & Heinemann, M. Metabolic heterogeneity in clonal microbial populations. *Curr. Opin. Microbiol.* **45,** 30–38 (2018).
90. Jacobsen, B. J., Zidack, N. K. & Larson, B. J. The role of Bacillus-based biological control agents in integrated pest management systems: Plant diseases. *Phytopathology* **94,** 1272–1275 (2004).
91. Lewis, K. I. M. & Strandwitz, P. Metabolic mischief as microbes target drugs. *Nature* **570,** 453–454 (2019).
92. Zimmermann, M., Zimmermann-Kogadeeva, M., Wegmann, R. & Goodman, A. L. Mapping human microbiome drug metabolism by gut bacteria and their genes. *Nature* **570,** 462–467 (2019).
93. Aoki, S. K. *et al.* Contact-dependent inhibition of growth in Escherichia coli. *Science* **309,** 1245–8 (2005).
94. Basler, M., Ho, B. T. & Mekalanos, J. J. Tit-for-tat: type VI secretion system counterattack during bacterial cell-cell interactions. *Cell* **152,** 884–94 (2013).
95. Murdoch, S. L. *et al.* The opportunistic pathogen Serratia marcescens utilizes type VI secretion to target bacterial competitors. *J. Bacteriol.* **193,** 6057–69 (2011).
96. Cornforth, D. M. & Foster, K. R. Competition sensing: the social side of bacterial stress responses. *Nat. Rev. Microbiol.* **11,** 285–293 (2013).
97. Brunelle, B. W., Bearson, B. L. & Bearson, S. M. D. Chloramphenicol and tetracycline decrease motility and increase invasion and attachment gene expression in specific isolates of multidrug-resistant Salmonella enterica serovar Typhimurium. *Front. Microbiol.* **5,** 1–12 (2015).
98. Müller, S. *et al.* Bacillaene and sporulation protect Bacillus subtilis from predation by Myxococcus xanthus. *Appl. Environ. Microbiol.* **80,** 5603–10 (2014).
99. Traxler, M. F. & Kolter, R. Natural products in soil microbe interactions and evolution. *Nat. Prod. Rep.* **32,** 956–970 (2015).
100. Molina, L. *et al.* Degradation of pathogen quorum-sensing molecules by soil bacteria: a preventive and curative biological control mechanism. *FEMS Microbiol. Ecol.* **45,** 71–81 (2003).
101. Lewis, K. Persister Cells. *Annu. Rev. Microbiol.* **64,** 357–372 (2010).
102. Oh, D.-C., Kauffman, C. A., Jensen, P. R. & Fenical, W. Induced Production of Emericellamides A and B from the Marine-Derived Fungus Emericella sp. in Competing Co-culture. *J. Nat. Prod.* **70,** 515–520 (2007).
103. Oh, D.-C., Jensen, P. R., Kauffman, C. A. & Fenical, W. Libertellenones A–D: Induction of cytotoxic diterpenoid biosynthesis by marine microbial competition. *Bioorg. Med. Chem.* **13,** 5267–5273 (2005).

104. Traxler, M. F., Watrous, J. D., Alexandrov, T., Dorrestein, P. C. & Kolter, R. Interspecies Interactions Stimulate Diversification of the Streptomyces coelicolor Secreted Metabolome. *MBio* **4,** e00459-13-e00459-13 (2013).
105. Schroeckh, V. *et al.* Intimate bacterial-fungal interaction triggers biosynthesis of archetypal polyketides in Aspergillus nidulans. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 14558–63 (2009).
106. Flannagan, R. S., Valvano, M. A. & Koval, S. F. Downregulation of the motA gene delays the escape of the obligate predator Bdellovibrio bacteriovorus 109J from bdelloplasts of bacterial prey cells. *Microbiology* **150,** 649–56 (2004).
107. An, D., Danhorn, T., Fuqua, C. & Parsek, M. R. Quorum sensing and motility mediate interactions between Pseudomonas aeruginosa and Agrobacterium tumefaciens in biofilm cocultures. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 3828–33 (2006).
108. Wadhams, G. H. & Armitage, J. P. Making sense of it all: bacterial chemotaxis. *Nat. Rev. Mol. Cell Biol.* **5,** 1024–1037 (2004).
109. Lai, S., Tremblay, J. & Déziel, E. Swarming motility: a multicellular behaviour conferring antimicrobial resistance. *Environ. Microbiol.* **11,** 126–136 (2009).
110. Calvio, C. *et al.* Swarming differentiation and swimming motility in Bacillus subtilis are controlled by swrA, a newly identified dicistronic operon. *J. Bacteriol.* **187,** 5356–66 (2005).
111. Kinsinger, R. F., Kearns, D. B., Hale, M. & Fall, R. Genetic Requirements for Potassium Ion-Dependent Colony Spreading in Bacillus subtilis. *J. Bacteriol.* **187,** 8462–8469 (2005).
112. Hoffman, L. R. *et al.* Aminoglycoside antibiotics induce bacterial biofilm formation. *Nature* **436,** 1171–5 (2005).
113. Jones, C., Allsopp, L., Horlick, J., Kulasekara, H. & Filloux, A. Subinhibitory Concentration of Kanamycin Induces the Pseudomonas aeruginosa type VI Secretion System. *PLoS One* **8,** e81132 (2013).
114. Bleich, R., Watrous, J. D., Dorrestein, P. C., Bowers, A. A. & Shank, E. A. Thiopeptide antibiotics stimulate biofilm formation in Bacillus subtilis. *Proc. Natl. Acad. Sci. U. S. A.* **112,** 3086–91 (2015).
115. Branda, S. S., Gonzalez-Pastor, J. E., Ben-Yehuda, S., Losick, R. & Kolter, R. Fruiting body formation by Bacillus subtilis. *Proc. Natl. Acad. Sci.* **98,** 11621–11626 (2001).
116. Fall, R., Kearns, D. B. & Nguyen, T. A defined medium to investigate sliding motility in a Bacillus subtilis flagella-less mutant. *BMC Microbiol.* **6,** 31 (2006).
117. Joys, T. M. & Frankel, R. W. Genetic control of flagellation in Bacillus subtilis. *J. Bacteriol.* **94,** 32–7 (1967).
118. Lino, T. Genetics of structure and function of bacterial flagella. *Annu. Rev. Genet.* **11,** 161–182 (1977).
119. Roux, D. *et al.* Identification of Poly-N-acetylglucosamine as a Major Polysaccharide Component of the Bacillus subtilis Biofilm Matrix. *J. Biol. Chem.* **290,** 19261–72 (2015).
120. Bald, R., Erdmann, V. A. & Pongs, O. Irreversible binding of chloramphenicol

analogues to E. coli ribosomes. *FEBS Lett.* **28,** 149–152 (1972).
121. Ehrlich, J., Bartz, Q. R., Smith, R. M., Joslyn, D. A. & Burkholder, P. R. Chloromycetin, a New Antibiotic From a Soil Actinomycete. *Science* **106,** 417 (1947).
122. Ehrlich, J. & Gottlieb, D. Streptomyces venezuelae, n. sp., the source of chloromycetin. *J. Bacteriol.* **56,** 467–77 (1948).
123. Sonenberg, N., Zamir, A. & Wilchek, M. Analogs of chloramphenicol and their application to labeling ribosomes. *Methods Enzymol.* **46,** 702–7 (1977).
124. Wilson, D. N. Ribosome-targeting antibiotics and mechanisms of bacterial resistance. *Nat. Rev. Microbiol.* **12,** 35–48 (2013).
125. Weisblum, B. Erythromycin resistance by ribosome modification. *Antimicrob. Agents Chemother.* **39,** 577–585 (1995).
126. Carter, A. P. *et al.* Functional insights from the structure of the 30S ribosomal subunit and its interactions with antibiotics. *Nature* **407,** 340–8 (2000).
127. Pioletti, M. *et al.* Crystal structures of complexes of the small ribosomal subunit with tetracycline, edeine and IF3. *EMBO J.* **20,** 1829–1839 (2001).
128. Shaw, W. V & Brodsky, R. F. Characterization of chloramphenicol acetyltransferase from chloramphenicol-resistant Staphylococcus aureus. *J. Bacteriol.* **95,** 28–36 (1968).
129. Leslie, A. G., Moody, P. C. & Shaw, W. V. Structure of chloramphenicol acetyltransferase at 1.75-A resolution. *Proc. Natl. Acad. Sci.* **85,** 4133–4137 (1988).
130. Bulkley, D., Innis, C. A., Blaha, G. & Steitz, T. A. Revisiting the structures of several antibiotics bound to the bacterial ribosome. *Proc. Natl. Acad. Sci.* **107,** 17158–17163 (2010).
131. Zhong, P. *et al.* Substrate requirements for ErmC' methyltransferase activity. *J. Bacteriol.* **177,** 4327–32 (1995).
132. Skinner, R., Cundliffe, E. & Schmidt, F. J. Site of action of a ribosomal RNA methylase responsible for resistance to erythromycin and other antibiotics. *J. Biol. Chem.* **258,** 12702–12706 (1983).
133. Reilman, E., Mars, R. A. T., van Dijl, J. M. & Denham, E. L. The multidrug ABC transporter BmrC/BmrD of Bacillus subtilis is regulated via a ribosome-mediated transcriptional attenuation mechanism. *Nucleic Acids Res.* **42,** 11393–11407 (2014).
134. Dar, D. *et al.* Term-seq reveals abundant ribo-regulation of antibiotics resistance in bacteria. *Science* **352,** aad9822 (2016).
135. Bui, L. M. G., Conlon, B. P. & Kidd, S. P. Antibiotic tolerance and the alternative lifestyles of Staphylococcus aureus. *Essays Biochem.* **61,** 71–79 (2017).
136. Kaplan, J. B. Antibiotic-induced biofilm formation. *Int. J. Artif. Organs* **34,** 737–751 (2011).
137. Dunkle, J. A., Xiong, L., Mankin, A. S. & Cate, J. H. D. Structures of the Escherichia coli ribosome with antibiotics bound near the peptidyl transferase center explain spectra of drug action. *Proc. Natl. Acad. Sci.* **107,** 17152–17157 (2010).

138. Davies, J., Gorini, L. & Davis, B. D. Misreading of RNA codewords induced by aminoglycoside antibiotics. *Mol. Pharmacol.* **1,** 93–106 (1965).
139. Davis, B. D., Chen, L. L. & Tai, P. C. Misread protein creates membrane channels: an essential step in the bactericidal action of aminoglycosides. *Proc. Natl. Acad. Sci. U. S. A.* **83,** 6164–8 (1986).
140. Kohanski, M. A., Dwyer, D. J. & Collins, J. J. How antibiotics kill bacteria: from targets to networks. *Nat. Rev. Microbiol.* **8,** 423–435 (2010).
141. Hol, F. J. H., Hubert, B., Dekker, C. & Keymer, J. E. Density-dependent adaptive resistance allows swimming bacteria to colonize an antibiotic gradient. *ISME J.* **10,** 30–38 (2016).
142. Levin-Reisman, I. *et al.* Antibiotic tolerance facilitates the evolution of resistance. *Science* **355,** 826–830 (2017).
143. Yasbin, R. E. & Young, F. E. Transduction in Bacillus subtilis by bacteriophage SPP1. *J. Virol.* **14,** 1343–8 (1974).
144. Brien, J. O. & Wright, G. D. An ecological perspective of microbial secondary metabolism. *Curr. Opin. Biotechnol.* **22,** 552–558 (2011).
145. Bader, M. W. *et al.* Regulation of Salmonella typhimurium virulence gene expression by cationic antimicrobial peptides. *Mol. Microbiol.* **50,** 219–230 (2003).
146. Straight, P. D., Willey, J. M. & Kolter, R. Interactions between Streptomyces coelicolor and Bacillus subtilis: Role of surfactants in raising aerial structures. *J Bacteriol* **188,** 4918–4925 (2006).
147. Orazi, G. & Toole, G. A. O. Pseudomonas aeruginosa Alters Staphylococcus aureus Sensitivity to Vancomycin in a Biofilm Model of Cystic Fibrosis Infection. *MBio* **8,** 1–17 (2017).
148. Alves, M. *et al.* Interaction between Staphylococcus aureus and Pseudomonas aeruginosa is beneficial for colonisation and pathogenicity in a mixed biofilm. *FEMS* **76,** 1–10 (2018).
149. Morales, D. K. *et al.* Control of Candida albicans Metabolism and Biofilm Formation by Pseudomonas aeruginosa Phenazines. *MBio* **4,** 1–9 (2013).
150. Shank, E. A., Klepac-ceraj, V., Collado-torres, L., Powers, G. E. & Losick, R. Interspecies interactions that result in Bacillus subtilis forming bio fi lms are mediated mainly by members of its own genus. *PNAS* **108,** E1236–E1243 (2011).
151. Sandoval-Motta, S. & Aldana, M. Adaptive resistance to antibiotics in bacteria: A systems biology perspective. *Wiley Interdiscip. Rev. Syst. Biol. Med.* **8,** 253–267 (2016).
152. Linares, J. F., Gustafsson, I., Baquero, F. & Martinez, J. L. Antibiotics as intermicrobiol signaling agents instead of weapons. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 19484–19489 (2006).
153. Klumpp, S., Zhang, Z. & Hwa, T. Growth Rate-Dependent Global Effects on Gene Expression in Bacteria. *Cell* **139,** 1366–1375 (2009).
154. Scott, M., Gunderson, C. W., Mateescu, E. M., Zhang, Z. & Hwa, T. Interdependence of Cell Growth. *Science.* **330,** 1099–1102 (2010).
155. Scott, M., Mateescu, E. M., Zhang, Z. & Hwa, T. Interdependence of Cell

Growth. *Science.* **330,** 1099–1103 (2010).
156. Bosdriesz, E., Molenaar, D., Teusink, B. & Bruggeman, F. J. How fast-growing bacteria robustly tune their ribosome concentration to approximate growth-rate maximization. *FEBS* **282,** 2029–2044 (2015).
157. Gillespie, A. J. A. D. Metabolic Events Occurring During Recovery from Prolonged Glucose Starvation in Escherichia coli. *J Bacteriol* **95,** 1030–1039 (1968).
158. Kaplan, R. & Apirion, D. Decay of Ribosomal Ribonucleic Acid in Escherichia coli Cells Starved for Various Nutrients. *J Biol Chem* **250,** 3174–3178 (1975).
159. Cashel, M. & Gallant, J. Two Compounds implicated in the Function of RC gene. *Nature* **221,** 838–841 (1969).
160. Hauryliuk, V., Atkinson, G. C., Murakami, K. S., Tenson, T. & Gerdes, K. Recent functional insights into the role of (p)ppGpp in bacterial physiology. *Nat. Rev. Microbiol.* **13,** 298–309 (2015).
161. Corrigan, R. M., Bellows, L. E., Wood, A. & Gründling, A. ppGpp negatively impacts ribosome assembly affecting growth and antimicrobial tolerance in Gram-positive bacteria. *Proc. Natl. Acad. Sci. U. S. A.* **113,** E1710-9 (2016).
162. Liu, K. *et al.* Molecular Mechanism and Evolution of Guanylate Kinase Regulation by (p) ppGpp Article Molecular Mechanism and Evolution of Guanylate Kinase Regulation by (p) ppGpp. *Mol. Cell* **57,** 735–749 (2015).
163. Kinsinger, R. F., Shirk, M. C. & Fall, R. Rapid surface motility in Bacillus subtilis is dependent on extracellular surfactin and potassium ion. *J. Bacteriol.* **185,** 5627–5631 (2003).
164. Kearns, D. B. A field guide to bacterial swarming motility. *Nat Rev Microbiol* **8,** 634–644 (2010).
165. Cremer, J. *et al.* Chemotaxis as a navigation strategy to boost range expansion. *Nature* **575,** 658–663 (2019).
166. Liu, W., Cremer, J., Li, D., Hwa, T. & Liu, C. An evolutionarily stable strategy to colonize spatially extended habitats. *Nature* **575,** 664–668 (2019).
167. Rosario, M. M. L., Fredrick, K. L., Ordal, G. W. & Helmann, J. D. Chemotaxis in Bacillus subtilis requires either of two functionally redundant CheW homologs. *J. Bacteriol.* **176,** 2736–2739 (1994).
168. Leizeaga, A., Estrany, M., Forn, I. & Sebastián, M. Using click-chemistry for visualizing in situ changes of translational activity in planktonic marine bacteria. *Front. Microbiol.* **8,** 1–11 (2017).
169. Couradeau, E. *et al.* Probing the active fraction of soil microbiomes using BONCAT-FACS. *Nat. Commun.* **10,** (2019).
170. Reilman, E., Mars, R. A. T., Van Dijl, J. M. & Denham, E. L. The multidrug ABC transporter BmrC/BmrD of Bacillus subtilis is regulated via a ribosome-mediated transcriptional attenuation mechanism. *Nucleic Acids Res.* **42,** 11393–11407 (2014).
171. Demain, A. L. & Sanchez, S. Microbial drug discovery : 80 years of progress. *J. Antibiot.* **62,** 5–16 (2009).
172. van Gestel, J., Vlamakis, H. & Kolter, R. From cell differentiation to cell

collectives: Bacillus subtilis uses division of labor to migrate. *PLoS Biol* **13,** e1002141 (2015).
173. Popp, P. F., Dotzler, M., Radeck, J., Bartels, J. & Mascher, T. The Bacillus BioBrick Box 2.0: Expanding the genetic toolbox for the standardized work with Bacillus subtilis. *Sci. Rep.* **7,** 1–13 (2017).
174. Brinsmade, S. R. *et al.* Hierarchical expression of genes controlled by the Bacillus subtilis global regulatory protein CodY. *Proc. Natl. Acad. Sci.* **111,** 8227–8232 (2014).
175. Liu, J. *et al.* Metabolic co-dependence gives rise to collective oscillations within biofilms. *Nature* **523,** 550–554 (2015).
176. Vargas-Bautista, C., Rahlwes, K. & Straight, P. Bacterial competition reveals differential regulation of the pks genes by Bacillus subtilis. *J Bacteriol* **196,** 717–728 (2014).
177. Bray, N. L., Pimentel, H., Melsted, P. & Pachter, L. Near-optimal probabilistic rna-seq quantification. *Nat. Biotechnol.* **34,** 4–8 (2016).
178. Nadler, S. G. *et al.* Differential expression and sequence-specific interaction of karyopherin α with nuclear localization sequences. *J. Biol. Chem.* **272,** 4310–4315 (1997).
179. Rosebrock, A. P. & Caudy, A. A. Metabolite Extraction from Saccharomyces cerevisiae for Liquid Chromatography – Mass Spectrometry. 1–6 (2017). doi:10.1101/pdb.prot089086
180. Blair, J. M. A., Webber, M. A., Baylay, A. J., Ogbolu, D. O. & Piddock, L. J. V. Molecular mechanisms of antibiotic resistance. *Nat. Rev. Microbiol.* **13,** 42–51 (2015).
181. Lavigne, J. P. *et al.* An adaptive response of Enterobacter aerogenes to imipenem: Regulation of porin balance in clinical isolates. *Int. J. Antimicrob. Agents* **41,** 130–136 (2013).
182. Deng, X. *et al.* Expression of multidrug resistance efflux pump gene norA Is iron responsive in Staphylococcus aureus. *J. Bacteriol.* **194,** 1753–1762 (2012).
183. Dar, D. *et al.* Term-seq reveals abundant ribo-regulation of antibiotics resistance in bacteria. *Science (80-.).* **352,** (2016).
184. Quentin, Y., Fichant, G. & Denizot, F. Inventory, assembly and analysis of Bacillus subtilis ABC transport systems. *J. Mol. Biol.* **287,** 467–484 (1999).
185. Torres, C., Galián, C., Freiberg, C., Fantino, J. R. & Jault, J. M. The YheI/YheH heterodimer from Bacillus subtilis is a multidrug ABC transporter. *Biochim. Biophys. Acta - Biomembr.* **1788,** 615–622 (2009).
186. Fukushima, S., Yoshimura, M., Chibazakura, T., Sato, T. & Yoshikawa, H. The putative ABC transporter YheH/YheI is involved in the signalling pathway that activates KinA during sporulation initiation. *FEMS Microbiol. Lett.* **256,** 90–97 (2006).
187. Reilman, E., Mars, R. A. T. T., van Dijl, J. M. & Denham, E. L. The multidrug ABC transporter BmrC/BmrD of Bacillus subtilis is regulated via a ribosome-mediated transcriptional attenuation mechanism. *Nucleic Acids Res.* **42,** 11393–11407 (2014).

188. Stubbendieck, R. M. & Straight, P. D. Multifaceted interfaces of bacterial competition. *J. Bacteriol.* **198,** 2145–2155 (2016).
189. Kumano, M. *et al.* Lincomycin resistance mutations in two regions immediately downstream of the -10 region of lmr promoter cause overexpression of a putative multidrug efflux pump in Bacillus subtilis mutants. *Antimicrob. Agents Chemother.* **47,** 432–435 (2003).
190. Crowe-McAuliffe, C. *et al.* Structural basis for antibiotic resistance mediated by the Bacillus subtilis ABCF ATPase VmlR. *Proc. Natl. Acad. Sci. U. S. A.* **115,** 8978–8983 (2018).
191. Yakhnin, H. *et al.* Nusg-dependent rna polymerase pausing and tylosin-dependent ribosome stalling are required for tylosin resistance by inducing 23s rrna methylation in bacillus subtilis. *MBio* **10,** 1–14 (2019).
192. Radeck, J. *et al.* Anatomy of the bacitracin resistance network in Bacillus subtilis. *Mol. Microbiol.* **100,** 607–620 (2016).
193. Alexieva, Z., Duvall, E. J., Ambulos, N. P., Kim, U. J. & Lovett, P. S. Chloramphenicol induction of cat-86 requires ribosome stalling at a specific site in the leader. *Proc. Natl. Acad. Sci. U. S. A.* **85,** 3057–3061 (1988).
194. Ramos, J. L. *et al.* Microbiol Mol Biol Rev 2005 RamosThe TetR family of transcriptional. *Society* **69,** 1–31 (2005).
195. Conformational alteration of mRNA structure and the posttranscriptional regulation of erythro- mycin-induced drug resistance. *Nucleic Acids Res.* **8,** 6081–6097 (1980).
196. Martínez, A., Torello, S. & Kolter, R. Sliding motility in mycobacteria. *J. Bacteriol.* **181,** 7331–7338 (1999).
197. Nawrocki, K. L., Edwards, A. N., Daou, N., Bouillaut, L. & McBride, S. M. CodY-dependent regulation of sporulation in Clostridium difficile. *J. Bacteriol.* **198,** 2113–2130 (2016).

APPENDIX A

SUPPLEMENTAL FIGURES

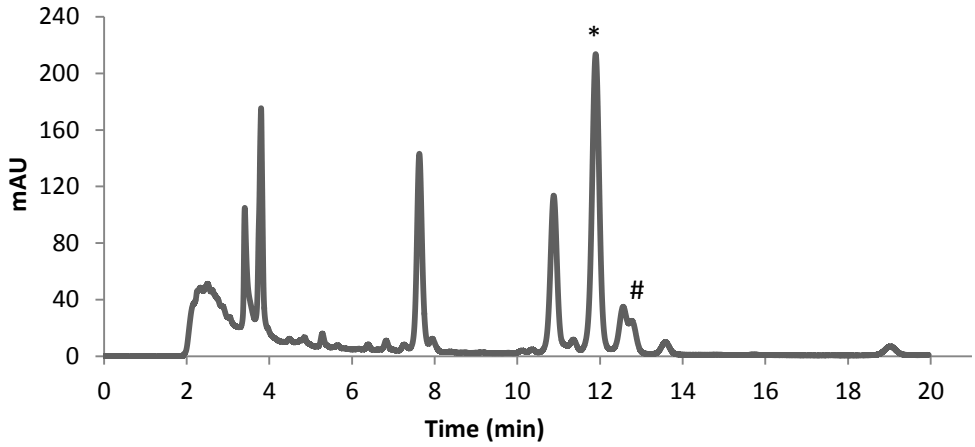
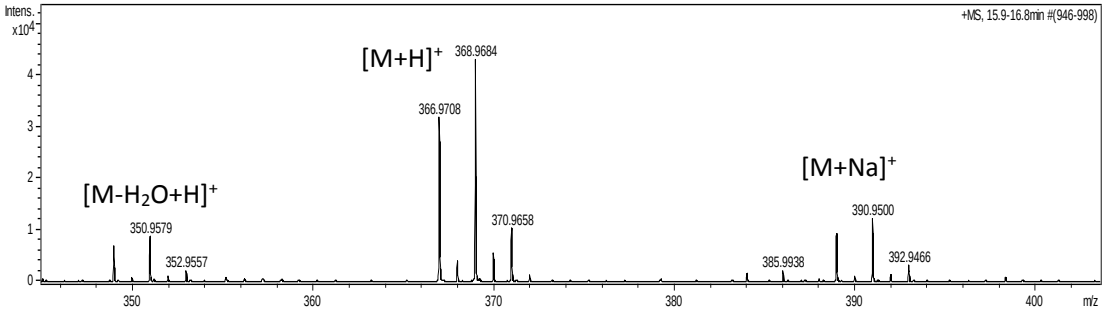
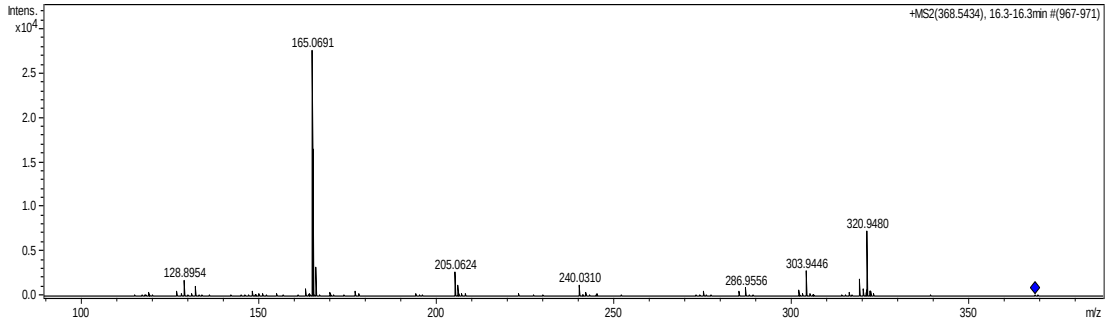


Figure II-S1 HPLC trace of 40% methanol fraction from crude extracts Crude extract was further fractionated. The active 40% methanol fraction was applied to HPLC for further separation at wavelength 254 nm. The peak corresponding to the inhibitory fraction was labeled with a star. The peak corresponding to the inducing fraction was labeled with a pound.

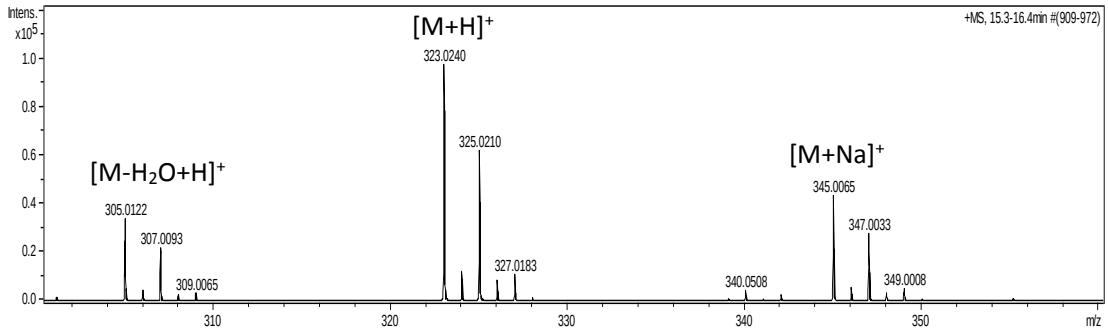
A



B



C



D

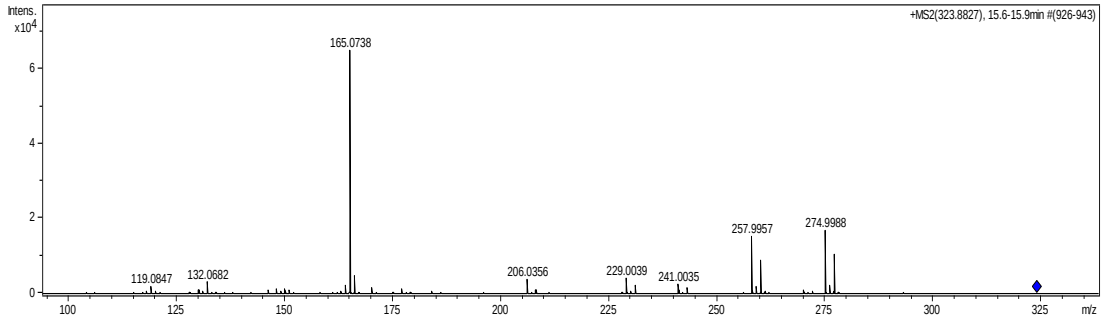


Figure II-S2 Identification of monobromamphenicol and chloramphenicol by HPLC-MS/MS. (A) The mass and isotope profile are consistent with monobromamphenicol. Different forms of parent ions are labeled in the MS1 spectrum. (B) The identity of monobromamphenicol was further confirmed by the fragment ions in the MS2 spectrum. (C) The mass and isotope profile are consistent with chloramphenicol. Different forms of parent ions are labeled in the MS1 spectrum. (D) The identity of chloramphenicol was further confirmed by the fragment ions in the MS2 spectrum.

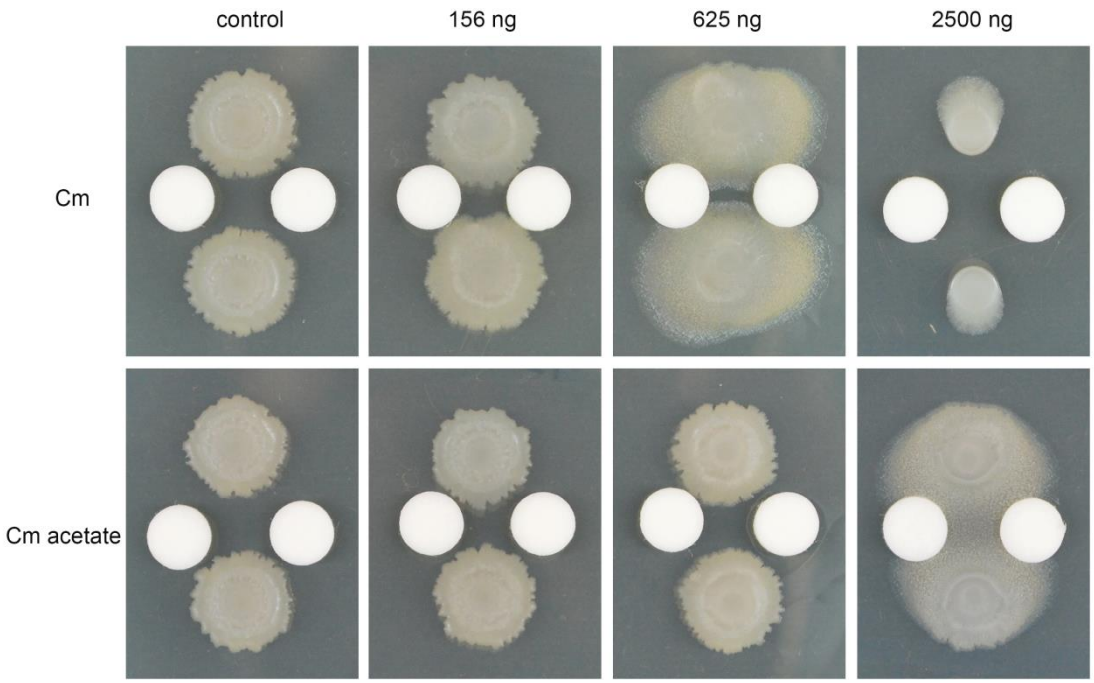


Figure II-S3. Chloramphenicol acetate does not induce sliding at the same amount as chloramphenicol Different amounts of Cm and Cm acetate were spotted on filter discs adjacent to *B. subtilis* colonies. Cm acetate did not induce sliding at an equivalent amount (625 ng) where Cm induced sliding. However, Cm acetate induced sliding with greater amount (2,500 ng) where Cm inhibited growth of *B. subtilis*. The solvent control for both Cm and Cm acetate is 10% ethanol (in H_2O). Pictures were taken at 24 h. Filter disc diameter, 0.6 cm

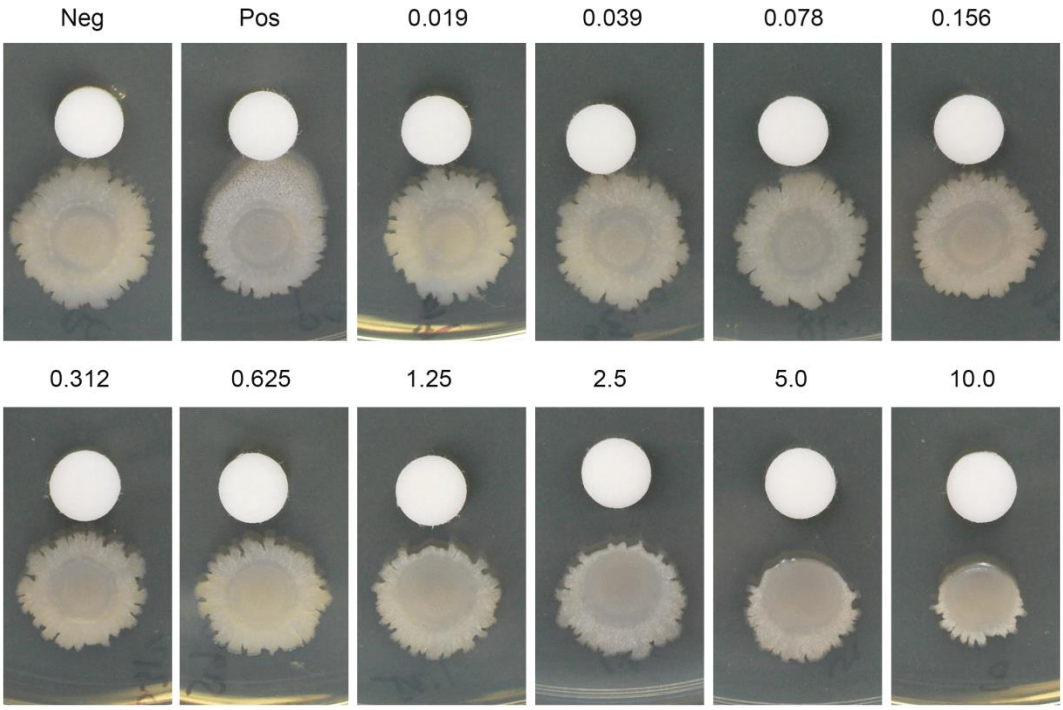


Figure II-S4. Lincomycin was unable to induce sliding response Different amounts of lincomycin (unit, µg/mL) were spotted on filter discs adjacent to *B. subtilis* colonies. The negative control is 10% ethanol solvent used in the assay and the positive control is 625 ng Cm. Pictures were taken at 24 h. Filter disc diameter, 0.6 cm

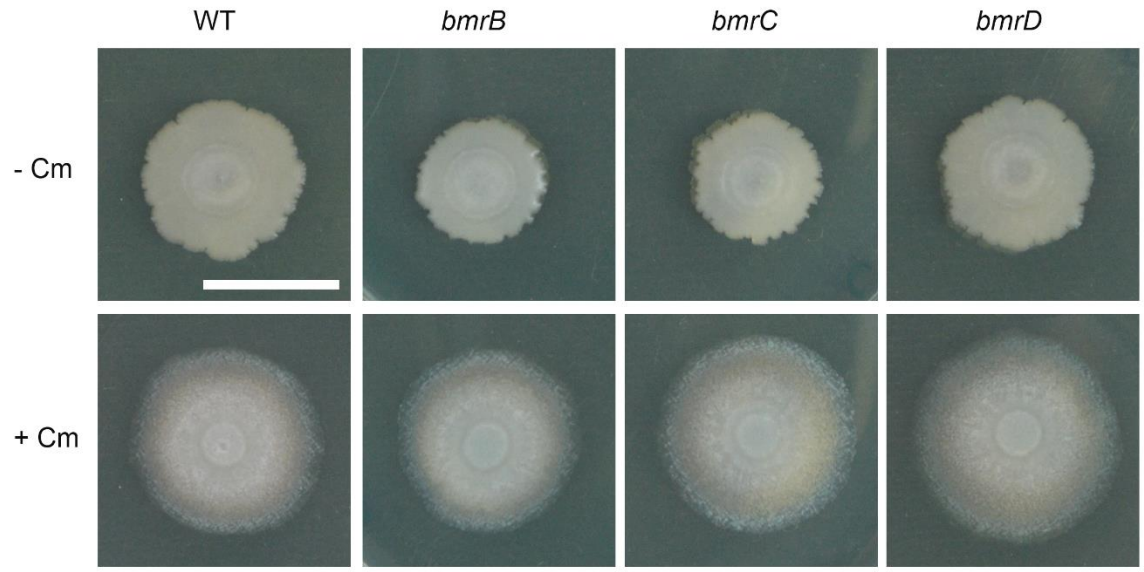
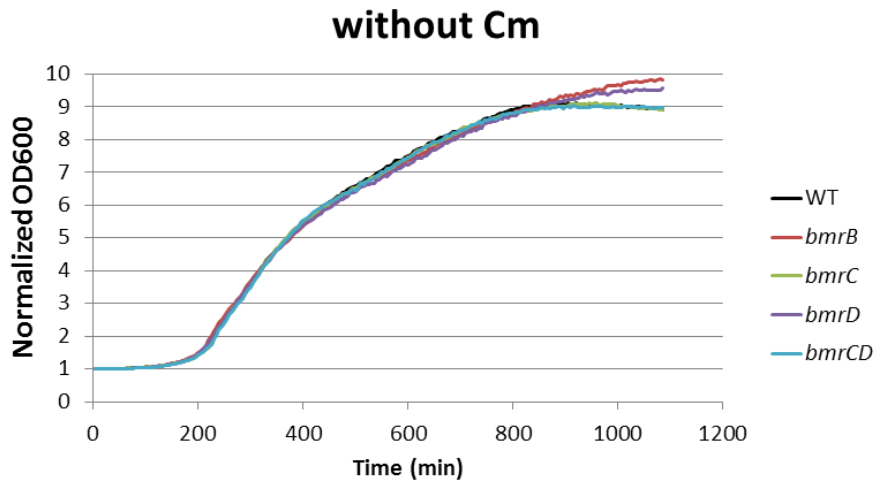
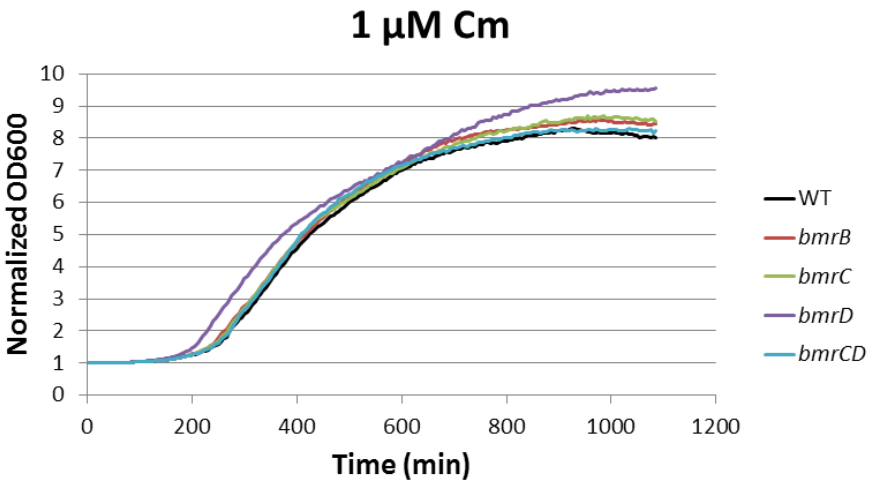
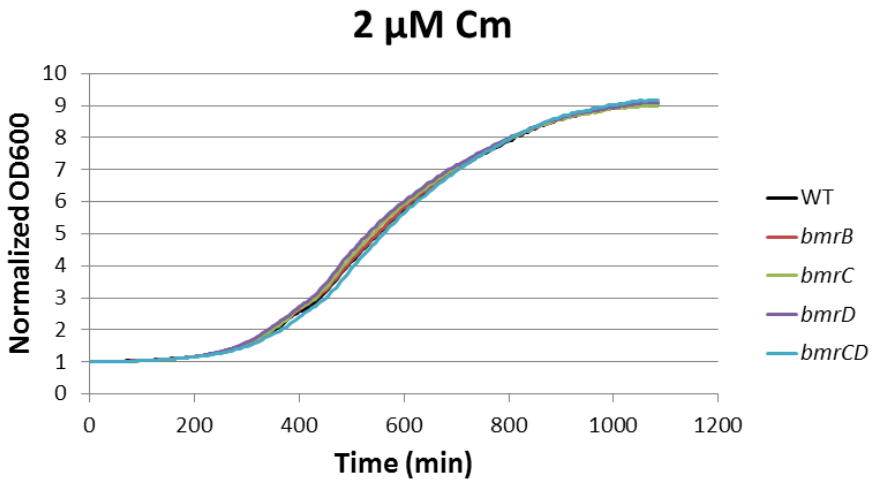
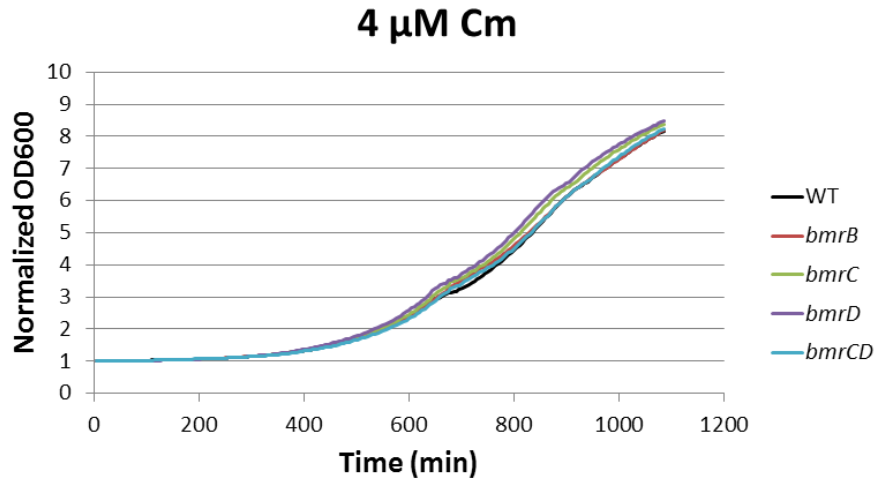


Figure S5 Sliding is *bmrBCD*-independent. Wild type (WT) *B. subtilis* NCIB3610, *bmrB*, *bmrC* and *bmrD* knockout strains were spotted on the agar plate in the absence or presence of Cm. Pictures were taken at 24h. Scale bar, 1 cm.


without Cm
Normalized OD600
Time (min)
WT
bmrB
bmrC
bmrD
bmrCD


1 µM Cm
Normalized OD600
Time (min)
WT
bmrB
bmrC
bmrD
bmrCD


2 µM Cm
Normalized OD600
Time (min)
WT
bmrB
bmrC
bmrD
bmrCD


4 µM Cm
Normalized OD600
Time (min)
WT
bmrB
bmrC
bmrD
bmrCD

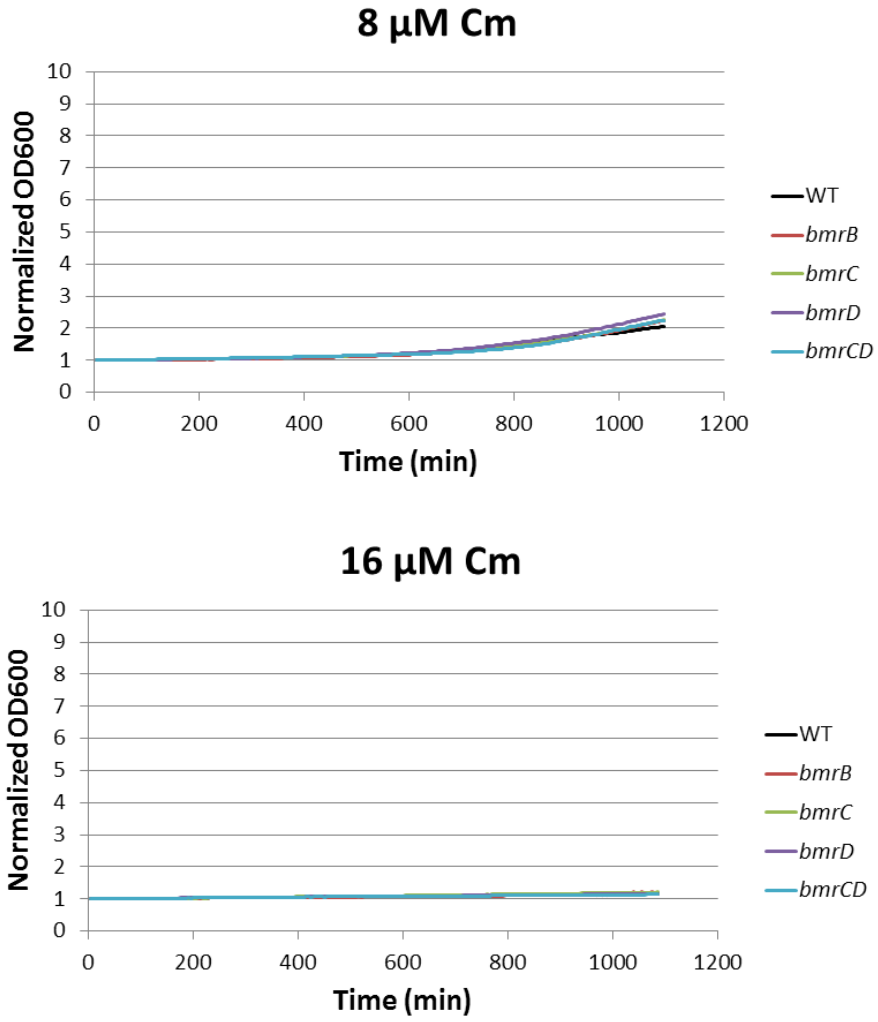


Figure S6. *bmrCD* knockout strain is not hypersensitive to chloramphenicol Growth curve of wild type (WT), *bmrB, bmrC, bmrD* and *bmrCD* knockout strains in response to different concentrations of Cm (0, 1, 2, 4, 8, 16 µM) in the period of 18 h.

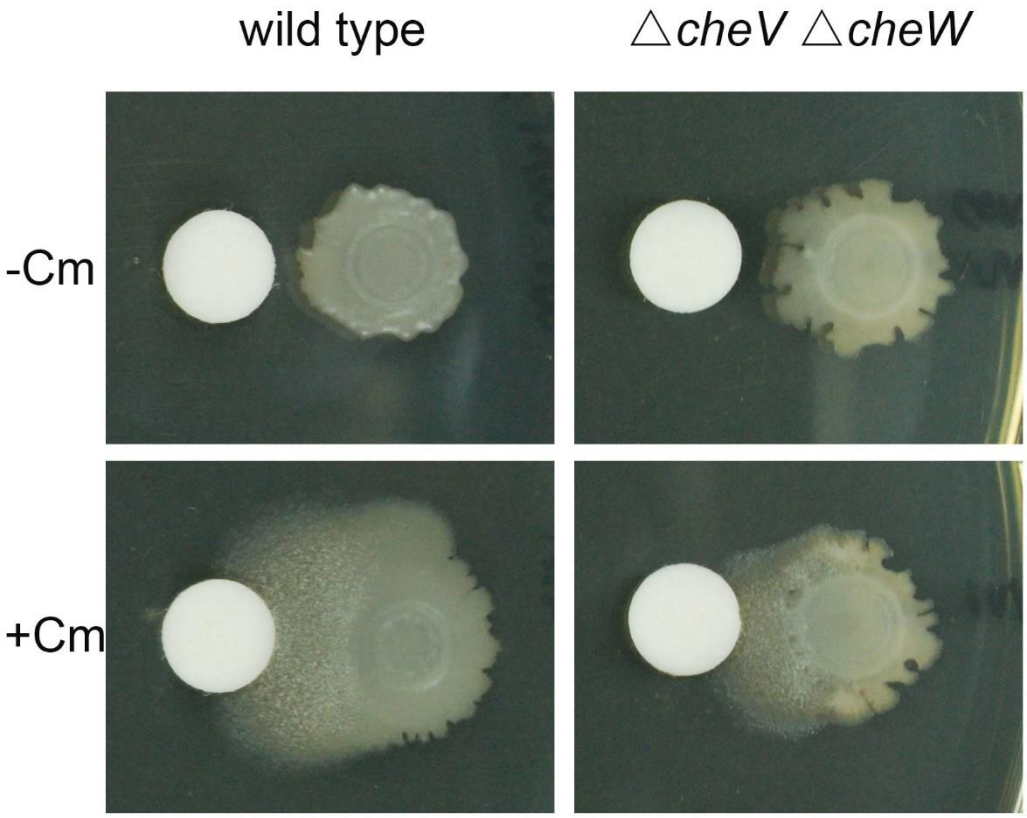


Figure III-S1. Cm-induced sliding motility in *B. subtilis* is not chemotaxis. **The Cm-induced motility is not a form of chemotaxis. *cheV* and *cheW*, the two genes required for chemotaxis in *B. subtilis* were deleted and tested for sliding motility in the absence (-) and presence (+) of Cm. 10 µL of 62.5 µg/ml Cm and solvent control was spotted on each disc. Pictures were taken at 24 h. Filter disc diameter, 6 mm.**

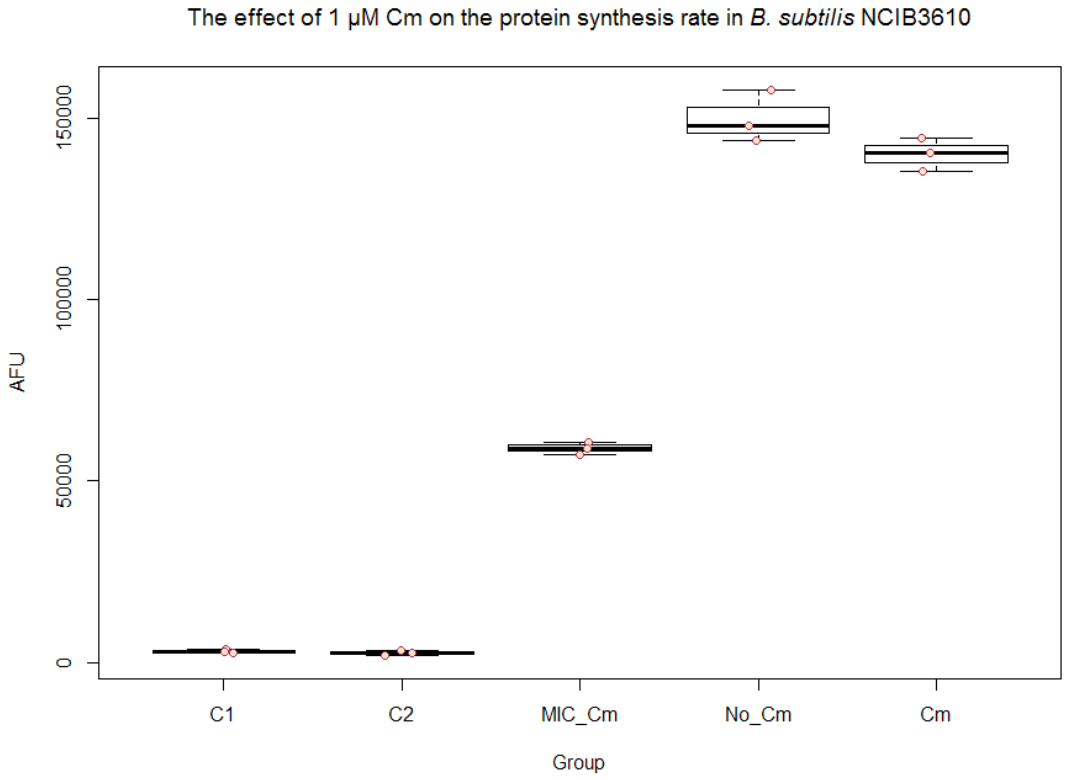


Figure III-S2. SubMIC Cm inhibits protein synthesis. The protein synthesis rate was reduced in the presence of 1 μM subinhibitory concentration of Cm by Click-iT assay. C1 is the control without HPG. C2 is the control without Alexa Fluor 488. MIC_Cm is the control of 1-fold MIC Cm. The decrease of Cm-treated group was calculated based on the average of No_Cm group. Each group contains three biological replicates (p value < 0.05 between No_Cm and Cm group).

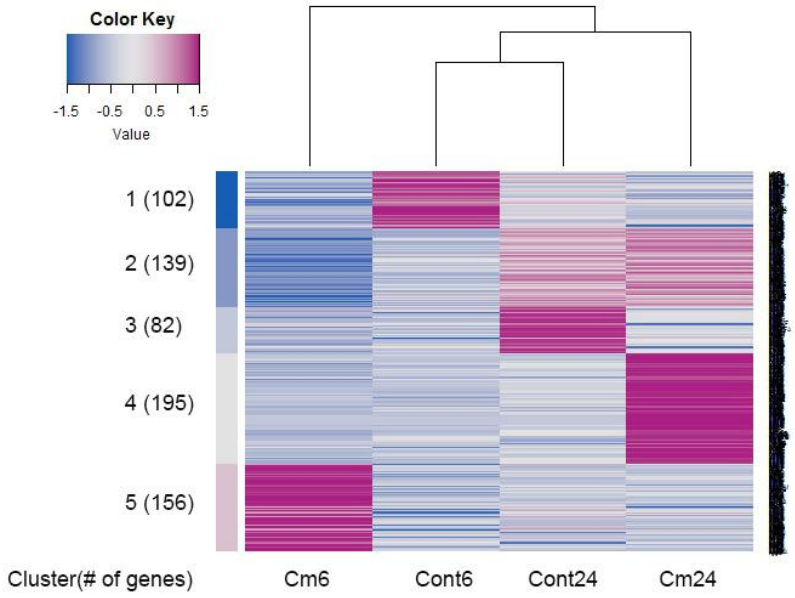


Figure III-S3. sub-MIC Cam triggers a wide range of changes in gene expression Heatmap of genes that change ≥two-fold with adjusted p value<0.05 across all four conditions (6 h and 24 h with or without Cm). Heatmap combined with K-mean clustering (K=5) was used to group the data with Z-scores based on the mean values calculated from normalized Deseq counts. Z-scores were calculated based on the formula: (X-Y)/Z (X: normalized counts of the sample; Y: average normalized counts of all four samples; Z: standard deviation for all four samples)

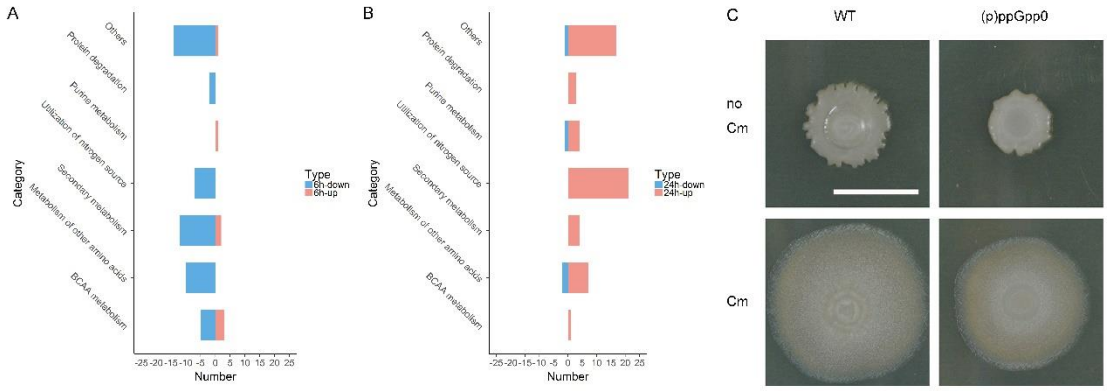


Figure III -S4. Shifting patterns in expression of CodY-regulated genes. A. Functional classification of CodY regulated genes that change ≥two-fold with adjusted p value<0.05 in response to Cm at 6 h. B. Functional classification of CodY regulated genes that change ≥two-fold with adjusted p value<0.05 in response to Cm at 24 h. C. (p)ppGpp is not required for sliding motility. WT (Wild type) and (p)ppGpp0 strains were spotted on the agar plate in the absence or presence of Cm. Pictures were taken at 24 h. Bar, 1 cm.

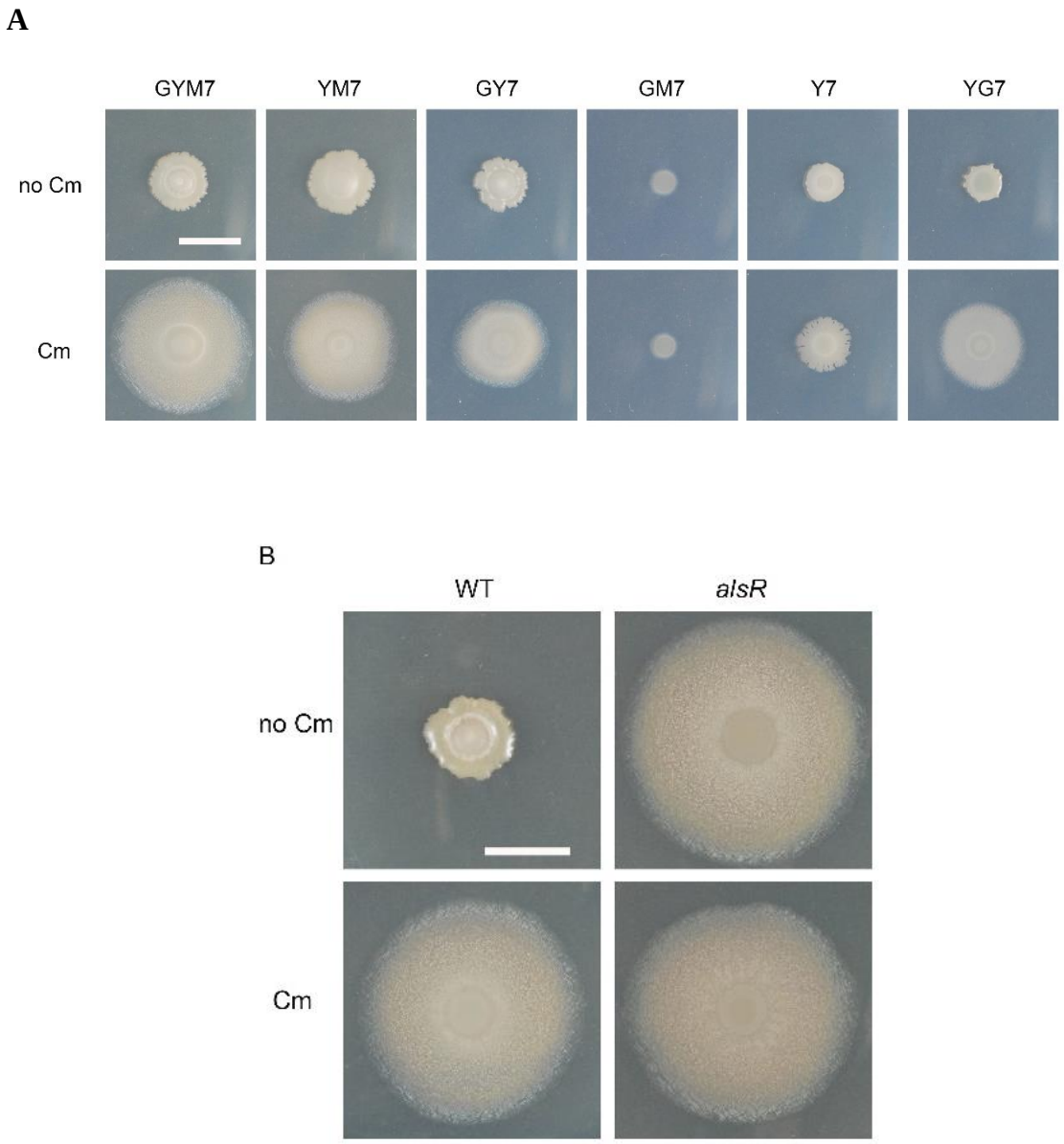


Figure III -S5. Addition of enough carbon source is key to sliding motility.
A. Wild type *B. subtilis* NCIB3610 were spotted on GYM7, YM7 (0.4% yeast extract, 1% malt extract, pH7.0), GY7 (0.4% glucose, 0.4% yeast extract, pH7.0), GM7 (0.4% glucose, 1% malt extract), Y7 (0.4% yeast extract, pH7.0) and YG7 (0.4% yeast extract and 0.5% glycerol, pH7.0) agar plate in the absence or presence of chloramphenicol (Cm). B. Wild type and *alsR B. subtilis* NCIB3610 were spotted on the GYM7 plate in the absence or presence of Cm. Pictures were taken at 24 h. Bar, 1 cm.

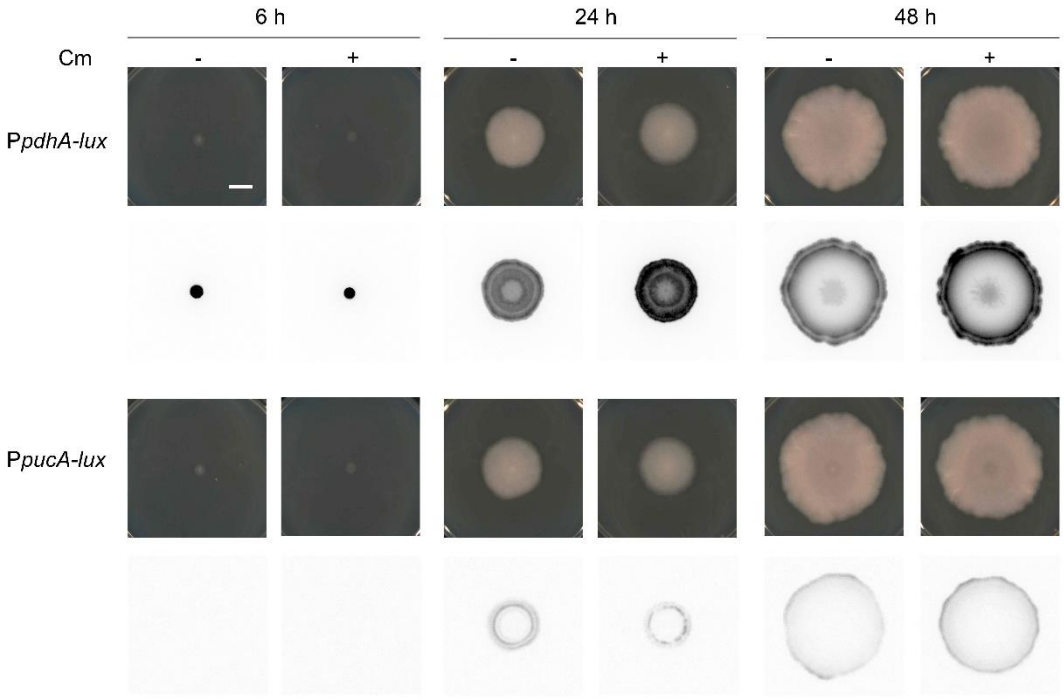


Figure III -S6. Disruption of the regulatory network leads to the change of spatial metabolic patterns. Reporter strains with luciferase operon *luxABCDE* fused to *pdhA* or *pucA* promoter in the *codY* deletion background were spotted on the agar plate without (-) or with Cm (+). Pictures were taken with phase contrast and chemiluminence mode at different time points: 6 h, 24 h and 48 h. Bar, 1 cm.

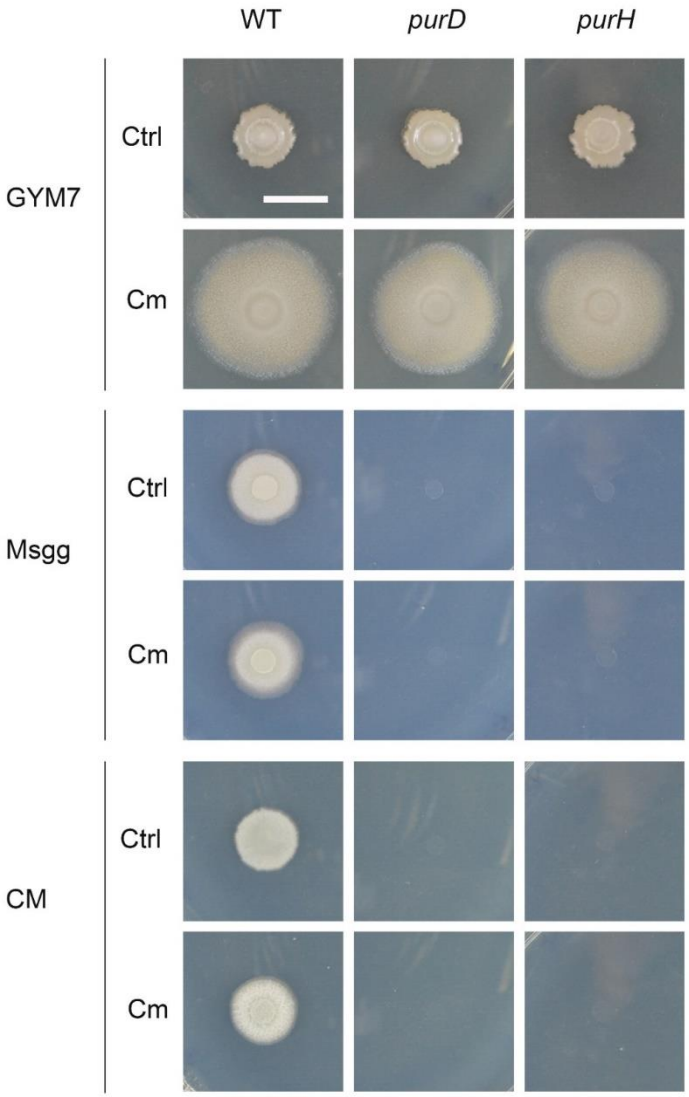


Figure III -S7. The effect of media on the sliding motility of mutant strains in purine de novo biosynthesis pathway. Wild type, *purD*, and *purH* deletion strains were spotted on different media (GYM7, Msgg and CM) in the absence or presence of Cm. Pictures were taken at 24 h. Bar, 1 cm.

Movie II-1 and Movie III-1. Competitive interaction between *B. subtilis* and *S. venezuelae*. Spots of each bacterial species on agar media captured by time-lapse video over 72 hours and reveal the pattern of sliding motility exhibited by *B. subtilis*. Initially, the *B. subtilis* move toward the proximal *S. venezuelae* spots (up to ~36 hours). Continued culture shows progression of the sliding population of *B. subtilis* outward, and deflected away from *S. venezuelae* (up to 72 hours). The agar plate is 8.4 cm in diameter. Movie II-1 and Movie III-1 were made using different setups.

Movie II-2 and Movie III-2. Induction of *B. subtilis* sliding motility by a subinhibitory concentration of chloramphenicol. A) A *B. subtilis* population cultured on media without chloramphenicol (left) for 72 hours at 30°C. B) A *B. subtilis* population cultured on the same medium as in (A) with supplementation of 0.3 µg/ml (~1 µM) chloramphenicol (right). The chloramphenicol induces migration by sliding motility. The agar plates are 8.4 cm in diameter. Movie II-2 and Movie III-2 were made using different setups.

Movie III-3. Tracking metabolic gene expression in the *B. subtilis* sliding population with P*pdhA-lux* and P*pucA-lux* reporter strains. The overnight cultures of P*pdhA-lux* reporter strain (left) and P*pucA-lux* reporter strain (right) were grown from OD600=0.08 to OD600=1.0 and spotted on GYM7 medium containing 1 µM chloramphenicol for 72 hours. The luminescence signal was captured by a Canon camera. The agar plates are 8.4 cm in diameter.

APPENDIX B

STRAIN AND PRIMER TABLES

Table II-S1. Bacterial strains used in chapter II.

Strain	Genotype	Source
PDS0066	*B. subtilis* NCIB 3610 wild type	Laboratory collection
PDS0611	*B. subtilis* NCIB 3610 Δ*epsH*::*kan*	[12]
PDS0060	*B. subtilis* NCIB 3610 Δ*srfAA*::*mls*	[50]
PDS0932	*B. subtilis* NCIB 3610 Δ*hag*	This study
PDS0934	*B. subtilis* NCIB 3610 Δ*bmrCD*::*kan*	This study
PDS0939	*B. subtilis* NCIB 3610 Δ*bmrB*	This study
PDS0940	*B. subtilis* NCIB 3610 Δ*bmrC*	This study
PDS0941	*B. subtilis* NCIB 3610 Δ*bmrD*	This study
PDS0207	*B. subtilis* NCIB 3610 Cm^R (*amyE*::P_{pksG}-*yfp* (*cat*))	Laboratory collection
PDS0234	*B. subtilis* NCIB 3610 Erm^R (Δ*yxe*::*mls*)	Laboratory collection
PDS0252	*Streptomyces venezuelae* ATCC 10712	John Innes Centre
PSK0531	*Streptomyces lividans* TK24	Laboratory collection
PSK0028	*Streptomyces coelicolor* M145	Laboratory collection
PDS0116	*Streptomyces aizunensis* NRRL B-11277	Laboratory collection

Table II-S2. Primers used in chapter II

Primer	Sequence(5'-3')
gyrB-qPCR-fwd	GGGCAACTCAGAAGCACGGACG
gyrB-qPCR-rev	GCCATTCTTGCTCTTGCCGCC
bmrC-qPCR-fwd	ATTATCCCGCTTCCAGTCAT
bmrC-qPCR-rev	TTCCAGCACTCTGTCATTCA
bmrC-up1000-fwd	GACAACACTTAAAAACAGCGGG
bmrC-up1000-rev	GCCAAGCTTTTTCAAAACTGAAAACAT
bmrD-down1000-fwd	CGCTCAAAAACCCAAAACAATCG
bmrD-down1000-rev	CGGGGTGTCATTCATCACC
kan-fwd	CAGCGAACCATTTGAGGTGATAGG
kan-rev	CGATACAAATTCCTCGTAGGCGCTCGG

Table III-1. Bacterial strains used in chapter III

Strain	Genotype	Source
PDS0066	*B. subtilis* NCIB 3610 wild type	Laboratory collection
PDS0972	*B. subtilis* NCIB 3610 Δ*codY*	This study
PDS0973	*B. subtilis* NCIB 3610 Δ*purR*	This study
PDS0974	*B. subtilis* NCIB 3610 Δ*pyrR*	This study
PDS0975	*B. subtilis* NCIB 3610 Δ*tnrA*	This study
PDS0976	*B. subtilis* NCIB 3610 Δ*sigB*	This study
PDS0977	*B. subtilis* NCIB 3610 Δ*pdhA*	This study
PDS0978	*B. subtilis* NCIB 3610 Δ*pucM*	This study
PDS0979	*B. subtilis* NCIB 3610 Δ*purH*	This study
PDS0980	*B. subtilis* NCIB 3610 Δ*pdhA* Δ*codY*	This study
PDS0981	*B. subtilis* NCIB 3610 Δ*amyE*::P*pdhA-luxABCDE-kan*	This study
PDS0982	*B. subtilis* NCIB 3610 Δ*amyE*::P*pucA-luxABCDE-kan*	This study
PDS0983	*B. subtilis* NCIB 3610 Δ*codY* Δ*amyE*::P*pdhA-luxABCDE-kan*	This study
PDS0984	*B. subtilis* NCIB 3610 Δ*codY* Δ*amyE*::P*pucA-luxABCDE-kan*	This study
PDS0985	*B. subtilis* NCIB 3610 Δ*relA* Δ*yjbM* Δ*ywaC*	This study
PDS0252	*Streptomyces venezuelae* ATCC 10712	John Innes Centre

Table III-2. Primers used in chapter III

Primer	Sequence(5'-3')
lux-fwd	AGGAGGCTAGCCTATGAAATTTGGAAACTTTTTGC
lux-rev	cacctcaaatggttcgctgTCAACTATCAAACGCTTC
P*pucA*(lux)-fwd	GGTCTGATCGAAATAGTACAAGAAAAGCTTGCGGAACTCC
P*pucA*(lux)-rev	ATTTCATAGGCTAGCCTCCTTGCCGCATCCTCCTCTCG
P*pdhA*(lux)-fwd	GGTCTGATCGAAATAGTACACTCGATCAGCTCTTTTTTTGAATG
P*pdhA*(lux)-rev	ATTTCATAGGCTAGCCTCCTACTAAGTCACCTCTTCCTTTC
kan-fwd	CAGCGAACCATTTGAGGTGA
kan-rev	CGATACAAATTCCTCGTAGGCG
amyE-Back-fwd	cctacgaggaatttgtatcgATCCGTTTAGGCTGGGC
amyE-Front-rev	TGTACTATTTCGATCAGACC

Table IV-1. Bacterial strains used in chapter IV

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
PDS0990	*B. subtilis* NCIB 3610 Δ*ytbE*	This study
PDS0991	*B. subtilis* NCIB 3610 Δ5::*kan*(Δ *vmlR*, Δ*bmrCD*::*kan*, Δ*yxjB*, Δ*mdr*, Δ*ytbD*)	This study
PDS0992	*B. subtilis* NCIB 3610 Δ*amyE*::P*hy-bmrCD-spec*	This study
PDS0993	*B. subtilis* NCIB 3610 Δ*bmrCD*::*kan* Δ*amyE*::P*hy-bmrCD-spec*	This study
PDS0994	*B. subtilis* NCIB 3610 Δ*amyE*::P*hy-bmrC*D*-spec* (For *bmrC*, mutate AAA to GCA; K377A; For *bmrD*, mutate AAA to GCT; K469A)	This study
PDS0995	*B. subtilis* NCIB 3610 Δ*bmrCD*::*kan* Δ*amyE*::P*hy-bmrC*D*-spec* (For *bmrC*, mutate AAA to GCA; K377A For *bmrD*, mutate AAA to GCT; K469A)	This study
PDS0252	*Streptomyces venezuelae* ATCC 10712	John Innes Centre
PDS0996	*Streptomyces venezuelae* ATCC 10712 Δ*cmlP*::*apr*	This study
PSK0524	*Streptomyces sviceus* 29083	Laboratory collection
PSK0497	*Streptomyces* sp. SPB74	Laboratory collection
PSK0332	*Streptomyces avermitilis* 31267	Laboratory collection
PSK0530	*Streptomyces lividans* TK24	Laboratory collection
PSK0028	*Streptomyces coelicolor* M145	Laboratory collection
PSK0528	*Streptomyces albus*	Laboratory collection
PSK0518	*Streptomyces filamentosus* 15998	Laboratory collection
PSK0522	*Streptomyces griseoflavus* Tu4000	Laboratory collection
PSK0534	*Streptomyces* sp. AA4	Laboratory collection

Table IV-2. Primers used in chapter IV

Primer	Sequence(5'-3')
spec-fwd	CAACGTTCTTGCCATTGC
spec-rev	GATCCCCCTATGCAAGGGT
RBS+*bmrC*-fwd	aggaggctagcctATGTTTTCAGTTTTGAAAAAGC
bmrD-rev	CAGCAATGGCAAGAACGTTGTTATGCAATGGAATGTTTCTG
bmrC1-mutation-rev	TTGTCGTTGCTCCGCTCCCGGTTTTAC
bmrC2-mutation-fwd	CGGGAGCGGAGCAACGACAATTATTAAGCAGC
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	AACCCTTGCATAGGGGGATCATCCGTTTAGGCTGGGC
	TGTACTATTTCGATCAGACC
amyE-Front-rev	TGTACTATTTCGATCAGACC