The molecular mechanisms of gene regulatory networks controlling cell fate decisions in *Bacillus subtilis*

A Dissertation Presented to

the Faculty of the Department of Biology and Biochemistry

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

By

Priyanka Srivastava

May 2019

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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my advisor, Dr. Masaya Fujita for giving me an opportunity to work and learn under his guidance. This dissertation work would not have been possible without his continuous encouragement and support. His dedication and passion towards his work and willingness to help his students makes him an outstanding mentor.

I would also like to thank my dissertation committee members, Dr. George Fox, Dr. William Widger, and Dr. Jason Rosenzweig for their valuable inputs and advice on my research work.

My heartfelt thanks to past Fujita Lab members- Brittany and Lindsey for helping me with my project during my initial days in the lab. I would also like to thank Anuradha, for being a wonderful lab-mate and my chit-chat companion. I am going to miss your home cooked meals. I wish you all the best for your research work. I would like to wish good luck to Brenda for her coursework and research. My best wishes to the undergrads in lab-Maria, Van and Thinh for their future endeavors.

Thank you Rachna and Savini for being great friends. I will always treasure our friendship.

This academic journey would not have been possible without the support of my family. I am grateful to my loving parents, Mrs. Anjali Srivastava and Mr. Vidhu Shekhar Srivastava for their love, motivation and moral support. I am also thankful to my in-laws for their incessant support and encouragement during my PhD. I would also like to mention my dear brothers and sisters-in-law, Mr. Kapil Shekhar,

Mrs. Swarnita Srivastava, Mr. Siddhartha Srivastava, and Mrs. Nikita Srivastava for their love, affection and long video calls on weekends.

My deepest gratitude to Mr. Vinod Nair and Mrs. Lakshmi Nair for providing a second-home to my daughter Jaya. We all will always cherish the sweet memories and good times she has at your place.

Above all, I am ever thankful and indebted to my dear husband, Dr. Sunil Kumar, and my precious daughter Jaya, for their unconditional love and faith in me.

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ABSTRACT

Under nutrient starvation conditions, the soil bacterium *Bacillus subtilis* differentiates to form biofilms or spores. Biofilm formation and sporulation are under the control of master transcription regulator Spo0A. Spo0A is activated when phosphorylated (Spo0A~P) through a phosphorelay system which includes histidine kinases KinABC, two phosphotransferases (Spo0Fand Spo0B), and Spo0A. It has been proposed that KinA is primarily responsible for activation of sporulation while KinC is involved in controlling biofilm formation. In this study, we aimed to uncover the mechanisms of how the two distinct cell types can be independently generated by two similar kinases. We constructed two strains harboring inducible KinA and KinC with reporter genes to monitor biofilm formation and sporulation, respectively.

Using these systems, we discovered that both *tapA* (marker gene for biofilm formation), and *spoIIG* (marker gene for sporulation) are induced more efficiently by KinA than KinC. Next, by analyzing the biofilm colony architecture using fluorescence microscopy, we demonstrated that distinct cell types, including motile, biofilm-forming, and sporulating cells are localized within distinct regions of a colony. We also provide evidence that biofilm formation and sporulation are mutually exclusive events, contrary to the previously published findings of sporulating cells deriving from biofilm forming cells. Further, we constructed a series of genetically engineered strains expressing reduced levels of Spo0A~P. Using these systems, we find that genes involved in biofilm formation are

sufficiently turned on, leading to biofilm formation, similar to the wild type strain. However, genes involved in sporulation are strongly impaired. We also determine the novel role of Spo0E, a Spo0A~P specific phosphatase, in modulating biofilm formation by controlling the Sp0A~P levels.

Based on these findings, we propose a new model in which genes involved in biofilm formation are controlled by relatively low cellular levels of Spo0A~P, while sporulation genes require relatively high levels of Spo0A~P through two kinases KinC and KinA, respectively. Thus, KinC is sufficient to induce biofilm formation by triggering the low-threshold of Spo0A~P at early times of starvation while KinA is required for triggering the high-threshold Spo0A~P to induce sporulation at relatively later times of starvation.

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Chapter 1: Introduction

"At the surface of the liquid… …The rods adhere together by their sides after the manner of the elements of columnar epithelium, but there is, I think, strong reason to believe that this adhesion is not direct, i.e. that they are not in actual contact but glued together by a viscous intermediary substance."

The "viscous intermediary substance described by Burton-Sanderson in 1870 (P. B. Beauregard, Y. Chai, H. Vlamakis, R. Losick, & R. Kolter, 2013)

I.i A short history of Biofilms

Biofilms are defined as a "structured consortium of microbial cells surrounded by a self-produced polymer matrix" (Hoiby, 2014). Even though the concept of 'biofilm' was established in 1930s, the term 'biofilm' was not coined until the year 1970. The first example of description of biofilms comes from Anthony van Leeuwenhoek where he described aggregated microorganisms in the 'scurf of the teeth' and particles scraped of his tongue as, "I saw an inconceivable number of exceeding small animalcules and these of divers sorts…….and most of these animalcules were abiding where the said matter from the tongue lay" (Leeuwenhoek & Dobell, 1960).

In the year 1933, Henrici found that when a clean glass slide was submerged into a pond or lake would result in bacterial community "eventually becoming so thick that individual cells may be distinguished with difficulty". He also noticed biofilm matrix and described it as, "In other cases, the groups of cells are evidently surrounded by a sheath of gum which also serves to fasten the colony to the glass" (Henrici, 1933). In the year 1935, ZoBell and Allen observed biofilms while studying biofouling and described it as, "it seems to take several minutes for the bacteria to

cement themselves to the glass. . . . But let the slide remained submerged for an hour or two . . .they will be found profusely, so firmly glued to the slide that running water will not detach them" (Zobell & Allen, 1935).

In the field of medicine, the first image of biofilm was published in 1977, from the cystic fibrosis patients whose lungs were infected with *Pseudomonas aeruginosa.* The term "biofilm" was first used by Bill Costerton in a technical microbiology report, and the term was later introduced to medical microbiology in 1985 (Costerton et al., 1987).

Before 1980, biofilms related studies were done using conventional light microscopy and electron microscopy. With the advancement of technologies after 1990, confocal laser scanning microscopy, automatic image analysis, Live-dead staining, various mutants of biofilm forming bacteria, crystal violet staining, and transcriptomic analysis. (Hoiby, 2014; Lawrence, Korber, Hoyle, Costerton, & Caldwell, 1991; Moller, Kristensen, Poulsen, Carstensen, & Molin, 1995) helped characterize the nature and various properties of biofilms and biofilm forming bacteria (O'Toole, Kaplan, & Kolter, 2000).

I.ii *Bacillus subtilis* as a Model Organism

Bacillus subtilis is a Gram-positive, soil bacterium, which has the ability to form biofilms and spores during nutrient starvation. *B. subtilis* was first identified as *Vibrio subtilis* in 1835 by Christian Gottfried Ehrenberg. It was later renamed as *Bacillus subtilis* by Ferdinand Cohen in 1872 (Brock, 1961). *B. subtilis* has excellent genetic amenability. It is non-pathogenic but shares similar genes

required for biofilm formation and sporulation in many disease causing bacteria such as *Bacillus anthracis*, *Clostridium acetobutylicum* which makes it an outstanding model system. Additionally, it also has the ability to differentiate into multiple cell types, such as motile cells, matrix - producing cells, spore - forming cells, and competent cells also (Figure I-1) making it an excellent model to study cell differentiation processes.

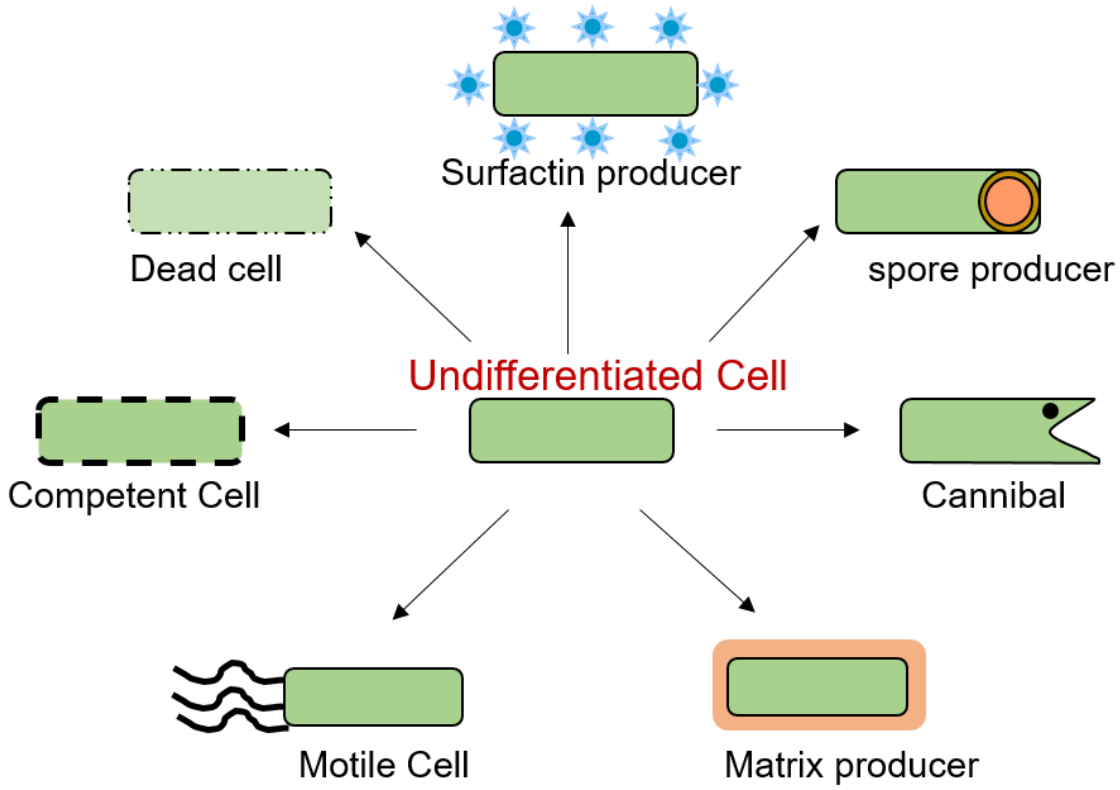


Figure I-1 Schematic representation of different cell types in *B. subtilis.*
Adapted from (Tasaki, Nakayama, & Shoji, 2017)

B. subtilis has been widely used for industrial scale enzyme production of proteases (van Dijl & Hecker, 2013) and antibiotics such as surfactin and bacilysin for industrial purposes (N. Mannanov & K. Sattarova, 2001). According to recent studies, *B. subtilis* is considered safe for human consumption and is being used in fermented soybean dishes such as *nattō* (Marco et al., 2017) and is also gaining popularity as a probiotic agent (Elshaghabee, Rokana, Gulhane, Sharma, & Panwar, 2017).

I.iii Spo0A and Phosphorelay network

Spo0A, a member of the response regulator family of DNA-binding proteins, is considered to be the master transcriptional regulator of biofilm formation and sporulation in *B. subtilis.* A multicomponent phosphorelay network is attributed to the activation of Spo0A in its phosphorylated form (Figure I-2). This phosphorelay network consists of five histidine autokinases namely-KinA, KinB, KinC, KinD, and KinE, and two phosphorelay proteins-Spo0F and Spo0B (Jiang, Shao, Perego, & Hoch, 2000). During nutrient starvation, any of these kinases become autophosphorylated by transferring a phosphoryl group from ATP to a conserved histidine residue. Upon autophosphorylation of the kinase, the phosphoryl group is transferred to one of the phosphotransfer proteins, Spo0F. Spo0F gets phosphorylated and then transfers its phosphoryl group to the second phosphotransfer protein, Spo0B. Spo0B ultimately transfers its phosphoryl group to Spo0A through a His-Asp-His-Asp cascade and becomes activated it in its

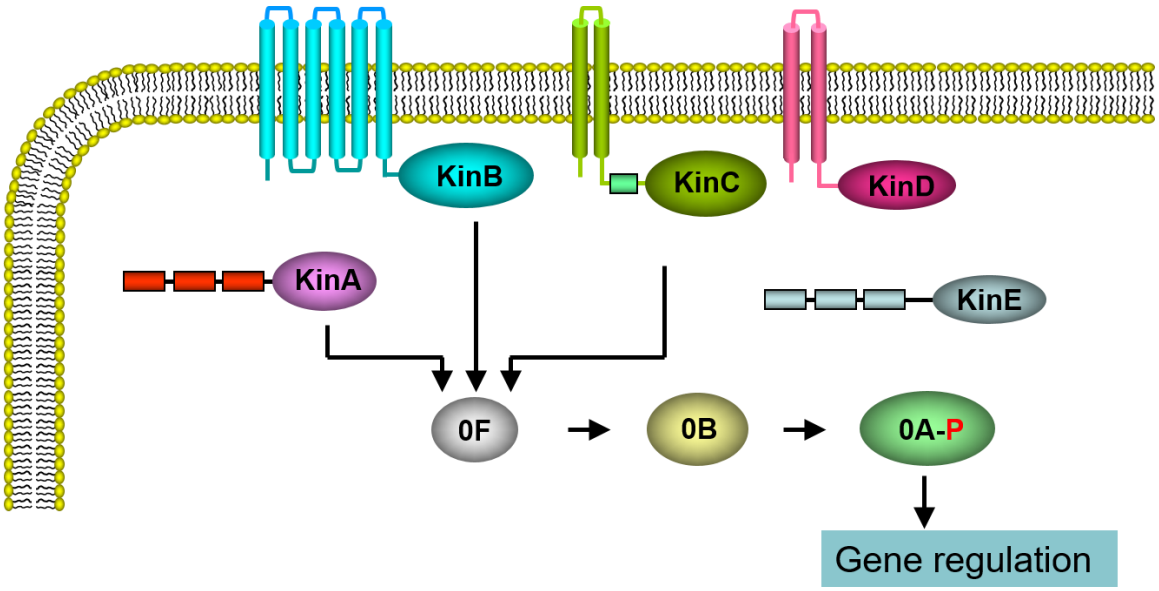


Figure I-2 Schematic representation of the multicomponent phosphorelay system in *B. subtilis*.
Histidine kinases KinA, KinB, KinC, KinD, and KinE can autophosphorylate. Then the phosphoryl group is transferred to phosphotransferases Spo0F and Spo0B respectively. The Spo0B transfers its phosphoryl group to Spo0A and activate it in its phosphorylated form (Spo0A~P).

phosphorylated form (Spo0A~P) (Burbulys, Trach, & Hoch, 1991). Interestingly, in the absence of Spo0F, KinC can phosphorylate Spo0A directly (Kobayashi et al., 1995; LeDeaux & Grossman, 1995).

While KinA and KinB are involved in initiation of sporulation (Hoch, 1993; Perego, Cole, Burbulys, Trach, & Hoch, 1989). KinC and KinD play a role in biofilm formation (Chen et al., 2012; Devi, Kiehler, Haggett, & Fujita, 2015), while KinE does not play any specific role (Jiang et al., 2000).

Orthologues of Spo0A have been identified in almost all spore forming bacteria in the *Bacillus* and *Clostridium* genera. However, the important components of

phosphorelay, Spo0F and Spo0B are missing in *Clostridium* species (Kanamaru, Stephenson, & Perego, 2002; Molle et al., 2003).

Two models have been proposed to describe the initiation of autophosphorylation. According to the widely accepted “Signal model,” the sensor domain on the histidine kinase receives an external starvation signal in response to nutrient starvation and triggers the autophosphorylation event. However, no starvation signal has been identified to date (Grossman, 1995; Hoch, 1993).

According to the “Threshold model,” the sensor kinase KinA is important for the initiation of autophosphorylation in response to nutrient depletion. Under the conditions of nutrient starvation, cell growth slows down, which leads to accumulation of KinA, which then leads to increased levels of Spo0A~P. Cells in which a “threshold” level of KinA is reached, enter into sporulation (Eswaramoorthy et al., 2010; Narula, Fujita, & Igoshin, 2016).

I.iii.1 0A boxes

Upon phosphorylation, Spo0A undergoes a conformational change by forming dimers which bind its target sequence. The consensus sequence for the binding site is TTTGTCRAA, known as the 0A box (Molle et al., 2003). Transcription of *spo0A* is under the control of two promoters-Pv and Ps. The upstream promoter, Pv, is regulated by the primary housekeeping sigma factor σ^A and is expressed during the exponential phase. As nutrients are depleted, cells enter into stationary phase, then, the downstream promoter Ps is expressed. Ps is under the control of stationary phase sigma factor $\sigma^{H.}$ During stationary phase, transcription from both

Pv and Ps leads to a gradual increase in the Spo0A~P levels, which is essential for the regulation of Spo0A controlled genes (Fujita, Gonzalez-Pastor, & Losick, 2005). Three 0A boxes-O1, O2, and O3 can be found between the Pv and Ps promoter regions. Out of these three 0A boxes, O1 represses Pv at the end of exponential phase, O2 represses Ps during growth, O3 activates Ps during stationary phase (Chastanet & Losick, 2011).

I.iii.2 The Spo0A regulon

There are 121 genes under direct control of Spo0A (Fujita et al., 2005; Molle et al., 2003). Many of these genes are regulated by Spo0A in a dose dependent manner. As shown in Figure I-3, genes that are regulated by Spo0A are divided into 2 categories-(1) Genes that are activated or repressed by low levels of Spo0A~P, known as Spo0A low-threshold genes and (2) genes that are activated or repressed by high levels of Spo0A~P, known as Spo0A high- threshold genes. The importance of this differential regulation by Spo0A is that different genes are activated or repressed at different times, thus, enabling the cells to adapt to the external environment. For example, under the conditions of nutrient starvation, genes involved in delaying the sporulation (*sdp*) and cannibalism (*skf*) are activated when Spo0A~P levels are low. Thus, prevents the cells from commiting to sporulation, which is an energy consuming process and the last resort to adapt to nutrient stress. However, when nutrient starvation continues, Spo0A~P levels

are high, genes involved in sporulation such as *spoIIA, spoIIE* and *spoIIG* are activated which are directly involved in sporulation (Fujita & Losick, 2005).

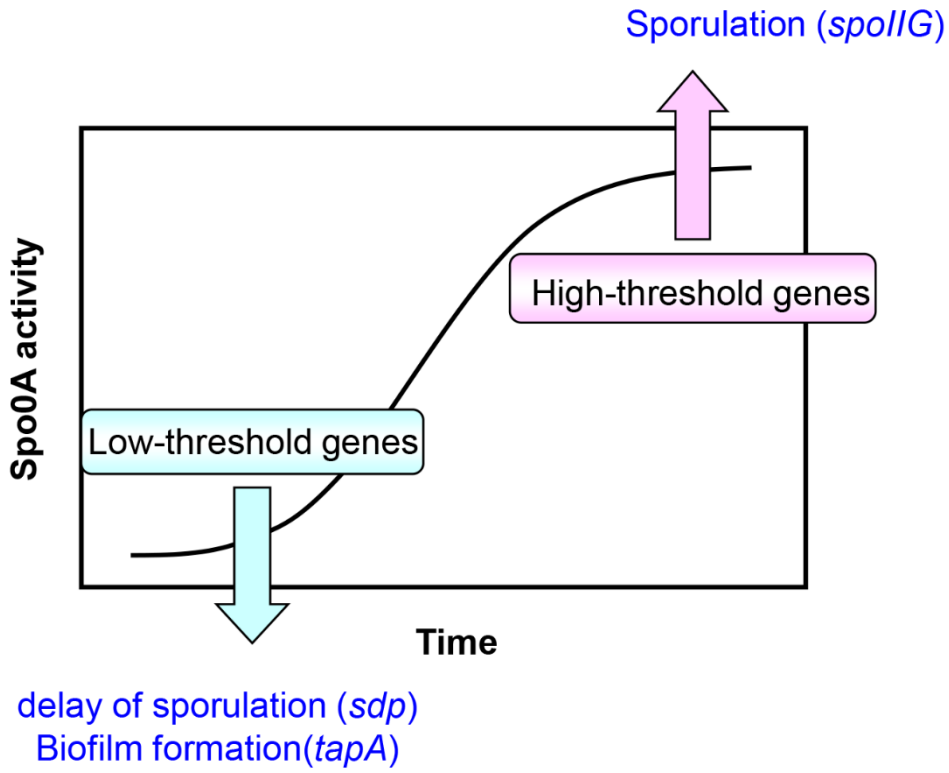


Figure I-3 Spo0A-low and high threshold gene expression.
Different genes are turned on /off depending on Spo0A~P levels. When Spo0A~P are low, genes that require lower doses of Spo0A~P such as those involved in cannibalism and biofilm are activated. As Spo0A~P levels increase gradually, genes that require higher level of Spo0A such as those involved in sporulation are activated.

I.iii.3 Regulatory networks involved in the control of phosphorelay

When phosphorelay becomes active, the transcription of *spo0A* is positively and negatively regulated by its own gene product Spo0A~P. These feedback loops affect the phosphorelay to regulate the levels of Spo0A~P (Figure I-4).

When the phosphorelay is activated, the increased levels of Spo0A~P represses the transcription of the transition phase regulator, *abrB.* This in turn leads to the

derepression of the transcription of the σ^H gene (an alternative sigma subunit of RNA Polymerase). Thus, σ^H levels begin to increase and further stimulate the expression of Spo0A~P. Besides, *spo0A*, σ^H also regulates the transcription of *kinA* and *spo0F* (Chastanet et al., 2010; Eswaramoorthy et al., 2010; Fujita & Sadaie, 1998; Predich, Nair, & Smith, 1992).

Spo0A~P also stimulates the transcription of its own gene *spo0A* through the Ps promoter and σ^H. Two phosphatases, RapA and Spo0E, also come into play in regulating the levels of Spo0A~P. RapA family of aspartate phosphatases, act by specifically dephosphorylating Spo0F, while Spo0E is a Spo0A~P specific phosphatase (Diaz et al., 2012). The phosphatase activity of RapA is modulated by PhrA, a peptide encoded by a gene adjacent to *rapA* (Perego, Glaser, & Hoch, 1996).

Spo0E is transcribed under three promoters. Promoters P1 and P2 (Perego & Hoch, 1991) and the third recently identified upstream promoter which is activated by ectopic expression of the alternative sigma factor, σ^B (Reder, Gerth, & Hecker, 2012) . σ^B is a key regulator of stress response in *B. subtilis*.

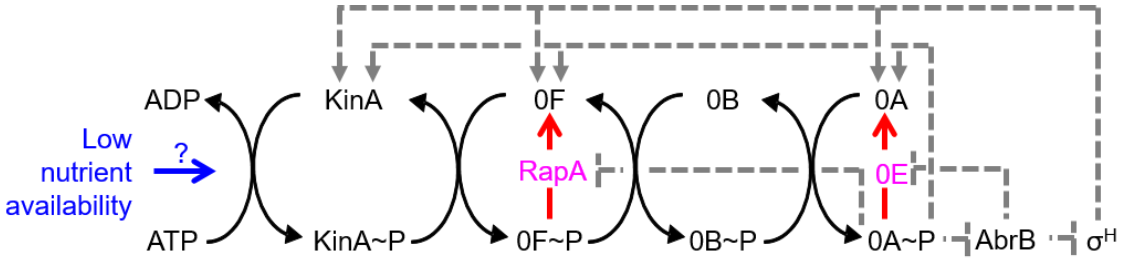


Figure I-4 Schematic representation of phosphorelay feedback loops.
Pointed and flat arrowheads denote positive regulation and negative regulation, respectively. Solid lines indicate direct interaction between proteins, and dashed lines indicate transcriptional regulation.

A recent study (Reder, Albrecht, Gerth, & Hecker, 2012) has shown that the transcription of *spo0E* is induced by σ^B under the conditions of ethanol stress.

I.iv Cell differentiation in *B. subtilis*

B. subtilis is considered a master of cellular differentiation processes. Cell differentiation can take place when cells are growing exponentially, in stationary phase, or under the conditions of nutritional stress (Daniel Lopez, Vlamakis, & Kolter, 2008). When cells are growing exponentially, a subpopulation of cells express sigma factor D, which is required for the production of the *flagellin,* gene responsible for cell motility. During the stationary phase, cells can differentiate into competent cells which have the ability to take up exogenous DNA (Ashikaga, Nanamiya, Ohashi, & Kawamura, 2000; Dubnau, 1991). Under the conditions of nutrient starvation, cells can differentiate into cannibalistic cells (José Eduardo González-Pastor, 2011; J. E. González-Pastor, Hobbs, & Losick, 2003), biofilm forming cells (Vlamakis, Aguilar, Losick, & Kolter, 2008; Vlamakis, Chai, Beauregard, Losick, & Kolter, 2013) , or spores (Sonenshein, 2000).

B. subtilis cell populations under any given experimental condition are heterogeneous. Thus, gene expression patterns in various subpopulations are different, and lead to divergent cell fates. This is called bistabilty. As a result of bistability, cells from the same genetic background can differentiate into multiple

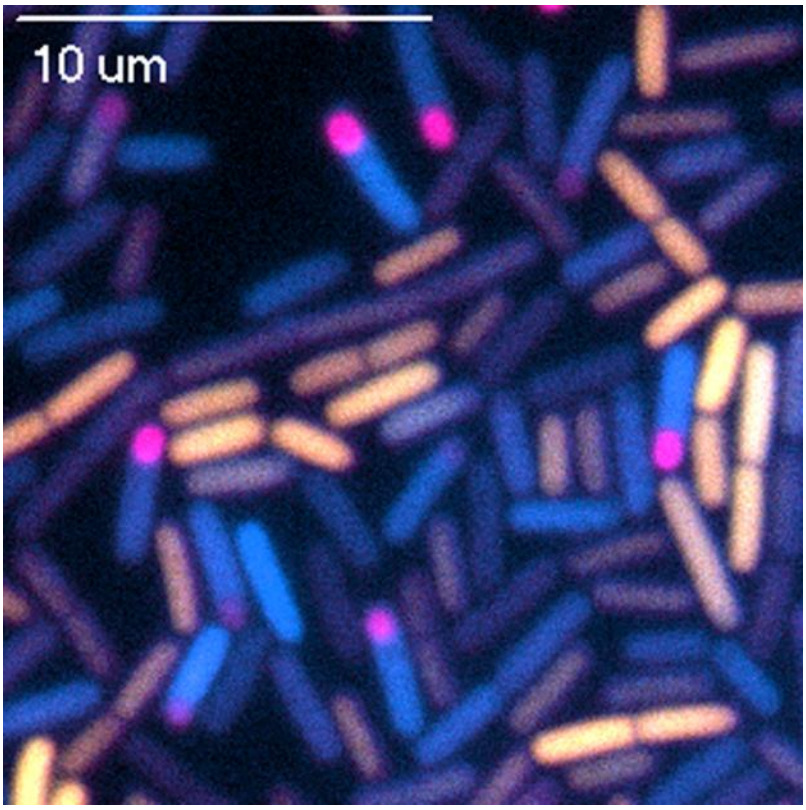


Figure I-5 Heterogeneity in a *B. subtilis* population.
Cells from the same genetic background can give rise to different cell fates. Here, three different cell types can be visualized in a same population. Cells expressing yellow fluorescent protein (YFP) under the control of the *PabrB* promoter are undergoing vegetative growth. Cells expressing cyan fluorescent protein (CFP) are from the sporulation promoter *PspoIIA*, while cells under the control of forespore specific promoter *PspoIIQ* are expressing the pink mCherry reporter and are initiating sporulation. Scale bar, 10 µm.

cell types such as motile cells, spore forming cells, cannibals, and biofilm forming cells as shown in Figure I-5 (Chai, Chu, Kolter, & Losick, 2008).

I.iv.1 Motile cells

An actively growing culture of *B. subtilis* consists of two kinds of cells-motile cells that can swim or non-motile cells that cannot swim and are found in chains. Motility in *B. subtilis* is under the control of *sigD*. The *sigD* encodes for alternative

sigma factor $\sigma^{D.}$ The *fla/chi* motility operon, under the control of σ^{D}, consists of genes for cell wall remodeling enzymes called autolysins as well as the genes required for the production of flagella-*flagellin* filament protein or *hag.* Cells in which σ^{D} is expressed, (σ^{D} ON), become motile and are separated from each other. In contrast, cells in which σ^{D} is not expressed, (σ^{D} OFF), become non-motile and form long chain of cells (Cozy & Kearns, 2010; Kearns & Losick, 2005). When Spo0A~P levels are low, most cells are in motile state (Dubnau & Losick, 2006).

A prior published study (Chai, Norman, Kolter, & Losick, 2010) demonstrated that the position of sigD gene within the operon is important to switch from the motile to non-motile state or vice versa. They also observed that the physiological states of the cell-motile or non-motile can be inherited epigenetically.

Motile cells have been demonstrated to be an important part of biofilm colonies. Motile cells are required to attach to a surface, inhibit the flagella, and differentiate into matrix producing cells.

I.iv.2 Biofilm and cannibalism

It has been known that majority of microbes such as *B. subtilis*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* are able to form architecturally complex, surface associated communities of cells enclosed in an extracellular matrix, known as biofilms (Branda, Vik, Friedman, & Kolter, 2005). Biofilms can be formed on liquid as well as solid surfaces. Biofilms which are formed on the interface of air and liquid are known as pellicles. A hallmark of biofilm formation is the presence

of wrinkles, colony architecture, and an extracellular matrix that acts as a glue to hold the colony together.

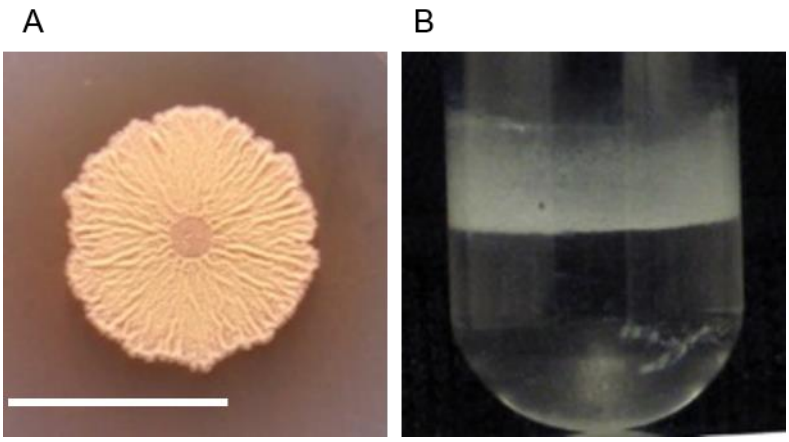


Figure I-6 Biofilm formation on solid agar surface (A) and Pellicle (B) in *B. subtilis.* Scale bar, 10 mm.

B. subtilis is able to form biofilms on solid surfaces and in the form of pellicles as shown in Figure I-6. The process of biofilm begins when the planktonic bacteria become attached to a biomaterial (Figure I-7). The initial attachment to the surface is reversible, but if environmental factors are favorable the attachment to the surface later becomes irreversible. The motile cells then differentiate into matrix producing cells, become encased in an extracellular matrix and develop into long chains of cells called cell chaining. Cell chaining is an essential step in biofilm formation (Vlamakis et al., 2013). *B. subtilis* mutants defective in cell chaining do not form biofilms. As the colony matures, some cells differentiate into spore forming cells and form spores. In the last stages of biofilm, some cells secrete D-amino acids and polyamines that disrupts the biofilm and the contents of the

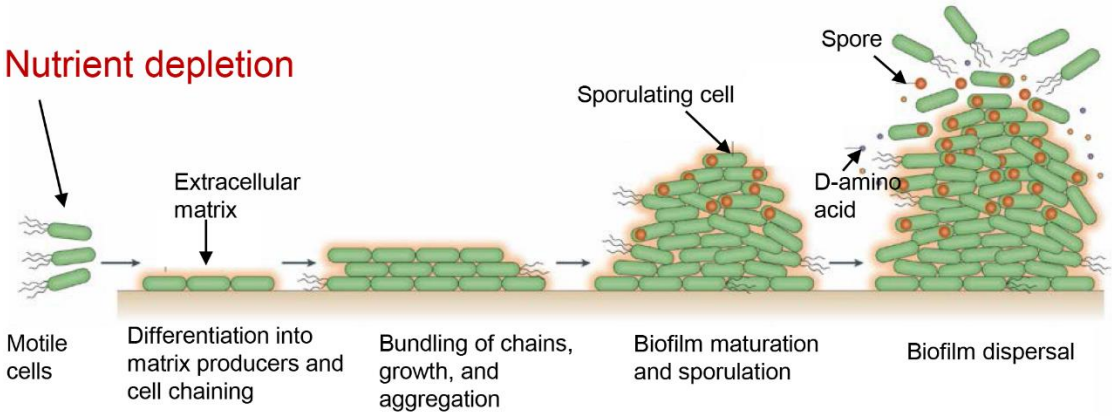


Figure I-7 Life cycle of biofilm formation in *B. subtilis.* Adapted from (Vlamakis et al., 2013).
The process of biofilm formation begins when the motile cells with flagella become attached to a surface. The motile cells then inhibit their flagella and differentiate into matrix producing cells. A hallmark of matrix producing cells is that they grow in long chains of cells and become enclosed in an extracellular matrix. As the biofilm matures, sporulation begins within the colony. As biofilm becomes old, some cells secrete D-amino acids and polyamines that disassemble the biofilm and the biofilm contents are dispersed in the environment.

biofilms are dispersed in the environment (Kostakioti, Hadjifrangiskou, & Hultgren, 2013). A mature biofilm displays a highly complex network of intertwined wrinkles and is extremely hydrophobic. The extracellular matrix consists of exopolysaccharides (product of *epsA-O* operon), proteins (TapA, TasA, and BslA), lipids, and extracellular DNA (eDNA) (Cairns, Hobley, & Stanley-Wall, 2014). The robustness and architecture of the colony varies depending on the *B. subtilis* strain and also the experimental conditions. For example, more robust biofilm formation occurs on LBGM media than the MSgg media, even though both media promote biofilm formation (Moshe Shemesh 2013). The most commonly used "domesticated" strain of *B. subtilis* does not form typical biofilm structures and is

considered to be biofilm deficient (Konkol, Blair, & Kearns, 2013; Zeigler et al., 2008). Besides forming biofilms under laboratory conditions, *B. subtilis* also forms biofilms under natural settings such as the rhizosphere of plant roots (Pascale B. Beauregard, Yunrong Chai, Hera Vlamakis, Richard Losick, & Roberto Kolter, 2013). It also forms biofilms with other bacterial species such as *Staphylococcus aureus.* It has been reported that a particular strain of *B. subtilis*, NDmed, isolated from the endoscope washer-disinfector, formed biofilms with *S. aureus* and protected it from biocides treatment (Bridier et al., 2012).

Biofilm formation in *B. subtilis* occurs under the conditions of nutrient starvation and is governed by the master transcription regulator Spo0A. Spo0A regulates the activity of another transcription regulator SinR (Figure I-8). SinR is the repressor for matrix producing operon *epsA-O*, amyloid synthesis operon *tapA-sipW-tasA,* and another regulatory gene *slrR*. SinI, the antirepressor for SinR , is under the control of Spo0A~P.

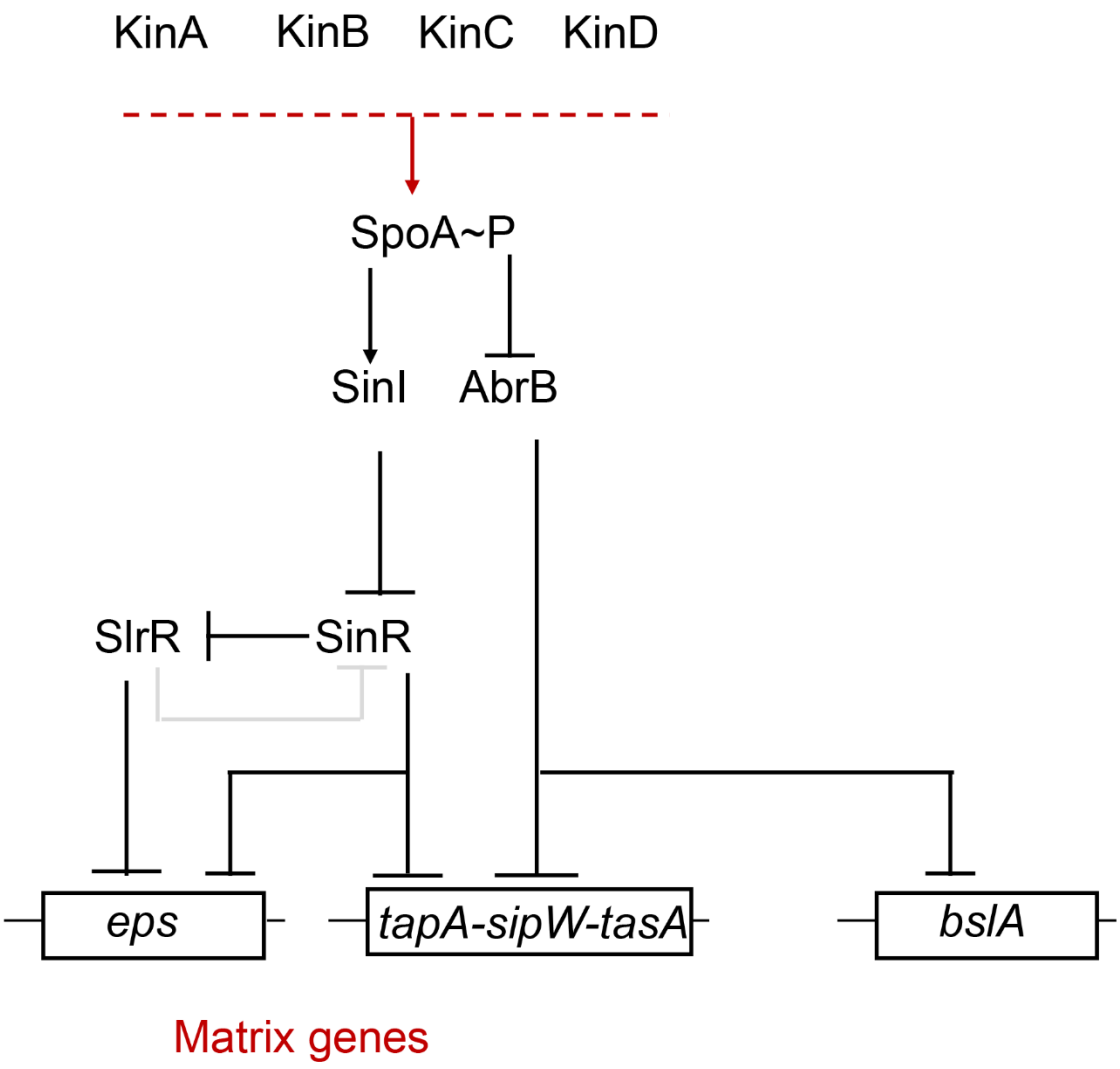


Figure I-8 Schematic representation of regulatory network that controls biofilm formation in *B. subtilis*. Adapted from (Vlamakis et al., 2013).
Arrows represent activation and T-bars represent repression of matrix genes expression depending on environmental condition. Dashed lines indicate indirect activity. The genes encoding components of extracellular matrix are represented as EPS (*epsA-O*), TasA (*tapA-sipW-tasA*), and BslA (*bslA*). Spo0A is activated by the kinases, KinA, KinB, KinC, and KinD. Spo0A governs the biofilm formation pathway by regulating the activity of the master regulator, SinR, and the transition phase regulator, AbrB. SinR represses the *epsA-O* and *tapA* operons. Spo0A activates the repressor of SinR, SinI, so the matrix genes are derepressed and matrix production takes place. SinR is under the control of another regulatory protein SlrR. AbrB represses both the *epsA-O* and *tapA* operons and *bslA*. Spo0A represses AbrB which in turn depresses epsA-O and tapA and bslA.

When *sinI* is expressed, derepression of matrix genes occurs, and matrix production is turned on (Chai et al., 2008; Chu, Kearns, Branda, Kolter, & Losick, 2006). Spo0A~P also represses another matrix repressor, AbrB. AbrB is a transition state regulator. It represses the amyloid protein synthesis operon *tapA-sipW-tasA* and the regulatory protein SlrR as shown in Figure I-9 (Hamon, Stanley, Britton, Grossman, & Lazazzera, 2004; M. Strauch, Webb, Spiegelman, & Hoch, 1990).

Thus, under initial stages of starvation, when Spo0A~P levels are low, SinI is turned on and blocks the SinR mediated repression of the matrix genes. Matrix genes are derepressed and matrix production and subsequently biofilm formation is turned on. In the meantime, Spo0A~P, also represses AbrB, resulting in derepression of amyloid synthesis operon *tapA-sipW-tasA* which is essential for biofilm formation. *sinI* is expressed only in a subpopulation of cells while *sinR* is expressed more or less in the entire population (Chai et al., 2008).

SlrR plays an important role in biofilm formation. It binds to SinR and prevents the repression of matrix producing genes *epsA-O* and *tapA-sipW-tasA.* The SinR-SlrR complex also repress the promoters for cell motility (*hag*) and autolysins involved in cell separation such as *lytABC,* and *lytF* as shown in Figure I-9 (Chai et al., 2010). As mentioned earlier, cell chaining is an essential part of biofilm formation. SlrR not only controls the matrix gene expression but also controls the genes responsible for cell motility and cell separation.

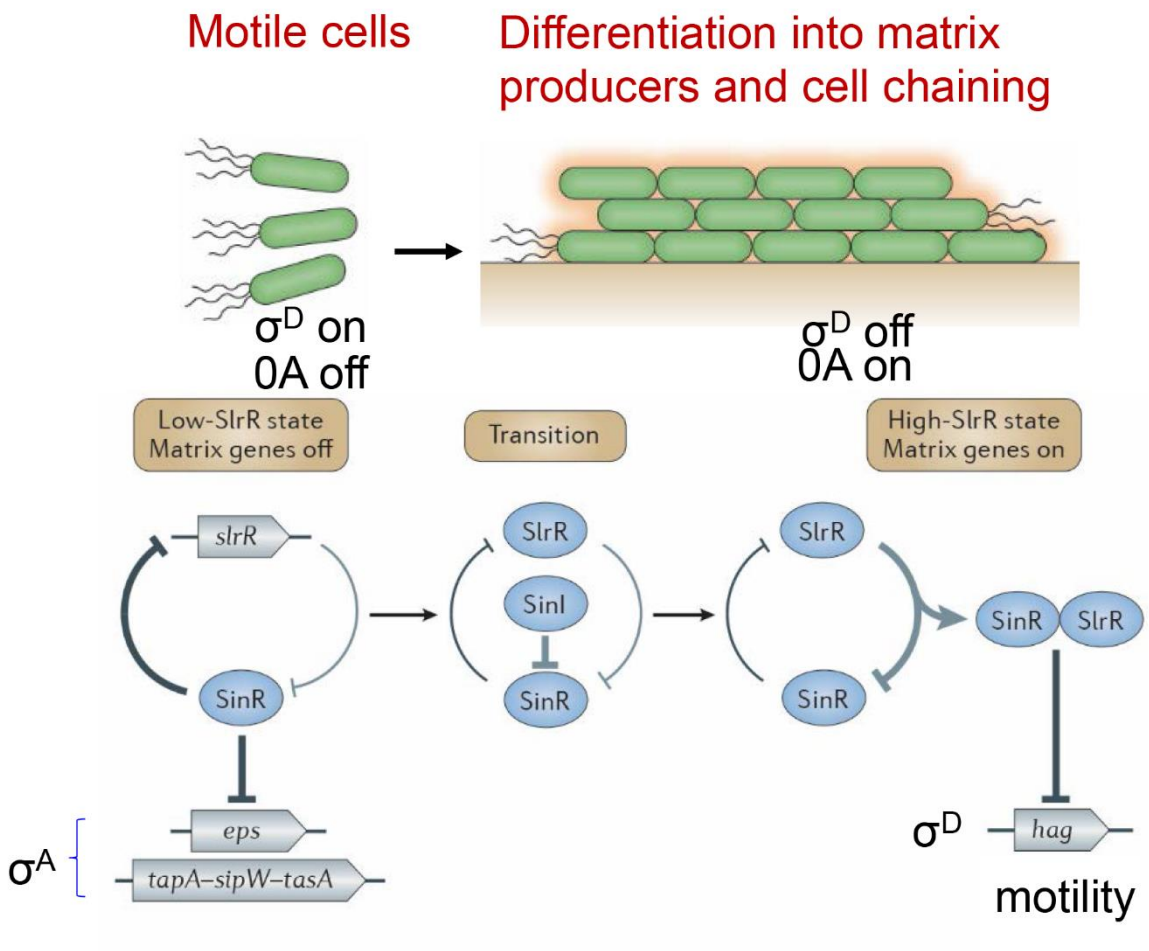


Figure I-9 The double negative feedback loop involving *slrR* gene, the SlrR protein and the SinR protein. Adapted from (Vlamakis et al., 2013).
The SinR-SlrR switch regulates matrix genes (*epsA-O* and *tapA* operons), and motility genes (*hag*) as well as the gene for SlrR itself. In the motile cells, in which σD is on and 0A is off, SlrR is in low state, *sinR* represses the *slrR* gene, and matrix genes are repressed. However, when motile cells switch to cell chaining and differentiate into matrix producing cells, σD is off and 0A is on, SlrR is in high state, SlrR binds to SinR which represses the motility genes.

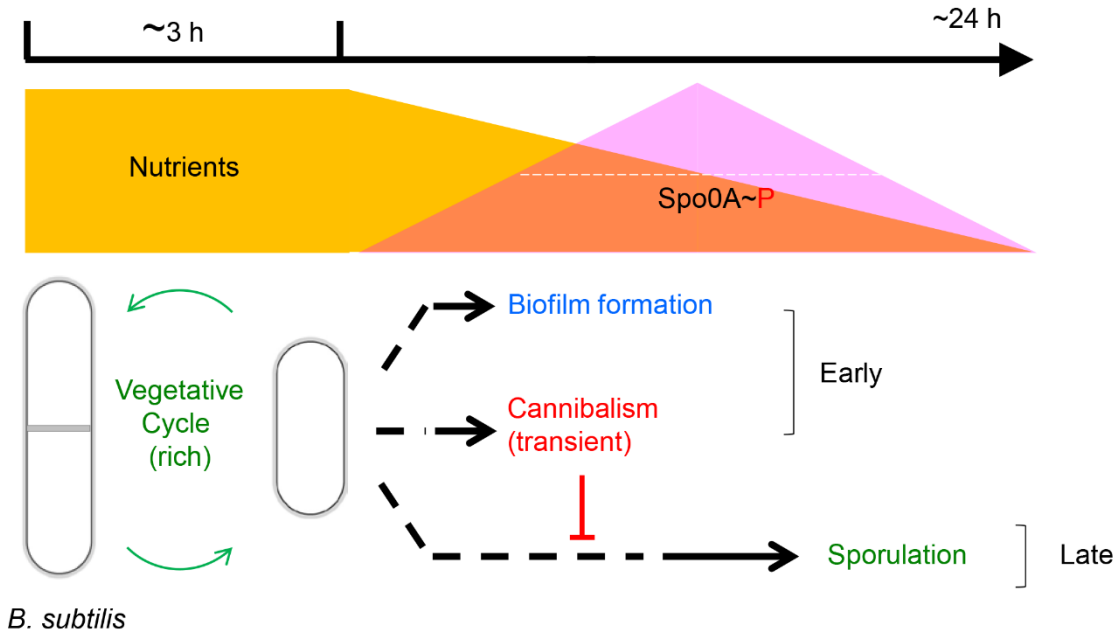


Figure I-10 Cell Differentiation in *B. subtilis* under nutrient starvation.
In a typical *B. subtilis* culture, cells undergo vegetative growth for a short period of time before nutrients are depleted. Spo0A levels gradually increase with the decreasing nutrient availability. At early stages of nutrient depletion, Spo0A~P levels are low and biofilm and cannibalism programs are triggered. Cannibalism pathway stalls the sporulation event but in the late stages of nutrient starvation, as nutrients levels continue to deplete, sporulation is activated at high levels of Spo0A~P.

Another cell differentiation pathway activated by low levels of Spo0A~P is cannibalism (Figure I-10). In response to low levels of Spo0A~P, cells express two cannibalism operons-*sdp* (sporulation delaying protein) and *skf* (sporulation killing factor) which secrete extracellular killing factors (Eichenberger et al., 2003; José Eduardo González-Pastor, 2011). The cells secreting the killing factors are themselves resistant to the effect of the toxic peptides (Ellermeier, Hobbs, González-Pastor, & Losick, 2006; Povolotsky, Orlova, Tamang, & Saier, 2010). These killing factors lyse the nonsporulating siblings that do not have immunity to the killing factors. Nutrients released by the dead cells are taken up by the

neighboring cells and sporulation is prevented in these cells. The *sdp* and *skf* operons are directly regulated by Spo0A and are highly expressed under sporulation conditions. The *sdp* operon is also repressed by the transition state regulator, AbrB. During early stages of sporulation, low levels of Spo0A~P repress *abrB* which in turn leads to derepression of the *sdp* operon and killing factors are expressed (Fujita et al., 2005). There is an overlap in between the matrix producing genes and cannibalism genes as they both are activated under low levels of Spo0A~P (Fujita & Losick, 2005; D. Lopez, Fischbach, Chu, Losick, & Kolter, 2009). The *sdp* and *skf* mutants sporulate faster than the wild type strain similar to the *kinC* mutant strain (José Eduardo González-Pastor, 2011). The expression of *sdp* is regulated partly by KinC (Devi, Kiehler, et al., 2015).

I.iv.3 Sporulation

The process of sporulation in *B. subtilis* is mainly induced under nutrient starvation conditions. Cannibalism can impede the sporulation process for some time but under late stages of nutrient starvation, cells commit to the more energy expensive process of sporulation (Figure I-10). The hallmark feature of sporulation in *B. subtilis* is the switch from vegetative cell division to an asymmetric cell division (Piggot & Coote, 1976; Piggot & Hilbert, 2004). As a result of asymmetric cell division, a larger compartment, called a mother cell, and a smaller compartment, called the forespore are formed as shown in Figure I-11. After DNA segregation, the mother cell gradually engulfs the forespore. Spore cortex, and the inner and outer coat are produced by the mother cell which helps the development of

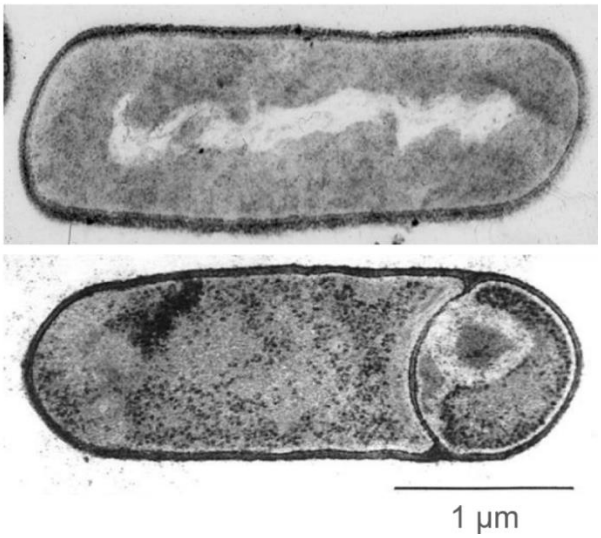


Figure I-11 Electron micrograph images of *B. subtilis* during vegetative growth (top) and sporulation (bottom).
The images were kindly provided by Dr. David Rudner (Harvard Medical School).

forespore into a spore. After the spore matures, the mother cell lyses and releases the mature spore (Higgins & Dworkin, 2012).

Sporulation in *B. subtilis* is under the control of the master transcriptional regulator Spo0A. As sporulation progresses, alternative sigma factors are expressed in a compartment-specific manner as shown in Figure I-12. During initial stages of sporulation, Spo0A~P levels are low. As a result, the stationary phase sigma factor, σ^H is activated, which triggers the activation of phosphorelay components, resulting in an increased expression of Spo0A as shown in Figures I-2 and I-5 (Fujita et al., 2005). As the polar septum is formed, the alternative sigma factor, σ^F controls the expression of forespore specific genes (X. Wang et al., 2009). The second sigma factor, σ^E, localized in the mother cell compartment, is activated by the product of σ^F (Eichenberger et al., 2004; Eichenberger et al., 2003) .

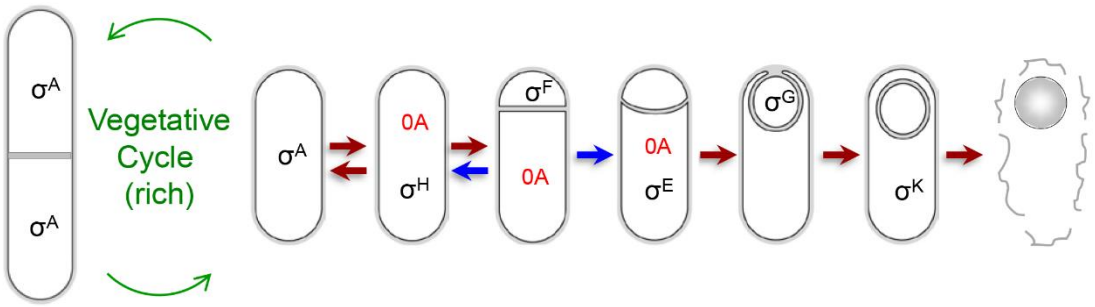


Figure I-12 Morphological changes during sporulation in *B. subtilis.*
The housekeeping sigma factor, σ^A controls the vegetative growth, during which two identical daughter cells are formed. In early stages of starvation, low levels of Spo0A~P activate the stationary phase factor σ^H. The polar septum is formed, giving rise to two unequal compartments. The larger mother cell compartment engulfs the smaller forespore compartment. The mother cell nurtures the forespore and once the spore matures, the mother cell lyses and releases the spore. During sporulation, each developmental stage is coupled to the activation of a different compartment-specific sigma factor. σ^F is activated only in the forespore, followed by σ^E in the mother cell which directs engulfment of the forespore. σ^G is expressed in the forespore and finally σ^K is expressed in the mother cell.

σ^E governs the expression of genes involved in the engulfment of the forespore compartment. After the forespore compartment is engulfed by the mother cell, σ^G and σ^K are activated in the forespore and mother cell compartment respectively (Cutting, Roels, & Losick, 1991; Eichenberger et al., 2004).

I.v Scope of this Dissertation

Throughout this Ph.D. study, my focus was on biofilms. I focused on how gene expression is regulated in regards to the cell-fate decision making process, what are the roles of histidine kinases, KinA and KinC, and what conditions are suitable to obtain reproducible results under laboratory conditions. There are many

research articles in these areas in the domesticated *B. subtilis* strain, but there is a dearth of knowledge in how these processes are affected in an undomesticated *B. subtilis* strain. So, in my Ph.D. project, I focused mainly on the undomesticated *B. subtilis* strain and tried to fill the gaps in our knowledge about these processes. The mechanisms of cell fate decision making process (whether to form a biofilm or a spore) are not well understood. Chapter 2 describes the methods used in order to gain a better understanding of cell-fate decision making process at the single cell level. By the use of a three color fluorescent reporter strain, I have described spatiotemporal gene regulation of three distinct cell types during the development of a biofilm colony. The methods described here are uniquely used during the course of development of a biofilm colony. The experimental conditions were kept the same. This is in contrast to other published results in which the experimental conditions were changed in order to favor one cell fate decision making process over another.

Chapter 3 describes the putative roles of histidine kinases-KinA and KinC in biofilm and sporulation pathways in an undomesticated *B. subtilis* strain. In this chapter, I analyzed the roles of KinA and KinC by artificially inducing their expression using an inducible promoter, and determined their effect on Spo0A low-threshold gene, *tapA* (required for biofilm formation) and Spo0A high-threshold gene *spoIIG* (required for sporulation).

Genes required for sporulation are Spo0A high-threshold genes, and the genes for biofilm formation such as *epsA*, and *tapA* are Spo0A low-threshold genes. To date,

there is no direct evidence demonstrating that low-threshold of Spo0A is sufficient for biofilm formation. Chapter 4 provides direct evidence for the first time that low-threshold of Spo0A is sufficient for biofilm formation but not for sporulation. Here, we also uncovered the novel role of a Spo0A specific phosphatase, Spo0E, in stimulating biofilm formation.

Chapter II: Establishment of the methods for single-cell analysis of cell fate decisions.

II.i Introduction

Under the conditions of nutrient starvation, *B. subtilis* cells differentiate into competent, biofilm forming, cannibalistic, and spore forming cells (José Eduardo González-Pastor, 2011; Piggot & Hilbert, 2004; Sonenshein, 2000). Biofilms are structurally architecture aggregates of surface-associated bacterial organisms which are enclosed in a self-produced matrix known as extracellular polymeric substances (EPS) (Branda et al., 2005; Mielich-Suss & Lopez, 2015). The EPS is composed of amyloid like fibers, proteins, DNA, and exopolysaccharides (Cairns et al., 2014). Besides holding the cells within the biofilm like a glue, the EPS also promotes the development of three-dimensional structures and protects the cells from extreme environmental conditions. Biofilm formation is a complex process which involves division of labor. During the development of a biofilm, genetically identical cells can differentiate into cells expressing diverse genetic backgrounds. These subpopulations of cells co-exist and contribute towards the development of biofilm (Vlamakis et al., 2013).

The three main distinct cell types during biofilm formation are-motile cells, matrix producing cells, and sporulating cells. While cell motility (*hag*) is under the control of sigma D (Cozy & Kearns, 2010), matrix production (*tapA*) is a Spo0A low threshold gene and is indirectly regulated by Spo0A. Sporulation gene (*spoIIA*) is a Spo0A high threshold gene an is under direct control of Spo0A (Fujita et al., 2005).The process of biofilm formation is a multistep process as shown in Figure II-1(Vlamakis et al., 2013). In the initial stages of biofilm formation, the motile cells

attached to a surface with impeded motility, differentiate into matrix producing cells. The matrix producing cells organize themselves into a long chain of cells which later becomes enclosed in an extracellular matrix. As the biofilm matures, the matrix producing cells differentiate into spore forming cells (Guttenplan & Kearns, 2013; Srinivasan et al., 2018; Vlamakis et al., 2013). *B. subtilis* serves as a model organism to study biofilm formation and cellular differentiation process. On agar surfaces, *B. subtilis* colonies exhibit a remarkable colony architecture.

In this Chapter, I have constructed a strain with fluorescent transcriptional reporters for cell motility, matrix production, and sporulation. Using these three-colored fluorescent reporter strain, I have studied the spatiotemporal gene regulation of three distinct cell types during the development of a biofilm colony. To this end, I have also constructed kinase mutants of the three color fluorescent reporter strain and compared the spatiotemporal gene regulation of cell motility, biofilm formation, and sporulation in these strains.

II.ii Materials and Methods

II.ii.1 Strains.

The parent strain for all experiments was the undomesticated and naturally competent DK1042ComI^{Q12L} (NCIB 3610). *B. subtilis* strains were constructed by transformation with either chromosomal DNA or plasmid DNA as described by Harwood & Cutting (Harwood and Cutting 1990). Standard recombinant DNA

techniques, including plasmid DNA construction and isolation using *Escherichia coli* DH5α were performed as described by Sambrook & Russell (Sambrook & Russell, 2001). All strains used in this study are described in Table II-1. Details of plasmid construction and oligonucleotide DNA primers used for PCR are described in Tables II-2 and II-3, respectively.

II.ii.2 Media and culture conditions.

Luria-Bertani medium (LB) was used for normal growth of *B. subtilis* (Sambrook & Russell, 2001). All experiments were performed in biofilm promoting MSgg medium [5 mM potassium phosphate, 100 mM MOPS (pH 7.0), 0.5% glycerol, 2 mM $MgCl_2.6H_2O$, 2 µM thiamine, 0.5% glutamate, 0.005% tryptophan, 0.005% phenylalanine, 700 µM $CaCl_2.2H_2O$, 50 µM $MnCl_2.4H2O$, 50 µM $FeCl_3.6H_2O$, and 1 µM $ZnCl_2$] (Branda, Gonzalez-Pastor, Ben-Yehuda, Losick, & Kolter, 2001). Strains harboring reporter genes at the *thr locus* were supplemented with 1 mg/ml of L-Threonine in the MSgg medium.

All biofilm colonies were grown on MSgg medium containing 1.5% agar. Approximately 25 ml MSgg media was poured into 9 mm diameter pertridish. Plates were prepared 24-48 h prior to the day of experiment and air dried for approximately 1 h before use. Cells were pre-cultured in nutrient-rich LB at 37 °C until an OD_{600} of 0.3 was reached. Cells were then serially diluted $1:10^{-8}$ in 1X T-base [15 mM $(NH_4)_2SO_4$, K_2HPO_4] (Harwood & Cutting, 1990)and 100 µl of culture was spread on an MSgg agar plate with glass beads. Plates were then incubated at 30 °C for 24-66 h.

II.ii.3 Image acquisition and data analysis

All strains harboring the three transcriptional fusion reporters were grown on MSgg agar as described above. Once the colonies become visible, camera images were acquired. After that the agar pad surrounding the colony was carefully cut and put on a glass slide. The colony was then observed using a 2X and 100X microscope objective (Olympus, model BX61). Images were taken every 2 h.

For colony resuspension assays, cells were taken from three different zones, designated as the peripheral (P), medial (M), and central (C) zones from the outside to the inside of the colony, resuspended in 1X T-base. The cell suspension was placed on an agarose pad containing 1X T-base and then observed under 100X magnification. The microscope system control and image processing were performed using SlideBook image analysis software (Intelligent Imaging Innovations, Inc.).

Table II-1. List of *B. subtilis* strains

Strain #	Genotype	Reference
MF8187	ΔkinA::tet comI^{Q12L} thrC::Phag-mCherry erm amyE::PtapA-cfp spc spoIIAΩPspoIIA-iyfp cm	This study
MF8188	ΔkinB comI^{Q12L} thrC::Phag-mCherry erm amyE::PtapA-cfp spc spoIIAΩPspoIIA-iyfp cm	This study
MF8189	ΔkinC comI^{Q12L} thrC::Phag-mCherry erm amyE::PtapA-cfp spc spoIIAΩPspoIIA-iyfp cm	This study
MF8196	comI^{Q12L} thrC::Phag-mCherry erm amyE::PtapA-cfp spc spoIIAΩPspoIIA-iyfp cm	This study
MF8730	amyE::PtapA-cfp spc thrC::Phag-mCherry erm rpoCΩrpoC-yfp kan comI^{Q12L}	This study

Table II-2. List of plasmids

Plasmid	Description	Reference
pDR244	temperature-sensitive plasmid with constitutively expressed Cre recombinase	(Koo et al., 2017)
pMF506	*rpoC-yfp spc*	This study
pMF642	*rpoC-yfp*	This study
pMF829	amyE::PtapA-cfp spc	This study
pMF875	thrC:: Phag-mcherry erm	This study

Table II-3. List of oligonucleotide primers

Primers	Sequence
om50	5'-gccaagcttacataaggaggaactact atggtttcaaaaggcgaagaactg-3'
om52	5'-gcgggatccttacttataaagttcgtccatgccaag-3'
om87	5'-gccaagcttacataaggaggaactact atggtcagcaagggagaggaagat-3'
om88	5'-gccggatccttatttgtataattcgtccattccacctgt-3'
om212	5'-cgcgaattcatgctgtcacccttttcttt-3'
om213	5'-cgcaagcttcttgatatgacaatcgttctctttaaagaac-3'
om329	5'-atggtcagcaagggagaggaagataatatg-3'
om330	5'-ttatttgtataattcgtccattccacctgt-3'
om381	5'-ggcgaattcggaattgacgccccaaagcatattg-3'
om382	5'-ggcaagcttgttgttaaggcacgtccttgtgccc-3'

II.iii Results

II.ii.4 Construction of a three color fluorescent reporter strain to visualize three distinct cell differentiation types in *B. subtilis*

A wild type strain, MF8196 (comI^{Q12L} thrC:: Phag-mCherry erm amyE::PtapA-cfp spc spoIIAΩPspoIIA-iyfp cm) was constructed in which three distinct cell types- motile cells, biofim forming cells, and sporulating cells could be visualized in a single biofilm forming *B. subtilis* strain. In this strain, *flagellin* expresssion in motile cells is reported by red flourescent protein mcherry, expresion of the matrix producing gene *tapA* is reported by cyan flourescent protein (CFP), and expression of the sporulating gene *spoIIA* is reported by yellow flourescent protein (YFP). Thus , by the use of this strain, cell motility, biofilm formation, and sporulation can be observed simultaneously in a growing biofilm colony of *B. subtilis* . Kinase mutants of the triple color wild-type strain were also constructed in which either *kinA*, *kinB,* or *kinC* were deleted. The wild-type strain and its kinase mutants were grown (as described in Materials and Methods) simultaneously and differences in gene expression profiles of the three distinct cell types were compared using flourescence microscopy.

II.ii.5 Measurement of gene expression at colony level

One of the salient features of biofilm colony development in *B. subtilis* is the differentiation of genetically identical cells into distinct cell types (Lemon, Earl, Vlamakis, Aguilar, & Kolter, 2008; van Gestel, Vlamakis, & Kolter, 2015). The

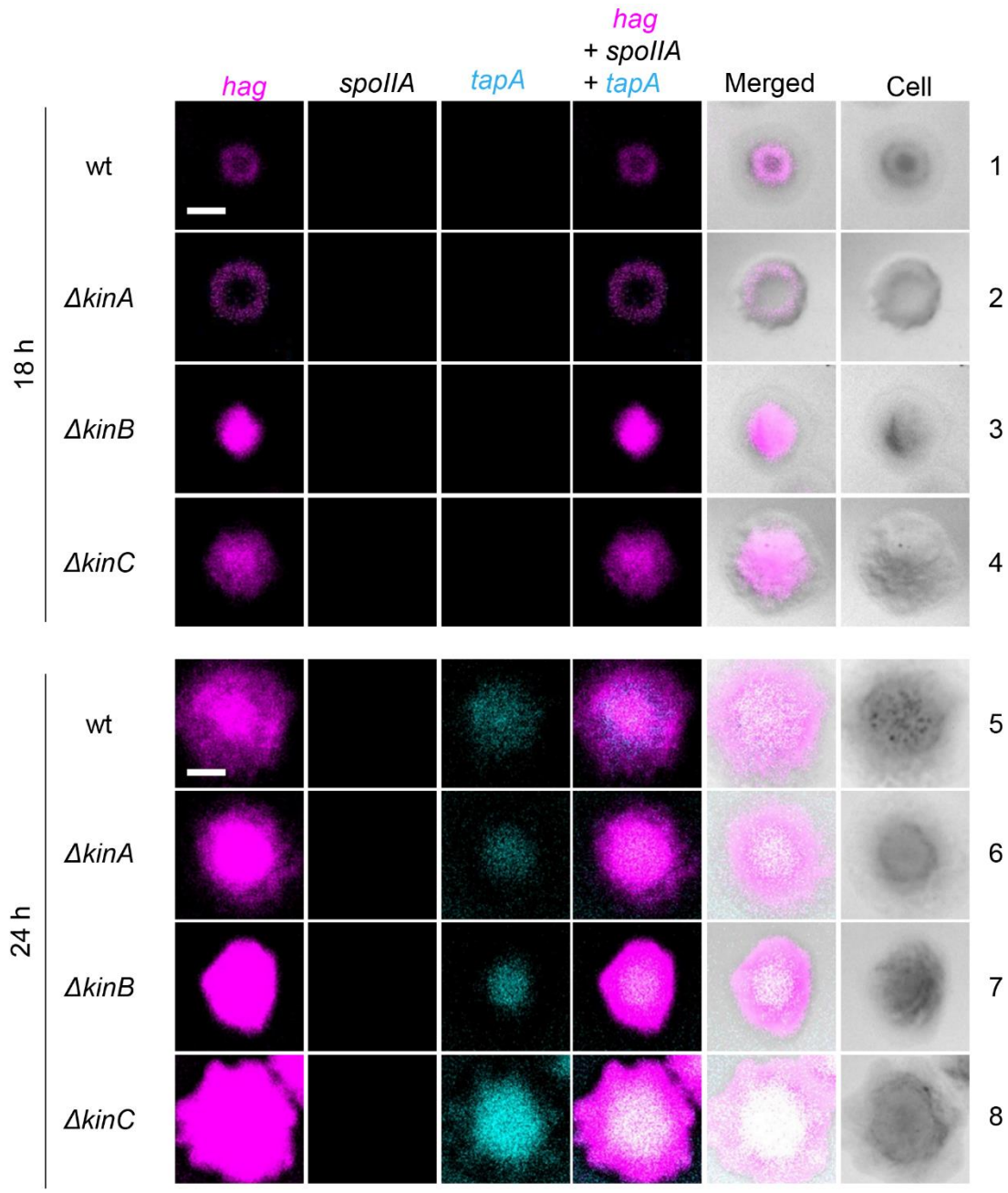


Figure II-1 Comparison of fluorescent reporters for motility (*Phag-mcherry*), sporulation (*PspoIIA-yfp*), and biofilm formation (*PtapA-cfp*) during *B. subtilis* biofilm colony development at 18 h and 24 h.
Cells expressing fluorescent reporters for motility, sporulation, and biofilm formation from the wild-type (wt) and various kinase mutants (*ΔkinA*, *ΔkinB*, and *ΔkinC*) were grown on MSgg agar plates. Images were taken at indicated time points with a 2X microscope objective lens. Scale bar, 1 mm.

subpopulations of these distinct cell types are spatiotemporally distributed within the biofilm and contribute towards the colony development. In this study, the spatiotemporal distribution of three distinct cell types-motile cells expressing the *flagellin/hag* gene, matrix producing cells expressing *tapA*, and sporulating cells expressing *spoIIA* was studied using flourescence microscopy.

Spatiotemporal expression and regulation of these distinct cell types was studied in *B. subtilis* biofilm colonies from 18-66 h of colony development by the use of three independent microscopic analysis-colony level, single-cell level, and resuspensions from different parts of colony. The colony level analysis was performed by using a 2X microscope objective, while single-cell and colony resuspensions were examined using 100X oil objective lens.

The biofilm colony development was divided into three stages-initail phase (18-24 h), mid phase (24-48 h), and late phase (48-66 h).

Flourescence microscopy analysis as shown in Figure II-1, panels 1-4, exihibited that in the initial phase of colony development at 18 h, the reporter for motility-*hag* was expressed in the wild type and the kinase mutants strains However, the reporters for matrix production-*tapA* and sporulation-*spoIIA* were not expressed. These results suggested that *hag* expression preceeded *tapA* and *spoIIA* expression during the development of a biofilm colony. These results further strengthened the notion that among the three distinct cell fates, motile cells are the vanguards for biofilm colony development. Notably, *hag* expression was localized in the central part of the colony but not at the peripheral region.

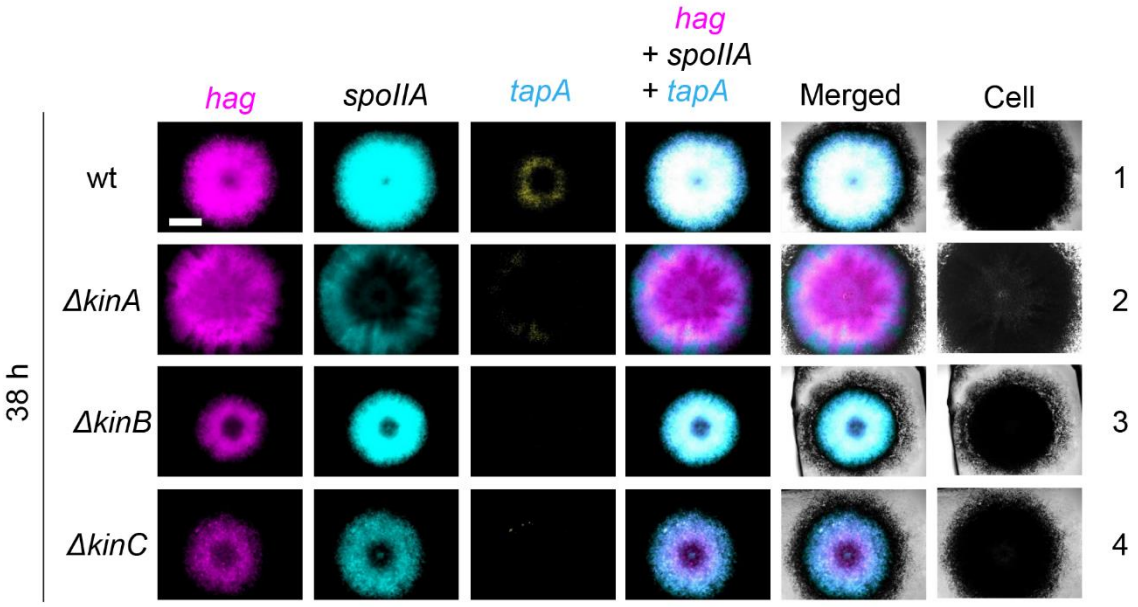


Figure II-2 Comparison of fluorescent reporters for motility (Phag-*mcherry*), sporulation (*PspoIIA-yfp*), and biofilm formation (*PtapA-cfp*) during *B. subtilis* biofilm colony development at 38 h.
Cells expressing fluorescent reporters for motility, sporulation, and biofilm formation from the wild-type (wt) and various kinase mutants (*ΔkinA*, *ΔkinB,* and *ΔkinC*) were grown on MSgg agar plates. Images were taken at indicated time points with a 2X microscope objective lens. Scale bar, 1 mm.

However, at 24 h of colony development, the reporter for matrix production-*tapA* was found to be expressed in the wild type as well as in the kinase mutant strains in addition to the motility reporter (Figure II-1, panels 5-8). Reporter for sporulation gene *spoIIA* was not found to be expressed at 24 h of colony development. Similar to *hag* expression, *tapA* expression was also localized in the central part of the colony, and its expression was excluded from the colony periphery.

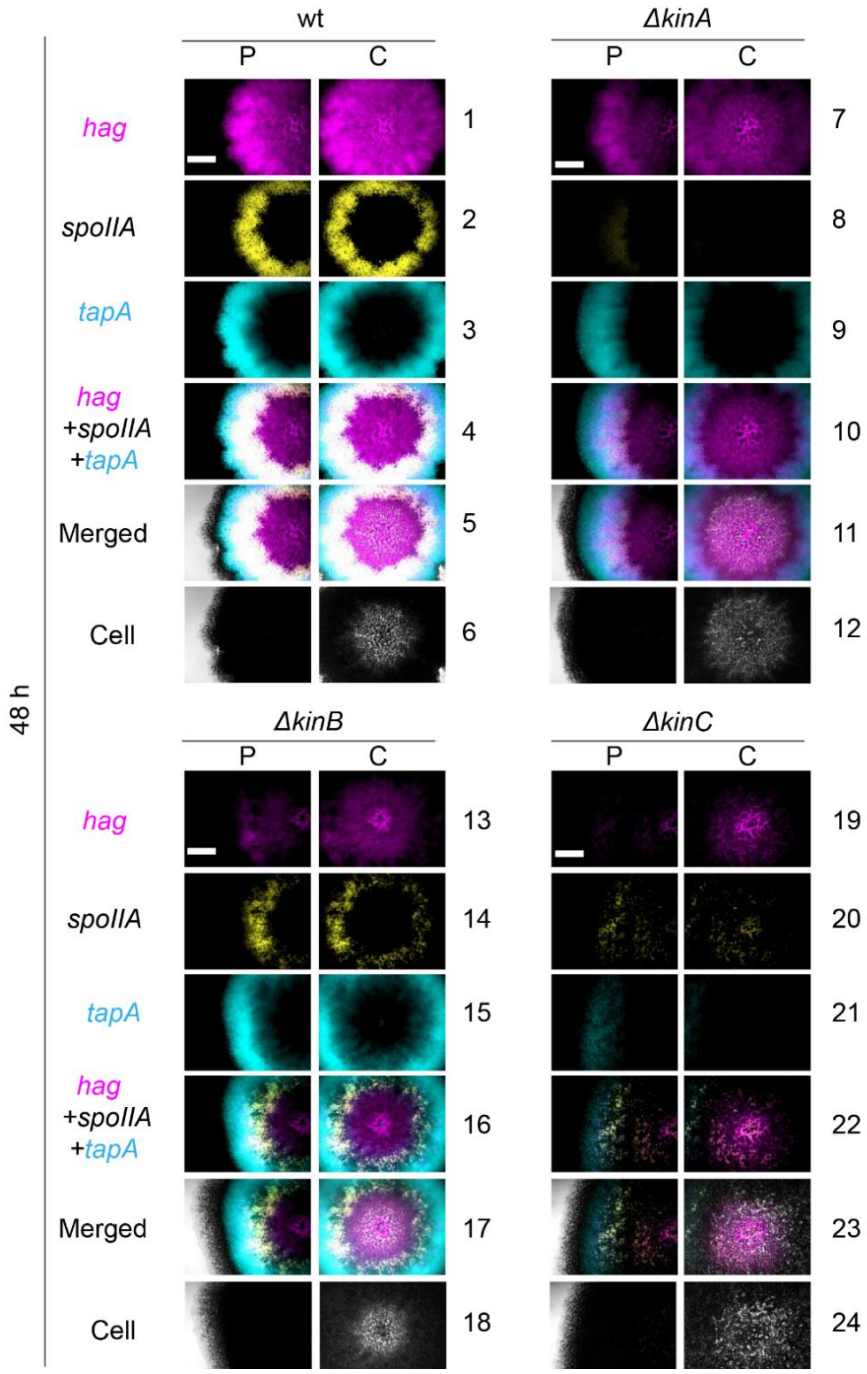


Figure II-3 Comparison of fluorescent reporters for motility (*Phag-mcherry*), sporulation (*PspoIIA-yfp*), and biofilm formation (*PtapA-cfp*) during *B. subtilis* biofilm colony development at 48 h.

Cells expressing fluorescent reporters for motility, sporulation, and biofilm formation from the wild-type (wt) and various kinase mutants (*ΔkinA*, *ΔkinB*, and *ΔkinC*) were grown on MSgg agar plates. Images were taken at indicated time points with a 2X microscope objective lens. Scale bar, 1 mm.

During the middle stage of colony development (38 h) , as shown in Figure II-2, panels 1-4, reporter activity for the sporulation gene, *spoIIA* was only seen in the wild type strain and not exhibited by any kinase mutants. Thus, at 38 h of colony development, only the wild type strain exhibited the expression of all three distinct cell types-motile cells, matrix producing cells and sporulating cells.

As shown in Figure II-3, panels 1-6, the wild type strain continued to express all three cell types. At 48 h of colony development, the *ΔkinB* deletion strains revealed *spoIIA* gene expression (Figure II-3, panel 14), similar to the wild type strain (Figure II-3, panel 2) in the form of a ring like structure localized in the medial part of the colony . However, in the *ΔkinC* deletion strain , low-level of *spoIIA* expression can be seen throughout the entire colony as shown in Figure II-3, panel 20. As mentioned earlier, the *kinA* is the major sporulation kinase, thus, as expected, the *ΔkinA* deletion strain did not demonstrate *spoIIA* expresison (Figure II-3, panel 8). Spatial localization for matrix production reporter-*tapA* was found to be analogous to sporulation reporter-*spoIIA,* in the form of a ring like structure in the medial part of colony, excluding the central most part of the colony. At late stages of colony development, reporters for motility and matrix production (*hag* and *tapA)* were expressed in the wild type strain while the sporulation reporter-s*poIIA* was not expressed at 62 h (Figure II-4, panels 1-6). Also, the spatial regulation of *hag* expressing cells altered from the earlier stages of colony development. At the later stages of colony development, *hag* expressing cells were confined to the central part of the colony, as compared to being present in the

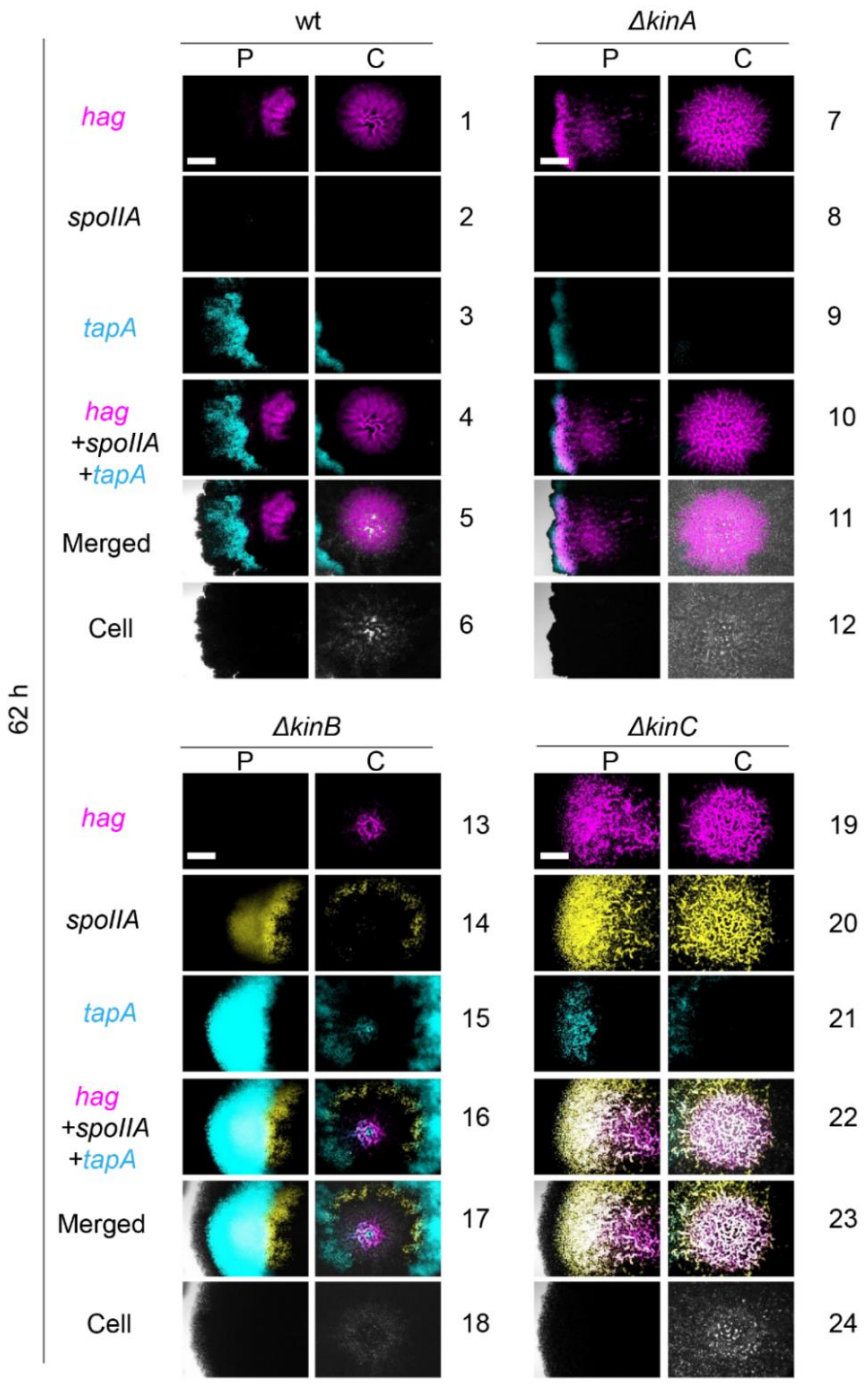


Figure II-4 Comparison of fluorescent reporters for motility (*Phag-mcherry*), sporulation (*PspoIIA-yfp*), and biofilm formation (*PtapA-cfp*) during *B. subtilis* biofilm colony development at 62 h.

Cells expressing fluorescent reporters for motility, sporulation, and biofilm formation from the wild-type (wt) and various kinase mutants (*ΔkinA*, *ΔkinB*, and *ΔkinC*) were grown on MSgg agar plates. Images were taken at indicated time points with a 2X microscope objective lens. Scale bar, 1 mm.

entire colony during the earlier stages of biofilm formation (Figure II-4, panels 1, 7, 13, and 19). In the *ΔkinB* deletion strain, *hag* expressing cells were mainly confined to the central most part of the colony (Figure II-4, panel 13). In contrast, the *ΔkinA* and *ΔkinC* strains displayed motile cells throughout the entire colony (Figure II-4, panels 7 and 19). The sporulation reporter-*spoIIA* was only expressed in the *ΔkinB* and *ΔkinC* strains (Figure II-4, panels 14 and 20). The spoIIA expression patterns in the *ΔkinB* and *ΔkinC* strains in the late stages of biofilm development followed the same expression patterns as the mid stage of colony development. Matrix production reporter–tapA was found to be preferentially localized towards the colony periphery in the wild type as well as the kinase mutant strains (Figures II-3 and II-4, panels 8 and 20).

Motile cells can be seen within 18 h (Figure II-2, panels 1-4), localized to central part of colony, and later at 36-48 h, they can be seen throughout the colony (Figure II-3, panels 1-4). At 62 h, motile cells were confined to the center most of the colony (Figure II-4, panels1, 7, 14, and 20). Matrix producing cells can be seen at 24 h of colony formation, localized to the central part of colony (Figure II-1, panels 5-8). These cells can be seen though out the colony at 36-48 h (Figure II-2, panels 1-4), and later restricted to the peripheral region of the colony at 62 h (Figure II-4, panels 3, 9, 15, and 21). Sporulating cells can only be seen at 38 h of colony development in the form of a ring in the medial part of the colony excluding the central part of the colony (Figure II-2, panel 1). Their expression pattern remained same until 62 h of biofilm formation (Figure II-4, panels 2, 8, 14, and 20). The results presented here are in stark contrast to a similar study (X. Wang, Meng, &

Han, 2017) which also investigated spatiotemporal gene regulation in *B. subtilis* biofilms. Their results indicated that motile cells were localized in the middle of colony, matrix producing cells were present at the colony periphery, and sporulating cells could be seen though out the colony except for the center.

Another recent report (Srinivasan et al., 2018), suggested that motile cells were mainly present in the central part of colony. Matrix producing cells were also expressed throughout the colony, but their expression was mainly localized to the peripheral region of the colony. Sporulating cells were throughout the colony.

II.ii.6 Technical difficulties in image acquisition and data analysis

As shown in all 2x microscopy images above (Figures II-1, II-2, II-3, and II-4), no gene expression could be visualized at the edges of the colony. This prompted us to identify whether this was due to the nature of biofilm colony development, or whether it was a technical limitation of the 2X microscopic analysis. In order to resolve the issue, a triple-color fluorescent strain (MF8730), in which constitutively expressed βeta-subunit of RNA Polymerase C (*rpoC)* tagged with YFP, matrix producing cells tagged with CFP, and motile cells tagged with mcherry was utilized. 2X microscopy revealed no gene expression at colony periphery (Figure II-5, 2X, panels 1-6).

In contrast, 100X analysis of the same colony revealed the expression of *rpoC* at the colony periphery as well as in the other parts of the colony (Figure II-5, 100X,

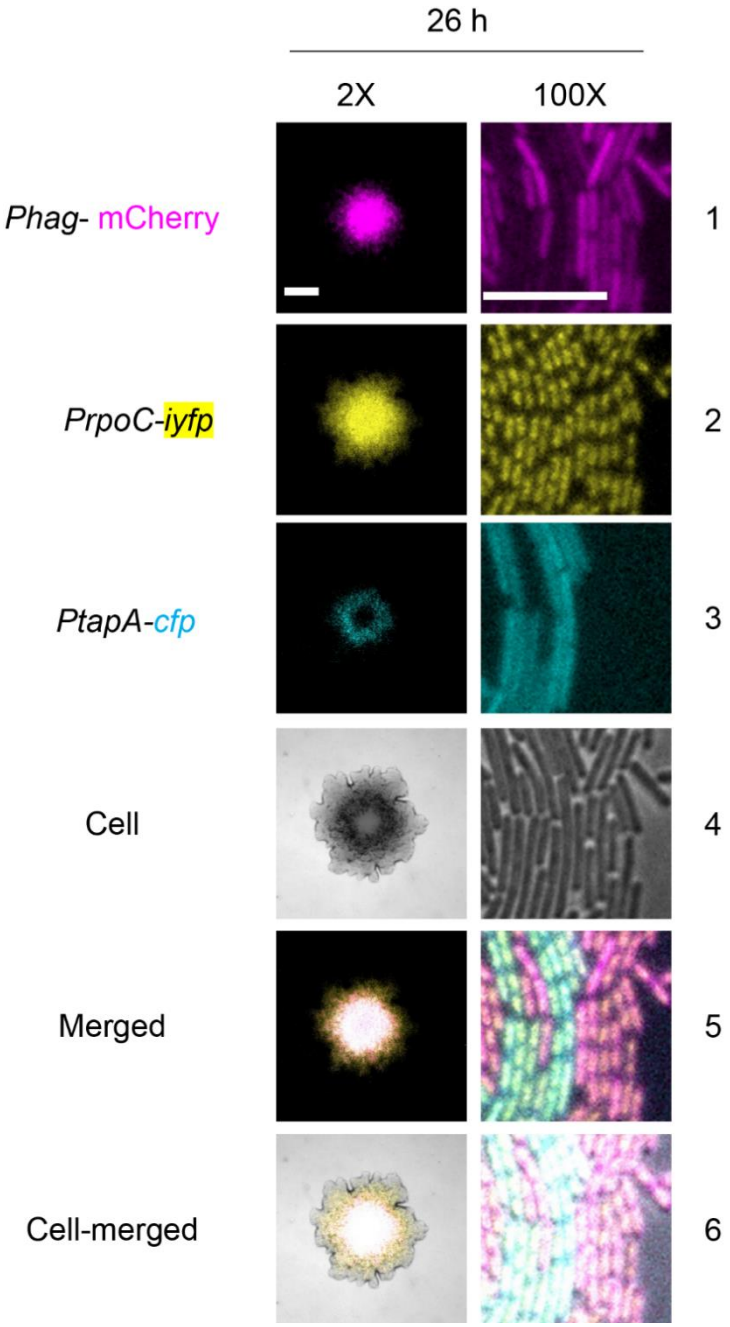


Figure II-5 Comparison of fluorescent reporters for motility (*Phag-mcherry*), RNA Polymerase C (*PrpoC-yfp*), and biofilm formation (*PtapA-cfp*) at colony level and single cell level during the development of a *B. subtilis* biofilm colony.
Cells expressing fluorescent reporters for motility, sporulation, and biofilm formation from the wild-type (wt) and various kinase mutants (*ΔkinA*, *ΔkinB*, and *ΔkinC*) were grown on MSgg agar plates. Images were taken at 26 h at colony level (2X) and single cell level (100X). Scale bar, 1 mm for colony level and 2 µm for single cell level.

panels 1-6). Thus, initially perceived lack of gene expression at the colony periphery was a technical limitation of 2X microscopic analysis. Since the cells within the biofilm were actively growing, only the cells at colony periphery were found in a monolayer, while the rest of the colony contained multilayered structures. Gene expression signals, visualized by 2X microscopy, depended on the number of cells in which the signal was expressed as well as the intensity of signal in those cells. We speculated that the number of cells accumulated within the monolayer did not have enough signal intensity to be visualized by 2X microscopy.

The second challenge faced during these analyses was heterogeneity in the colony sizes. At any given time point, there were several different colony sizes (Figure II-6, panels 1-8) and, thus, different spatiotemporal gene regulation in the colonies. For example, as seen in Figure II-3, (panel 2) at 48 h of colony development, the reporter for sporulation gene *spoIIA* was seen in the form of a ring like structure in the median part of colony. This ring like expression of s*poIIA* disappeared at 62 h of colony development (Figure II-5, panel 3). This suggested that the sporulation reporter was not expressed at 62 h. However, when colony resuspension analysis was done, it was found that sporulation reporter *spoIIA* was expressed in the median and central part of the colony (Figure II-11). Another issue faced during data analysis of the 2X microscopic observations of the colony was to differentiate between the real and false positive signals. As the colony grew older, matrix production increased and had auto-fluorescence.

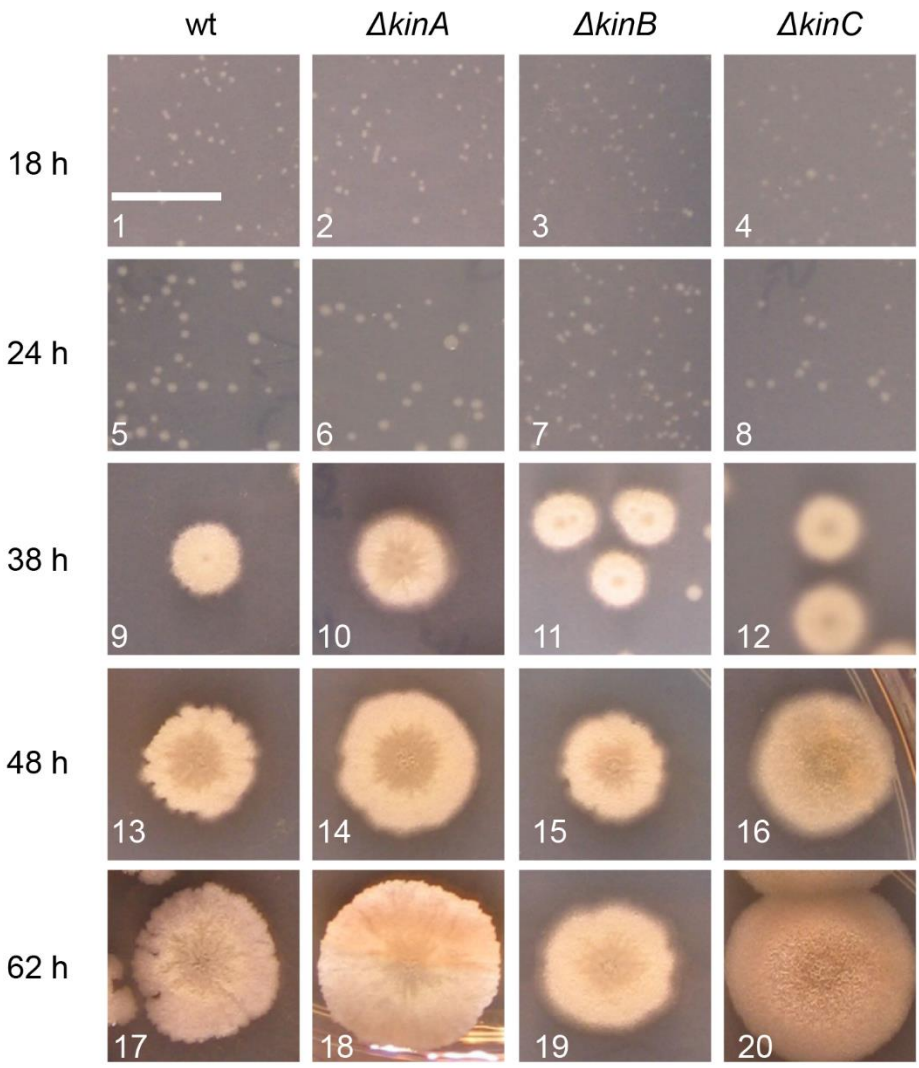


Figure II-6 Comparison of colony morphologies of the wild type (wt) and various kinase mutants.

Cells expressing fluorescent reporters for motility, sporulation, and biofilm formation from the wild type (wt) and various kinase mutants (*ΔkinA*, *ΔkinB*, and *ΔkinC*) were grown on MSgg agar plates. Camera images were taken at indicated time points. Scale bar, 10 mm.

In order to overcome this issue, a wild type strain with no reporters (MF5609) was used as a negative control. The signal intensities obtained from the no reporter strain for the three fluorescent reporters-mcherry, CFP, and YFP were subtracted from the signal intensities of the strain containing these reporters to obtain the real intensity.

II.ii.7 Measurement of gene expression at single cell level

To visualize the spatiotemporal gene regulation at single cell level in developing biofilm colonies, microscopic observations were performed with the help of a 100X oil objective. A rectangular piece of agar containing the colony was cut and placed on a glass slide. The colony was then covered with a poly-L-lysine treated glass coverslip and observed under 100X oil objective lens. As mentioned earlier, cells found at the edges of the colony were in monolayer, and the rest of the colony was formed with multilayered structures. Through the use of current 100X oil objective lens, it was not possible to decipher the spatiotemporal gene regulation at single cell level within these multilayered structures. Thus, the results presented here pertain to single cell analysis at the peripheral region of the colony only.

The single cell analysis of colony edges at 38 h of colony development revealed that the reporters for motility and matrix production were localized at the edges of the colony while the sporulation reporter could not be visualized (Figure II-7). Heterogeneity in expression of different fluorescent reporters was also observed. Only a subset of entire population expressed matrix producing cells while another subset expressed motile cells.

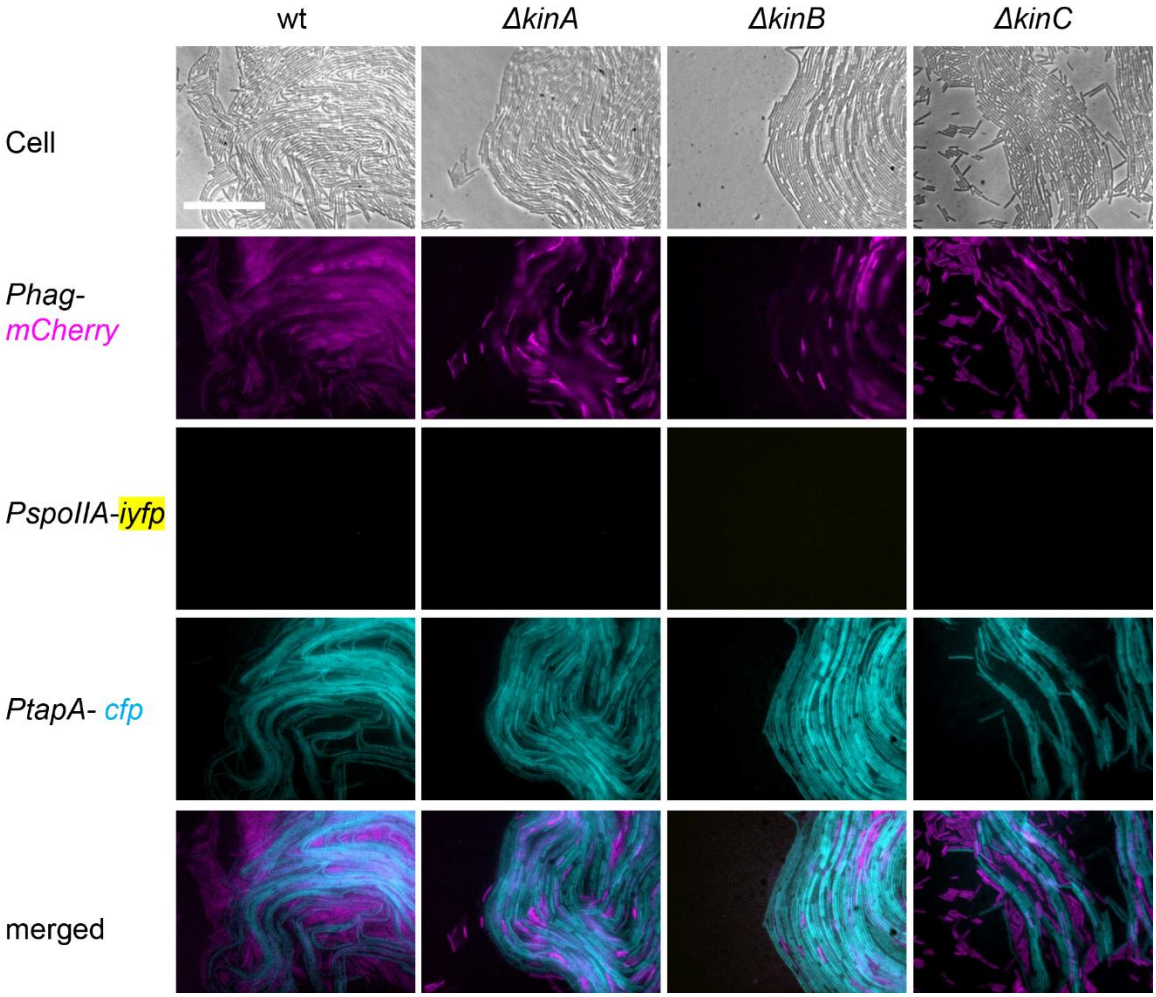


Figure II-7 Comparison of fluorescent reporter expression profiles at the peripheral regions of the colony in the wild type and various kinase mutant strains.
Cells expressing fluorescent reporters for motility (*Phag-mcherry*), sporulation (*PspoIIA-yfp*), and biofilm formation (*PtapA-cfp*) from the wild-type (wt) and various kinase mutants (*ΔkinA*, *ΔkinB*, and *ΔkinC*) were grown on MSgg agar plates. Images were taken at 24 h at the peripheral region of the colony with the help of a 100X oil objective lens. Scale bar, 10 µm.

A striking characteristic of biofilm formation-cell chaining, can also be visualized at the edges of the colony (Figure II-7). The wild-type, *ΔkinA*, and *ΔkinB* strains had similar levels of cell chaining. In contrast, the *ΔkinC* strain exhibited decreased cell chaining, as it is deficient in biofilm formation. Another noteworthy observation was that the expression of the reporters for cell motility and biofilm formation were mutually exclusive. We also observed that *tapA* was preferentially expressed in chaining cells, while *hag* expression did not require cell chaining and was expressed in single cells. During the course of experiments studied in this chapter, *tapA* and *hag* were not found to be co-expressed within the same cell.

II.ii.8 Analyzing spatiotemporal gene regulation of motility, matrix production and sporulation in developing biofilm colonies by colony resuspension assay

As mentioned earlier, a developing biofilm colony is made up of multilayered structures. In order to understand spatiotemporal gene regulation within the biofilm colony, it was essential to dissect these multilayered structures. It was not possible to obtain an in-depth analysis of these multilayered structures using our 100x oil objective lenses. So, to overcome the limitation, a novel technique was developed. In this technique, the biofilm colony was hypothetically divided into three different zones-peripheral (P), medial (M), and central (C) from outside to inside of colony. Some cells were scratched from these three regions and resuspended in buffer.

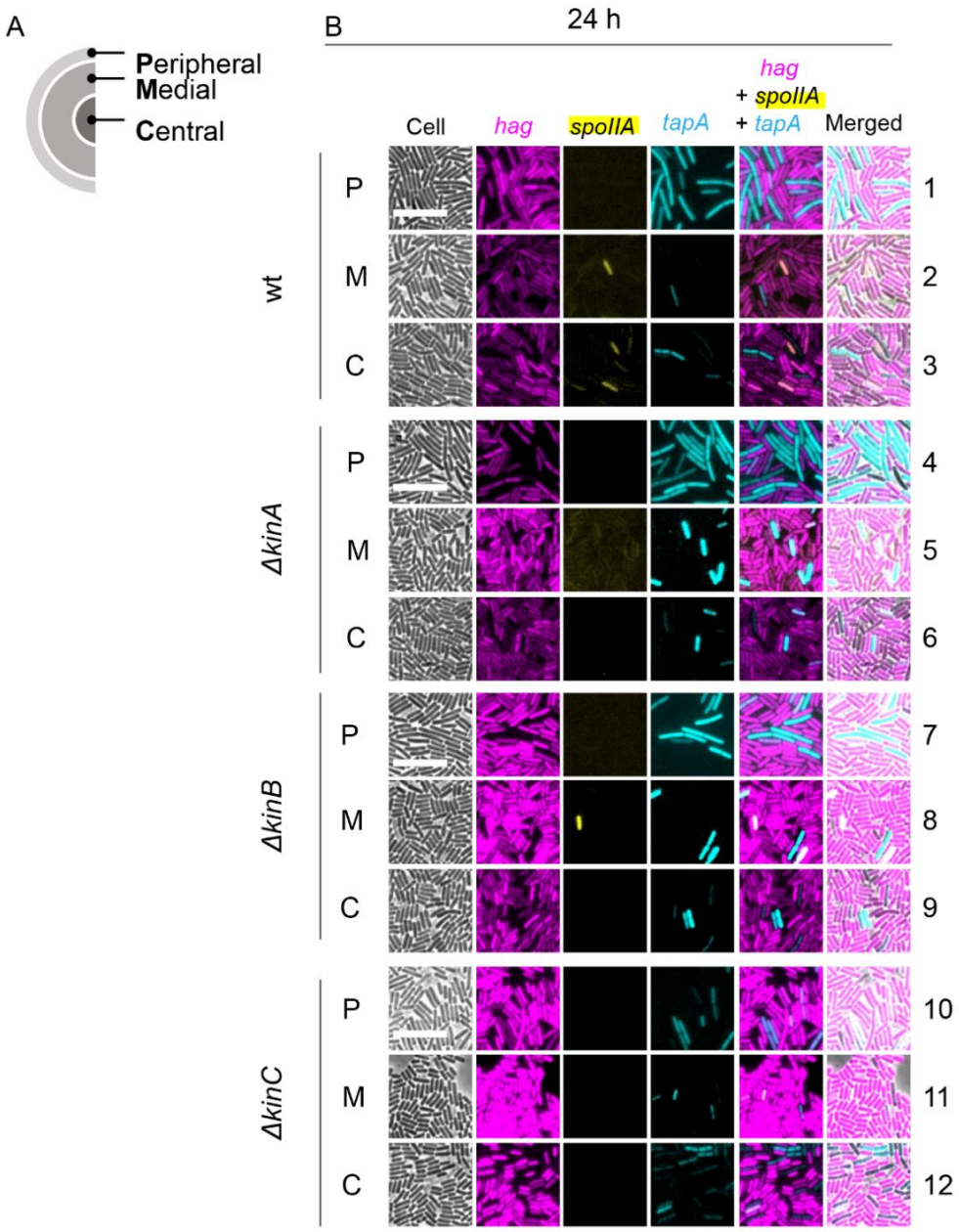


Figure II-8 Comparison of fluorescent reporters for motility, sporulation, and biofilm formation during *B. subtilis* biofilm colony development at 24 h.
Cells expressing fluorescent reporters for motility (*Phag-mcherry*), sporulation (*PspoIIA-yfp*), and biofilm formation (*PtapA-cfp*) from the wild-type (wt) and various kinase mutants (*ΔkinA*, *ΔkinB*, and *ΔkinC*) were grown on MSgg agar plates. (A) Schematic representation of colony architecture. Colonies grown on MSgg agar plates were divided into three concentric zones-peripheral (P), media (M), and central (C). Cells taken from the three designated zones of MSgg colonies were observed under the fluorescent microscope at 24 h. Scale bar, 10 μm.

The multilayered structures within these cells were disrupted by vigorously vortexing the cells. Cells were then observed under 100x oil objective. Thus, this technique made it possible to dissect these multilayered structures and analyze the spatiotemporal gene regulation within these structures.

The spatiotemporal regulation for cell motility, matrix production, and sporulation was analyzed from 24-66 h of colony development. Before 24 h, the colony size was small and the peripheral, medial, and central regions of the colony were not distinguishable.

At 24 h of colony development, it was observed that the expression of motility reporter in the wild type as well as in all the kinase mutant strains was seen throughout the colony (Figure II-8, panels 1-12). The reporter for matrix production was also observed throughout the colony. It was expressed to a similar extent in the wild type, *ΔkinA*, and *ΔkinB* mutant strains (Figure II-8, panels 1-9). Since *ΔkinC* strain is deficient in biofilm formation, as expected, fewer matrix producing cells were seen (Figure II-8, panels 10-12). The matrix producing cells were also seen towards the peripheral region of the colony (Figure II-8, panels 1, 4, 7, and 10). The reporter for sporulation was mainly observed in the medial and central parts of colony, excluding the peripheral region. At 24 h, low level expression of the sporulation reporter was observed only in the wild type and *ΔkinB* strain (Figure II-8, panels 2, 3, and 8).

At 36 h of colony development, all strains expressed the reporters for motility, matrix production, and sporulation (Figure II-9, panels 1-12). The reporter for

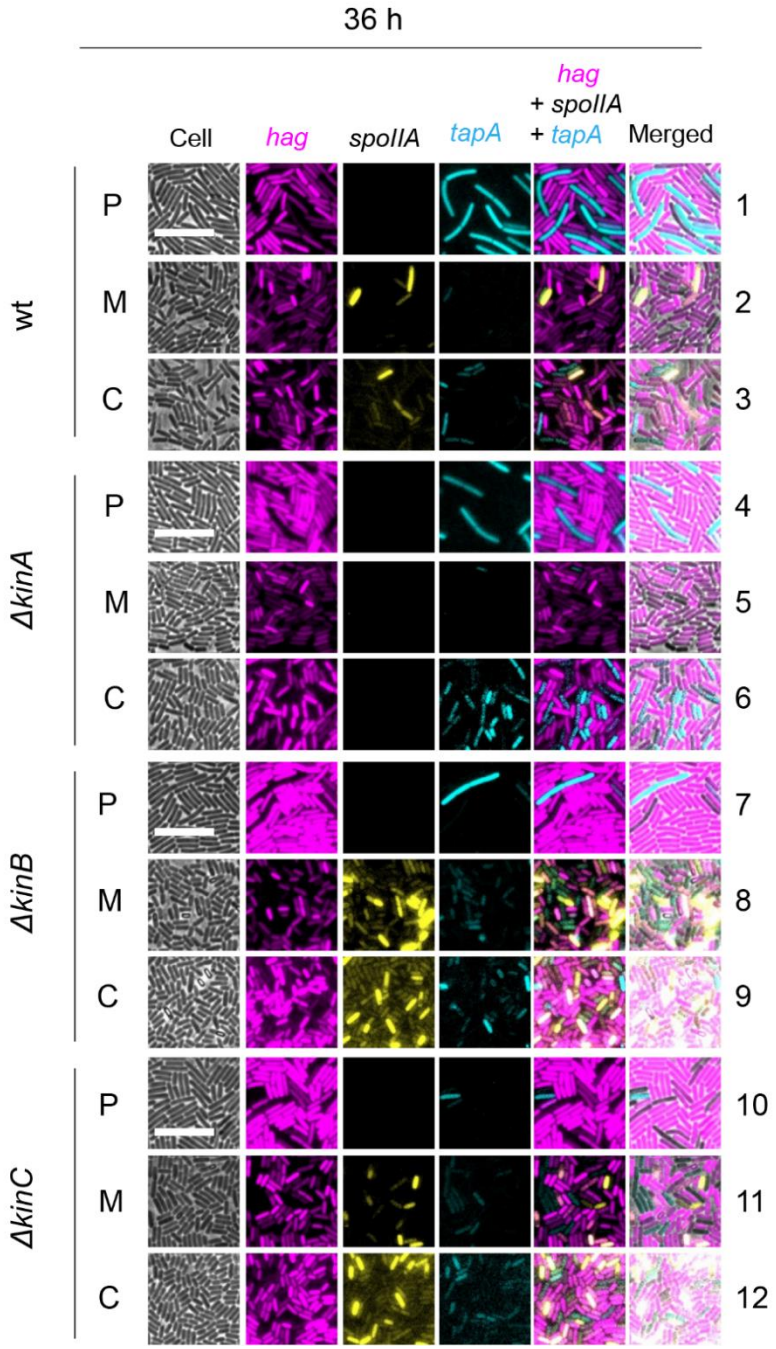


Figure II-9 Comparison of fluorescent reporters for motility, sporulation, and biofilm formation during *B. subtilis* biofilm colony development at 36 h.
Cells expressing fluorescent reporters for motility (*Phag-mcherry*), sporulation (*PspoIIA-yfp*), and biofilm formation (*PtapA-cfp*) from the wild-type (wt) and various kinase mutants (*ΔkinA*, *ΔkinB*, and *ΔkinC*) were grown on MSgg agar plates. Colonies grown on MSgg agar plates were divided into three concentric zones-peripheral (P), media (M), and central (C). Cells taken from the three designated zones of MSgg colonies were observed under the fluorescent microscope at 36 h. Scale bar, 10 μm.

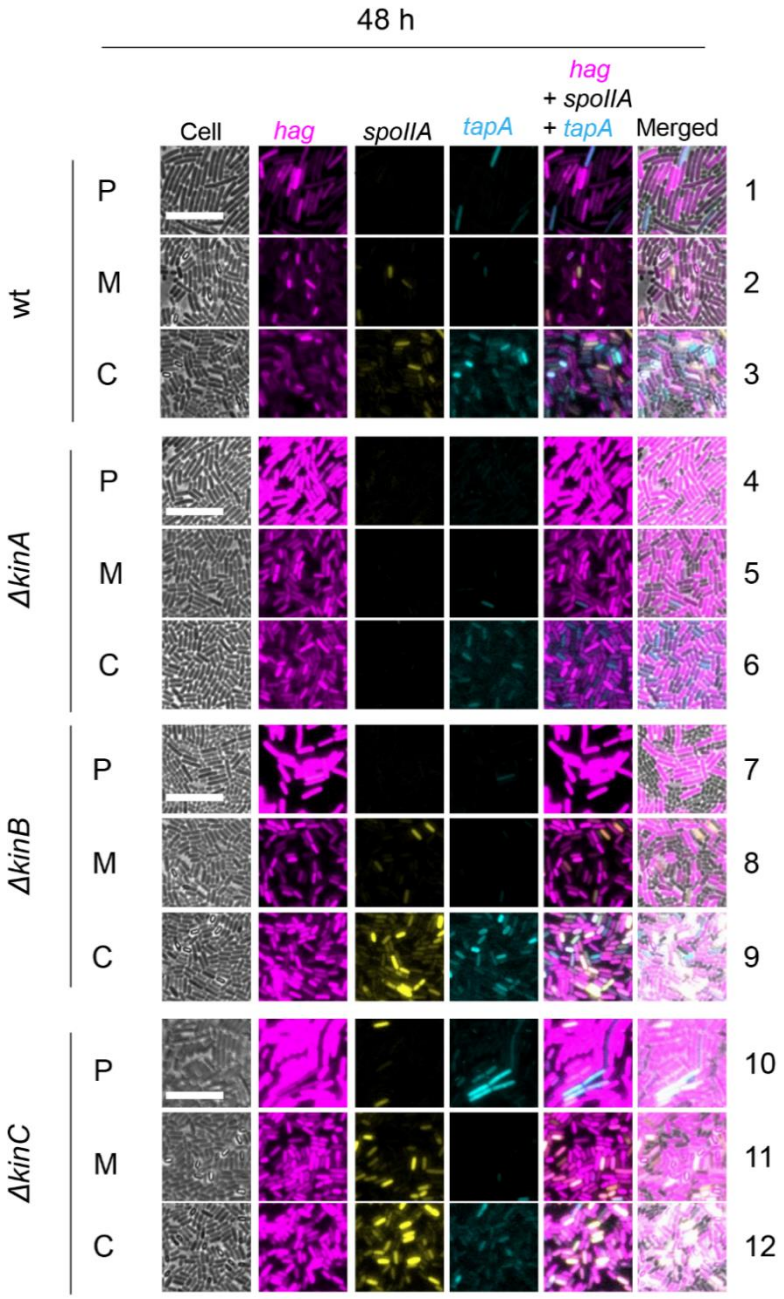


Figure II-10 Comparison of fluorescent reporters for motility, sporulation, and biofilm formation during *B. subtilis* biofilm colony development at 48 h.
Cells expressing fluorescent reporters for motility (*Phag-mcherry*), sporulation (*PspoIIA-yfp*), and biofilm formation (*PtapA-cfp*) from the wild-type (wt) and various kinase mutants (*ΔkinA*, *ΔkinB*, and *ΔkinC*) were grown on MSgg agar plates. Colonies grown on MSgg agar plates were divided into three concentric zones-peripheral (P), media (M), and central (C). Cells taken from the three designated zones of MSgg colonies were observed under the fluorescent microscope at 48 h. Scale bar, 10 μm.

motility can be visualized throughout the colony. A large subset of the population expressed the motility reporter in all the strains. The reporter for matrix production was mainly visible at the peripheral regions of the colony in the wild type, *ΔkinA*, and *ΔkinB* strains (Figure II-9, panels 1, 4, and 7). The *ΔkinC* strain had an overall low level of expression for the matrix production reporter (Figure II-9, panels 10-12). The reporter for sporulation was localized in the medial and central part of the colony. The sporulation reporter as well as the phase bright spores were only expressed in the wild type (Figure II-9, panels 2, and 3), *ΔkinB* (Figure II-9, panels 8, and 9) and *kinC* (Figure II-10, panels 11, and 12) strains. As *ΔkinA* strain is deficient in sporulation, the sporulation reporters were not expressed in this strain (Figure II-9, panels 4-6).

At 48 h of colony development, as the colony size increased and nutrients were exhausted, the motility reporter was expressed throughout the colony (Figure II-10, panels 1-12). A large subset of the population showed expression of the motility reporter in the *ΔkinA*, *ΔkinB,* and *ΔkinC* strains. However, in the wild type strain only a small subset of the population expressed the motility reporter (Figure II-10, panels 1-3). The reporter for matrix production showed an overall low-level expression and was localized at the peripheral and central regions of the colony (Figure II-10, panels 1, 3, 4, 7, 9, 10, and 12). As shown earlier (Figure II-9, panels 2, 3 8, 9, 11, and 12), the reporter for sporulation was only seen in the wild type, *ΔkinB*, and *ΔkinC* deletion strains (Figure II-10, panels 2, 3 8, 9, 11, and 12).

The wild type strain exhibited lower levels of expression of the sporulation reporter as compared to the *ΔkinB,* and *ΔkinC* deletion strains.

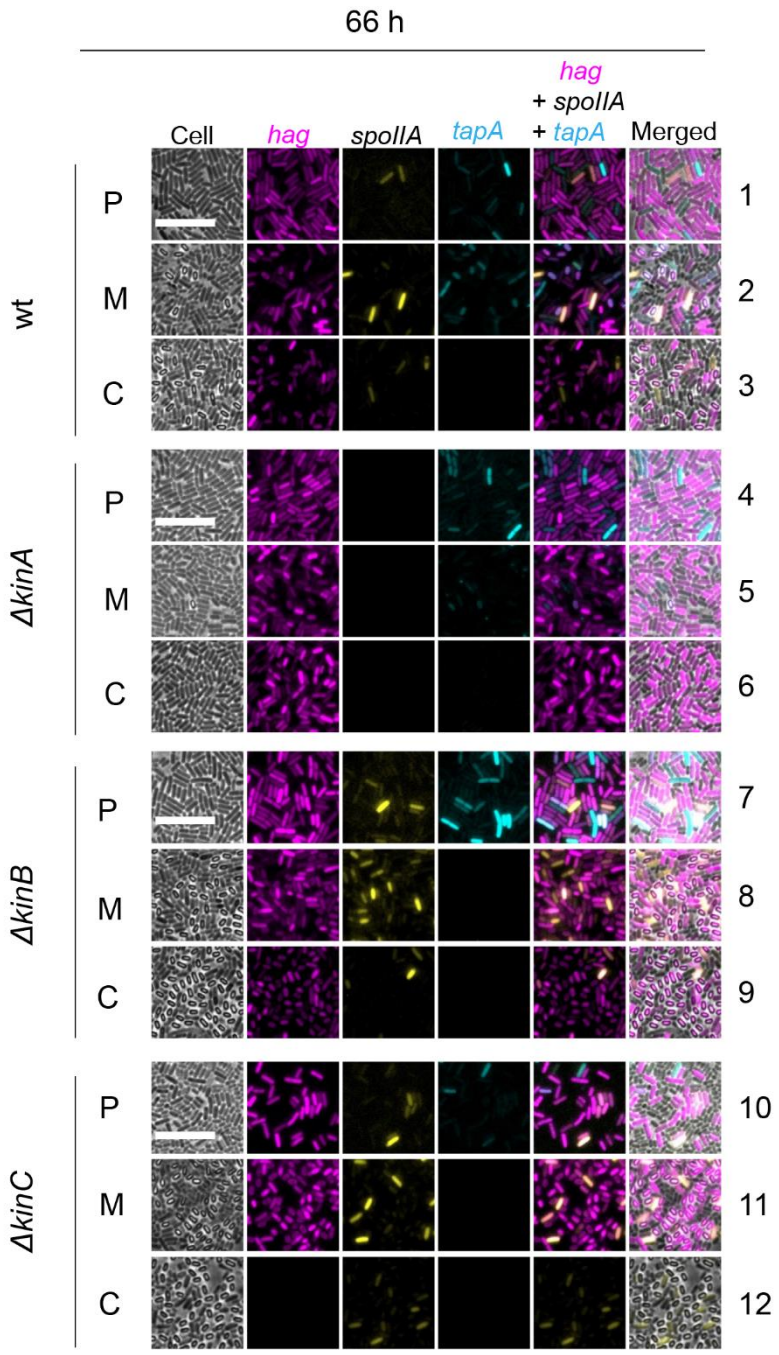


Figure II-11 Comparison of fluorescent reporters for motility, sporulation, and biofilm formation during *B. subtilis* biofilm colony development at 66 h.
Cells expressing fluorescent reporters for motility (*Phag-mcherry*), sporulation (*PspoIIA-yfp*), and biofilm formation (*PtapA-cfp*) from the wild-type (wt) and various kinase mutants (*ΔkinA*, *ΔkinB*, and *ΔkinC*) were grown on MSgg agar plates. Colonies grown on MSgg agar plates were divided into three concentric zones-peripheral (P), media (M), and central (C). Cells taken from the three designated zones of MSgg colonies were observed under the fluorescent microscope at 66 h. Scale bar, 10 μm.

The number of phase bright spores and released spores increased from 38-48 h. The *ΔkinC* strain showed the highest number of phase bright spores in the medial and central regions of the colony as compared to the wild type and *ΔkinB* strains (Figure II-10, panels 2, 3, 8, 9, 11, and 12). This suggested an alternative cell fate pathway for the strain in which *kinC* was deleted.

At late stages of colony development, at 66 h, the reporter for motility was seen throughout the colony (Figure II-11, panels 1-12). Matrix producing cells were mainly localized at the peripheral region (Figure II-11, panels 1, 4, 7, and 10) and either no expression or very low level of expression were observed at the medial and central parts of the colony. At 66 h, the reporter for sporulation was observed for the first time at the peripheral region in the wild type, *ΔkinB,* and *ΔkinC* strains along with its expression in the medial and central parts of the colony. Phase bright spores constituted a large subset of population in the medial and central parts of the colony.

II.iv Discussion

It has been known that various subpopulations expressing different cell types coexist within a developing biofilm colony. In this Chapter, I intended to determine the spatiotemporal gene regulation of three distinct cell types-motile cells, matrix producing cells, and sporulating cells in a biofilm colony by using a three color fluorescent reporter strain. The spatiotemporal gene expression was also compared between the wild type and various kinase mutants. The results presented in this Chapter are the first of its kind as all of the previous studies

focused on the spatiotemporal gene regulation in the wild type strain only. By the use of 2X microscopic analysis, it was found that the cell motility genes were the first set of genes to be expressed in the development of a biofilm colony. The results also demonstrated that the expression of motile genes preceded the expression of matrix production genes and sporulation genes. Sporulation genes are expressed in the late stages of colony development.

Technical difficulties were also experienced during the 2x microscopic analysis. Some of these technical difficulties were (1) no gene expression was observed at the colony periphery which was later attributed to the low cell numbers, and (2) heterogeneity in colony sizes which led to different spatiotemporal gene regulation in the colonies.

The single cell analysis revealed that cell motility and matrix production are mutually exclusive events. During the course of my experiments, no overlapping expression in the genes for cell motility and matrix production was observed. My results also indicated that cell chaining is essential for the expression of genes required for matrix production. KinC is suggested to be the main kinase for biofilm formation (Devi, Kiehler, et al., 2015). It was found that the *ΔkinC* strain was deficient in cell chaining and matrix production which further strengthened the important role of *kinC* during biofilm formation. A novel technique was utilized to study spatiotemporal gene regulation by the use of colony resuspension in developing biofilm colonies (as mentioned in Materials and Methods section). In all strains, the reporter for motility was expressed throughout the colony beginning at 24 h of colony development (Figure II-12) and continued until 66 h of colony

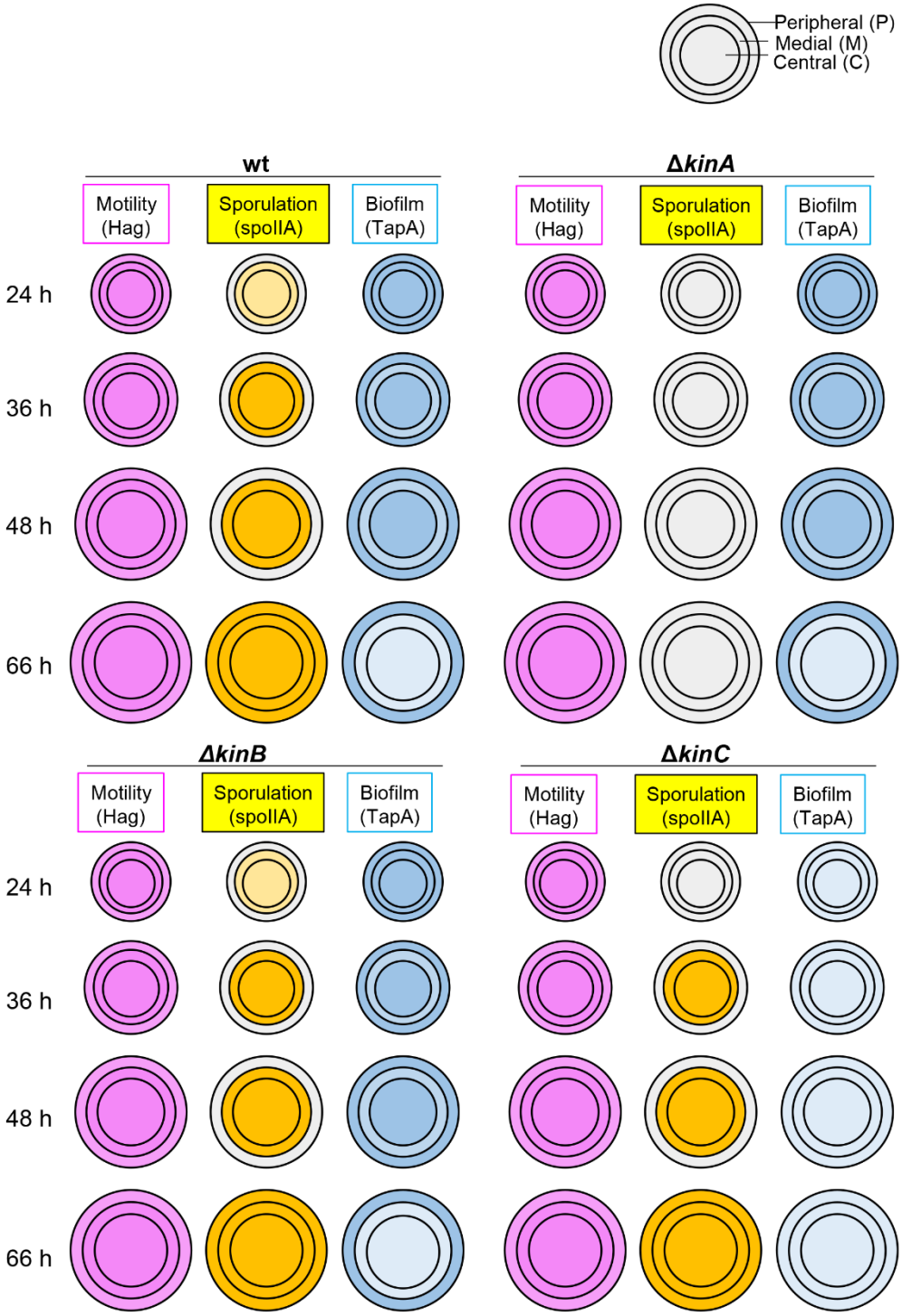


Figure II-12 Schematic representation of expression profiles of the reporters for cell motility (hag), sporulation (spoIIA), and biofilm formation (tapA) in the wild type (wt) and various kinase mutant strains (*ΔkinA, ΔkinB, and ΔkinC*) during the development of *B. subtilis* biofilm colony from 24-66 h.

development. The reporter for sporulation was localized in the medial and central parts of colony in the wild type, *ΔkinB,* and *ΔkinC* strains. As KinA is the main sporulation kinase, (Eswaramoorthy et al., 2011; Narula, Devi, Fujita, & Igoshin, 2012) *ΔkinA* strain did not exhibit the expression of sporulation reporter *spoIIA*.

At late stages of colony development, the sporulation reporter was also expressed in the peripheral region in addition to the medial and central regions of the colony. The reporter for matrix production was expressed throughout the colony at 24 h of colony development in the wild-type, *ΔkinA,* and *ΔkinB* mutant strains. Its expression was later (36-48 h) localized mainly to the peripheral and central regions and finally it was confined to the peripheral region at 66 h. Wrinkles and colony architecture were not observed in the colony of the *ΔkinC* mutant strain (Figure II-6, panels 12-20). Notably, the *ΔkinC* strain exhibited very low expression of matrix production overall, whereas sporulation reporter was expressed in more number of cells than the wild type strain. These results are in contrast to the results presented in a separate study (Vlamakis et al., 2008) in which a Δ*tasA* strain (*tasA* is required for matrix production), was severely defective in colony architecture and had reduced spore formation. However, my results indicated that the *kinC* mutant strain which completely lacked colony architecture, had greater number of spores than the wild type strain. My results also pointed out that colony architecture was not essential for sporulation, as suggested in the previous study.

Another study mentioned that the aerial projections, which are a part of colony architecture, are the major site for spore formation (Branda et al., 2001). However, my colony resuspension studies revealed that the spores were not only seen in the

medial and central parts of the colony but also at the peripheral region at later stages of colony development. Also, the *kinC* mutant strain that showed no colony architecture, demonstrated the presence of a large number of spores in the median and central parts of the colony. The results presented in this Chapter do not support the notion that aerial structures are the primary sites for spore formation.

A recent study (Srinivasan et al., 2018), that employed microscopic methods and mathematical modelling demonstrated that in a developing biofilm, matrix production is localized in the peripheral region in the form of a ring-shaped front while sporulating cells were present in the form of another front at some distance from the interior regions of the colony. In agreement with this study, I found that at later stages of colony development, matrix production is localized mainly to the peripheral region. Within a biofilm, several microenvironments are created due to the concentration gradients of oxygen, nutrients, waste products, and signaling molecules (Stewart & Franklin, 2008). Thus, the presence of matrix producing cells at the periphery of the colony will serve as direct communicators of the changes in microenvironment around the colony (Srinivasan et al., 2018).

The termination factor Rho also affects cell motility, biofilm formation and sporulation. While rho inactivation impaired the cell motility and biofilm formation, its overexpression inhibited sporulation and stimulated motility (Bidnenko et al., 2017).

Another study (Habib et al., 2017) demonstrated that matrix production was reduced in a biofilm colony which was exposed to reactive oxygen species. Thus, the matrix producing cells may act as sensors to the environmental changes

around the colony and enable the colony to adapt to these changes. This strategy may come in handy as *B. subtilis* is a soil bacterium and within its natural environment it is exposed to environmental stresses such as desiccation, changes in pH, salinity, and oxidative stress. Thus, the ability of the cells to switch on /off matrix production in order to adapt to the local environment will provide better defense strategies against environmental stresses. Future studies to determine cellular differentiation such as live-cell imaging will provide us more detailed information about these processes.

Chapter III: Analyzing the roles of multiple kinases (KinA and KinC) in regulating the dynamics of master transcription factor Spo0A.

III.i Introduction

B. subtilis has the ability to endure a highly orchestrated cell-differentiation program when it is exposed to nutrient limitation conditions. This cell-differentiation program consists of different developmental phases including competence, cannibalism, biofilm formation and sporulation (José Eduardo González-Pastor, 2011; Piggot & Hilbert, 2004). At the core of these genetic pathways is a response regulator, Spo0A, which is the master-transcriptional regulator for biofilm formation, and sporulation. Spo0A is activated to its phosphorylated form (Spo0A~P) by a multicomponent phosphorelay pathway. This phosphorelay consists of at least four histidine kinases namely-KinA, KinB, KinC, and KinD, two intermediate phosphotransferases-Spo0F and Spo0B and a response regulator Spo0A. These histidine kinases (KinA-KinD) can autophosphorylate and transfer a phosphoryl group from ATP to one of their conserved histidine residues. Upon autophosphorylation, any of these kinases can pass their phosphoryl group to Spo0F and phosphorylate it. Spo0F~P, then transfers its phosphoryl group to Spo0B and activates its phosphorylated form Spo0B~P. Ultimately, Spo0B~P transfers its phosphoryl group to the response regulator Spo0A and activates its phosphorylated form, Spo0A~P. Spo0A~P then binds to a DNA sequence element box known as 0A box and triggers activation or repression of many genes (Burbulys et al., 1991; Hoch, 1993; Kanamaru et al., 2002).

During the initial phase of starvation, the Spo0A protein and its activity are at relatively low levels, resulting in the switching on/off of the Spo0A~P low-threshold

genes, mainly the genes involved in cannibalism (*sdp*) and biofilm formation (*tapA* and *epsA*). During the later stages of starvation, when the Spo0A~P increases gradually and reaches a threshold level, it can regulate the Spo0A~P high-threshold genes such as the genes required for sporulation (*spoIIG* and *spoIIA*) (Fujita & Losick, 2005). Thus, regulation of cell fate -biofilm formation or sporulation is dependent on the level of phosphorylation of Spo0A.

Of these kinases, KinA and KinB are mainly responsible for initiation of sporulation processes (Jiang et al., 2000; LeDeaux & Grossman, 1995) while KinC and KinD activates the biofilm pathway (Devi, Kiehler, et al., 2015). Biofilm formation and sporulation are complex gene networking events. There are many possible pathways that can lead to biofilm formation and sporulation as phosphorylation levels of 0A are controlled by many positive and negative feedback loops (Eswaramoorthy et al., 2010; Fujita & Sadaie, 1998; Piggot & Hilbert, 2004; Sonenshein, 2000; M. A. Strauch, Wu, Jonas, & Hoch, 1993) . Thus, rewiring these complex genetic networks and decoupling the feedback loops can provide a better understanding of functioning of cell fate decision making processes. As previously reported, (Devi, Kiehler, et al., 2015; Eswaramoorthy et al., 2010; Fujita & Losick, 2005; Vishnoi et al., 2013) by constructing a series of genetically engineered strains, which either express KinA or KinC under an IPTG (isopropyl-β-D-thiogalactopyranoside)- inducible hyper-spank promoter, the phosphorylation of Spo0A can be artificially regulated. Cells expressing KinA under an IPTG- inducible promoter were found to sporulate efficiently under both nutrient rich and starvation conditions in a manner independent of Spo0A phosphorylation

level. (Eswaramoorthy et al., 2010; Eswaramoorthy, Guo, & Fujita, 2009). Similar results were obtained when cells expressing artificially induced KinC triggered sporulation under nutrient rich conditions. However, the sporulation efficiency decreased when the level of KinC induction was high (Vishnoi et al., 2013). These published results focused on sporulation pathways in the domesticated strain of *B. subtilis* and showed that the gradual accumulation of Spo0A~P is crucial for the expression of Spo0A regulated genes. How biofilm and sporulation pathways are effected in response to artificial induction of KinA and KinC in an undomesticated biofilm-forming *B. subtilis* strain is not well understood. In this Chapter, the effect of artificially inducing KinA and KinC on Spo0A low-threshold gene *tapA*, which is required for biofilm formation, and Spo0A high-threshold gene *spoIIG,* which is required for sporulation in the undomesticated *B. subtilis* strain is explored. In this Chapter, I also provide evidence that artificial induction of KinA and KinC has a profound effect on low-threshold gene *tapA* than on Spo0A high-threshold gene *spoIIG.*

III.ii Materials and Methods

III.ii.1 Strains

The parent strain for all experiments was the undomesticated and naturally competent DK1042ComI^{Q12L} (NCIB 3610). *B. subtilis* strains were constructed by transformation with either chromosomal DNA or plasmid DNA as described by Harwood & Cutting (Harwood & Cutting, 1990). Standard recombinant DNA

techniques including plasmid DNA construction and isolation using *Escherichia coli* DH5a were performed as described by Sambrook & Russell (Sambrook & Russell, 2001). All strains used in this study are described in Table III-1.

III.ii.2 Media and culture conditions

Luria-Bertani medium (LB) was used for normal growth of *B. subtilis* (Sambrook & Russell, 2001). All experiments were performed in MSgg medium (Branda et al., 2001). Briefly, cells were pre-cultured in nutrient-rich LB at 37 °C and then transferred to MSgg medium and shaken at 30 °C. Strains harboring reporter genes at the non-essential *thr locus* were supplemented with 40 µg ml^{-1} of L-threonine in the MSgg media. 0-10 µM IPTG was added to the MSgg cultures as needed after an OD_{600} of 0.2 was reached. For sporulation, *B. subtilis* undomesticated strains were grown in MSgg medium overnight at 30 °C. Sporulating colonies were examined on solid DSM supplemented with 1.5% agar as described by Harwood & Cutting (Harwood & Cutting, 1990). When appropriate, antibiotics were included at the following concentrations: 10 µg ml^{-1} of tetracycline, 100 µg ml^{-1} of spectinomycin, 20 µg ml^{-1} of kanamycin, 5 µg ml^{-1} chloramphenicol and 1 µg ml^{-1} of erythromycin.

III.ii.3 Immunoblot analysis

B. subtilis undomesticated strains and various mutants were grown in MSgg medium at 30 °C in the presence of required concentration of IPTG (10 µM). Cells

were harvested at indicated time points. Whole cell lysates were prepared by sonication. Protein concentration was measured by the Bradford method. Total proteins were fractionated by SDS-PAGE (16% acrylamide) and transferred to a nitrocellulose filter. Immunoblot analysis was carried out using polyclonal anti-green fluorescent protein (GFP) and sigma A (σ^A) antibodies. The primary antibodies were detected using an alkaline phosphatase coupled secondary antibody (Anti-rabbit IgG). A colorimetric detection system using nitro blue tetrazolium-5bromo-4-chloro-3-indoyl phosphate (NBT/BCIP) as a substrate was utilized to detect the proteins of interest. σ^A was used as a loading control.

III.ii.4 β–galactosidase assay

β-galactosidase assays were performed as described (Harwood & Cutting, 1990), from the samples collected for *B. subtilis* undomesticated strains grown under MSgg medium as required at 30 °C.

III.iii Results

III.iii.1 Construction of an inducible KinA and KinC expression system

It has been reported that KinA triggers the sporulation process while KinC contributes towards the biofilm and cannibalism pathways by activating the same master regulator Spo0A in its phosphorylated from SpoA~P. However, it is still

unclear how these two kinases (KinA and KinC) can regulate two distinct cell fates In order to answer, how the dynamics of Spo0A~P is regulated by KinA and KinC, genetically engineered strains were constructed in which the synthesis of KinA and KinC was artificially induced using an IPTG-inducible promoter. In these engineered strains, the native promoter of *kinA* was replaced by the IPTG inducible hyper-spank promoter (Phy-spank) and then other kinases, *kinB* and *kinC* were deleted. Similarly, in the other strain, the native promoter of *kinC* was also replaced by the Phy-spank promoter and the other kinases, *kinA* and *kinB* were deleted. Using these systems, the genetic networks governing the sporulation and biofilm processes can be rewired and accurate measurement for the activities of KinA and KinC can be determined without the involvement of other kinases. These strains produced different levels of *kinA* and *kinC* mRNA in response to various concentration of IPTG. By utilizing these systems, the effect of accelerated accumulation of Spo0A~P levels on biofilm formation and sporulation can be precisely evaluated in a chemically defined MSgg media. *B. subtilis* cells harboring the Phy-spank promoters were grown under biofilm promoting medium MSgg medium at 30 °C and the reporter activities for Spo0A low-threshold biofilm reporter (*PtapA*) and Spo0A high-threshold sporulation reporter (*PspoIIG)* were measured using beta galactosidase assay.

Table III-I. List of *B. subtilis* strains

Strain #	Genotype	References
	Beta galactosidase assay	
MF5616	thrC::PspoIIG-lacZ erm comI^{Q12L}	This study
MF5763	thrC::PtapA-lacZ erm comI^{Q12L}	This study
MF6961	thrC::PspoIIG-lacZ erm ΔkinB::kan ΔkinC::cm kinAΩPhy-spank-kinA spc comI^{Q12L}	This study
MF6963	thrC::PspoIIG-lacZ erm ΔkinA::tet ΔkinB::kan kinCΩPhy-spank-kinC spc comI^{Q12L}	This study
MF6964	thrC::PtapA-lacZ erm ΔkinB::kan ΔkinC::cm kinAΩPhy-spank-kinA spc comI^{Q12L}	This study
MF6966	thrC::PtapA-lacZ erm ΔkinA::tet ΔkinB::kan kinCΩPhy-spank-kinC spc comI^{Q12L}	This study
	Immunoblot assay	
MF7076	kinAΩPhy-spank-kinB-gfp spc kan comI^{Q12L}	This study
MF7078	kinCΩPhy-spank-kinC-gfp spc kan comI^{Q12L}	This study
MF7710	kinAΩkinA-gfp spc kan comI^{Q12L}	This study
MF6701	kinCΩkinC-gfp spc kan comI^{Q12L}	(Devi *et al*., 2015)
	Viable and spore counts	
MF5609	DK1042 comIQ12L (competent 3610) Bs Undomesticated Wild type strain	
MF5697	ΔkinB::kan ΔkinC::cm kinAΩPhy-spank-kinA spc comI^{Q12L}	This study
MF5712	ΔkinA::tet ΔkinB::kan kinCΩPhy-spank-kinC spc comI^{Q12L}	This study

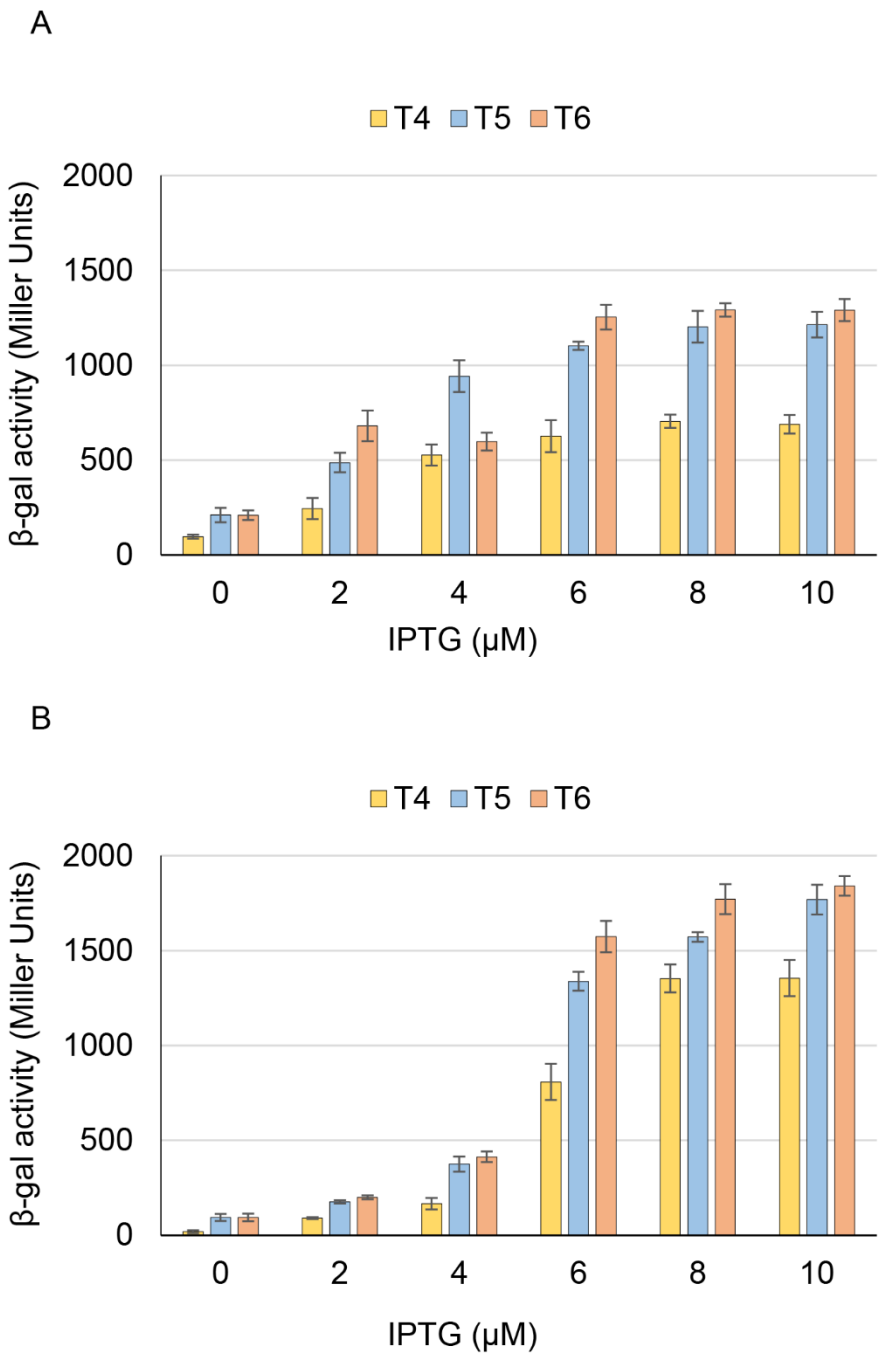


Figure III-1 Reporter activities for biofilm formation (*PtapA-lacZ*) under various concentrations of IPTG.

β-galactosidase activities from the *PtapA-lacZ* were measured in the strains harboring (A) Phy-spank-KinA and (B) Phy-spank-KinC constructs. Cells were grown in MSgg medium at 30 °C in the presence of 0-10 μM IPTG. T0 is time of IPTG addition. T4, T5, and T6 represent 4, 5, and 6 h respectively after IPTG addition. Samples were collected at indicated time points and assayed for β-galactosidase activity.

III.iii.2 Determining the effects of various IPTG concentrations on biofilm and sporulation in artificially inducible KinA and KinC strain

As shown in Figures III-1A and III-1B, the β-galactosidase activity of the Spo0A low-threshold biofilm reporter *tapA* increased in response to an increase in IPTG concentration (2-10 μM IPTG) under KinC as well as KinA induction. However, under lower IPTG concentrations (2-4 μM), the β-galactosidase reporter activity from *tapA* promoter was found to be at least two times higher (β-galactosidase activity of ~942 miller units) under the conditions of KinA induction and saturation levels were achieved at 8-10 μM. In contrast, under KinC induction, at lower concentrations of IPTG (2-4 μM), the β-galactosidase reporter activity from *tapA* promoter were low (β-galactosidase activity of ~ 413 miller units) but the reporter activity continued to increase at 6-10 μM. These results suggested that both *kinA* and *kinC* can influence the low-threshold gene *tapA* for biofilm formation but higher IPTG concentrations were required for biofilm formation under artificial induction of *kinC*. As shown in Figure III-2, the β-galactosidase activity for the *tapA* reporter in the wild type strain did not increase in the presence of 10 μM IPTG.

As shown in Figures III-3A and III-3B, it was found that the artificial induction of both KinA and KinC can also impact sporulation as measured by the β-galactosidase activity of the Spo0A high-threshold sporulation reporter *spoIIG.* The sporulation process is triggered mainly by KinA and KinB but not by KinC (Jiang et al., 2000) . In the case of KinA induction, β-galactosidase reporter activity from *spoIIG* promoter gradually increased with an increase in IPTG concentration (2-10 μM IPTG). However, the number of spores (spores ml^{-1}) was not found to be

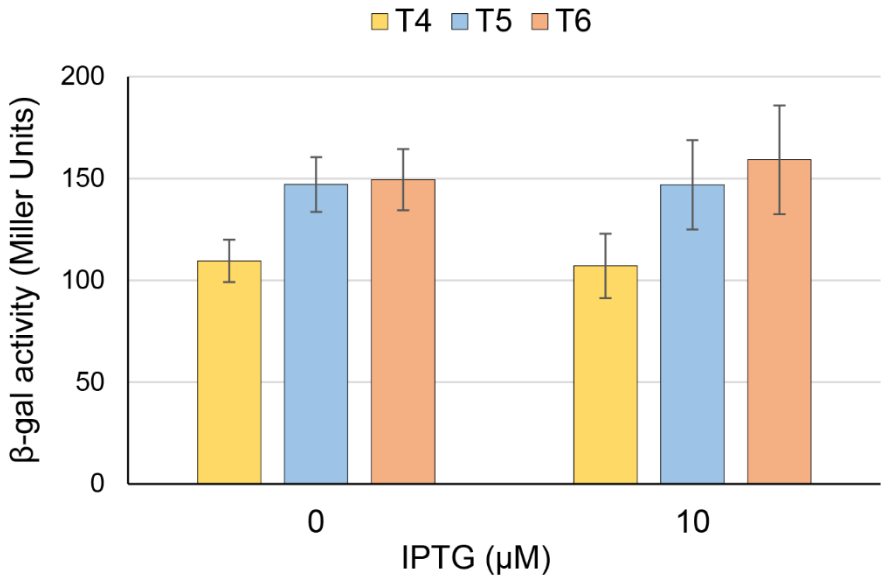


Figure III-2 Reporter activities for biofilm formation (*PtapA-lacZ*) for the wild type strain under various concentrations of IPTG.
β-galactosidase activities from the *PtapA-lacZ* were measured in the wild type strain. Cells were grown in MSgg medium at 30 °C in the presence of 0-10 μM IPTG. T0 is time of IPTG addition. T4, T5, and T6 represent 4, 5, and 6 h respectively after IPTG addition. Samples were collected at indicated time points and assayed for β-galactosidase activity.

affected by an increase in IPTG concentration as seen in Figure III-5. The role of KinC in sporulation process is the production of sporulation delaying factors (Devi, Kiehler, et al., 2015) and killing factors that delay the entry into sporulation. In contrast, under artificial induction of KinC, β-galactosidase reporter activity from *spoIIG* was found to increase only at higher IPTG concentrations (6-10 μM). Thus, low concentrations of IPTG (2-4 μM) did not affect sporulation in the artificially induced KinC strain.

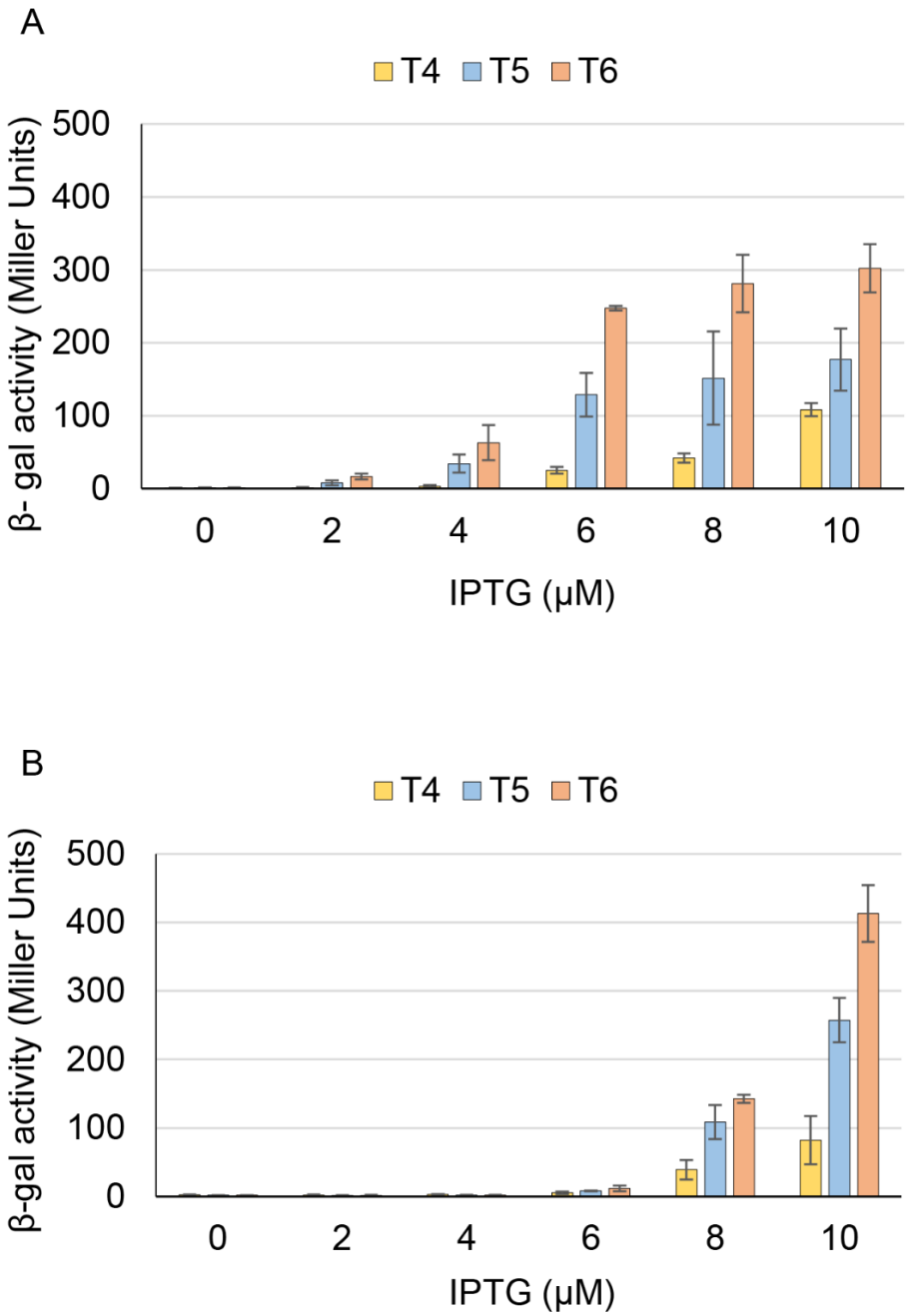


Figure III-3 Reporter activities for the sporulation reporter (P*spoIIG*-lacZ) under various concentrations of IPTG.

β-galactosidase activities from the *PspoIIG-lacZ* were measured in the strains harboring (A) Phy-spank-KinA and (B) Phy-spank-KinC constructs. Cells were grown in MSgg medium at 30 °C in the presence of 0-10 μM IPTG. T0 is time of IPTG addition. T4, T5, and T6 represent 4, 5, and 6 h respectively after IPTG addition. Samples were collected at indicated time points and assayed for β-galactosidase activity.

III.iii.3 Accelerated accumulation of Spo0A exhibits vigorous biofilm formation

We further verified the optimum IPTG concentration for biofilm formation and sporulation. The cells harboring the artificially inducible KinA and KinC promoters were cultured under biofilm conditions in MSgg and sporulation conditions in DSM in the presence of varying IPTG concentrations (0-10 µM). It was found that sporulation in the artificially inducible KinA and KinC strains were similar to wild type strain when the media was supplemented with 10 µM IPTG (~10^8 cells ml^{-1}) as shown in Figure III-4. Thus, for all future experiments, cultures were supplemented with 10 µM IPTG. It has been reported earlier that a gradual increase in levels of activation of Spo0A is required for efficient biofilm and sporulation processes. Here, we investigated how the dynamics of Spo0A~P is regulated when the activation of Sp0A is accelerated as a result of artificially inducible KinA and KinC. Cells harboring the Phy-spank promoter for KinA and KinC were cultured in MSgg medium supplemented with 10 µM IPTG and grown at 30 °C. As shown in Figure III-5, β-galactosidase reporter activity of the Spo0A low-threshold *tapA* promoter in the strain harboring Phy-spank KinA exhibited a gradual increase in *tapA* expression at 3-6 h and saturation levels were achieved at 8 h after IPTG addition. However, β-galactosidase reporter activity of *tapA* promoter in the strain harboring the Phy-spank KinC displayed a rapid increase in *tapA* expression at 2-3 h after IPTG addition. The *tapA* expression increased continuously until 7 h and saturation levels were achieved at 8 h after IPTG addition. Reporter activities of the *tapA* promoter in the Phy-spank KinC strain was also found to be much higher than that of Phy-spank KinA strain.

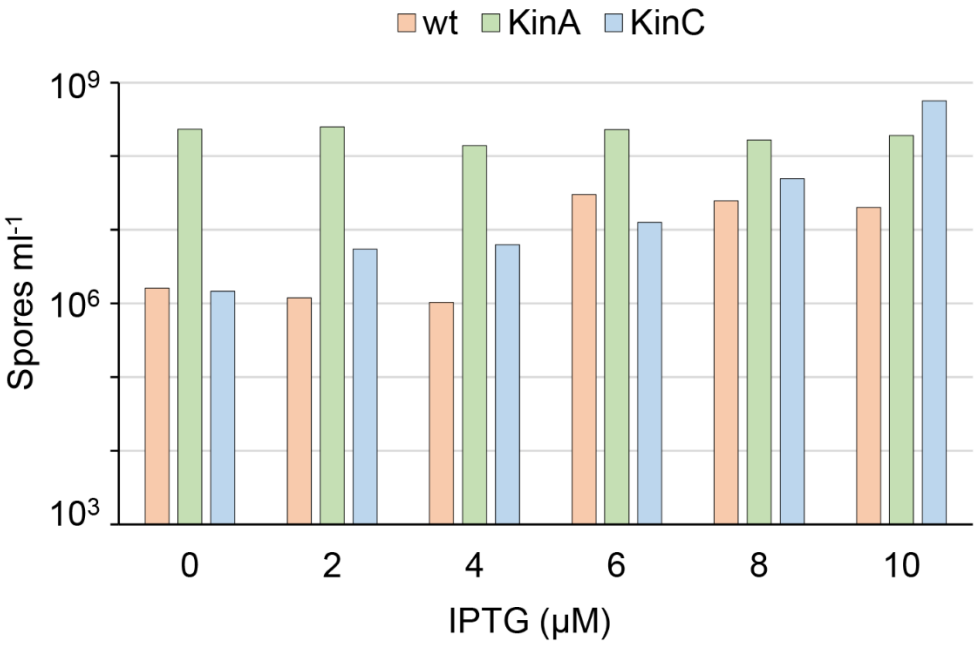


Figure III-4 Spores ml^{-1} in the wild type strain, Phy-spank KinA, and Phy-spank KinC constructs at various IPTG concentrations.
Wild type cells and the cells harboring Phy-spank-kinA and Phy-spank-kinC constructs were grown in MSgg medium at 30 °C. Number of spores ml^{-1} was determined as Colony Forming Units (CFU) per ml after heat treatment by incubation at 80 °C for 10 min.

It was also evident that artificially induced *kinA* and *kinC* were “super active “when compared with the wild type strain as suggested by high reporter activities of the biofilm reporter *tapA* (β-galactosidase activity of ~1200 and ~2000 Miller units, respectively).

As indicated in Figure III-5B, the β-galactosidase activities of the Spo0A high-threshold sporulation reporter *spoIIG* indicated that its expression levels were similar in the Phy-spank KinA and KinC strains and was found to increase in a gradual manner. Control experiments were performed in the absence of IPTG. As shown in Figure III-6, KinA and KinC inducible strains showed higher *PtapA*

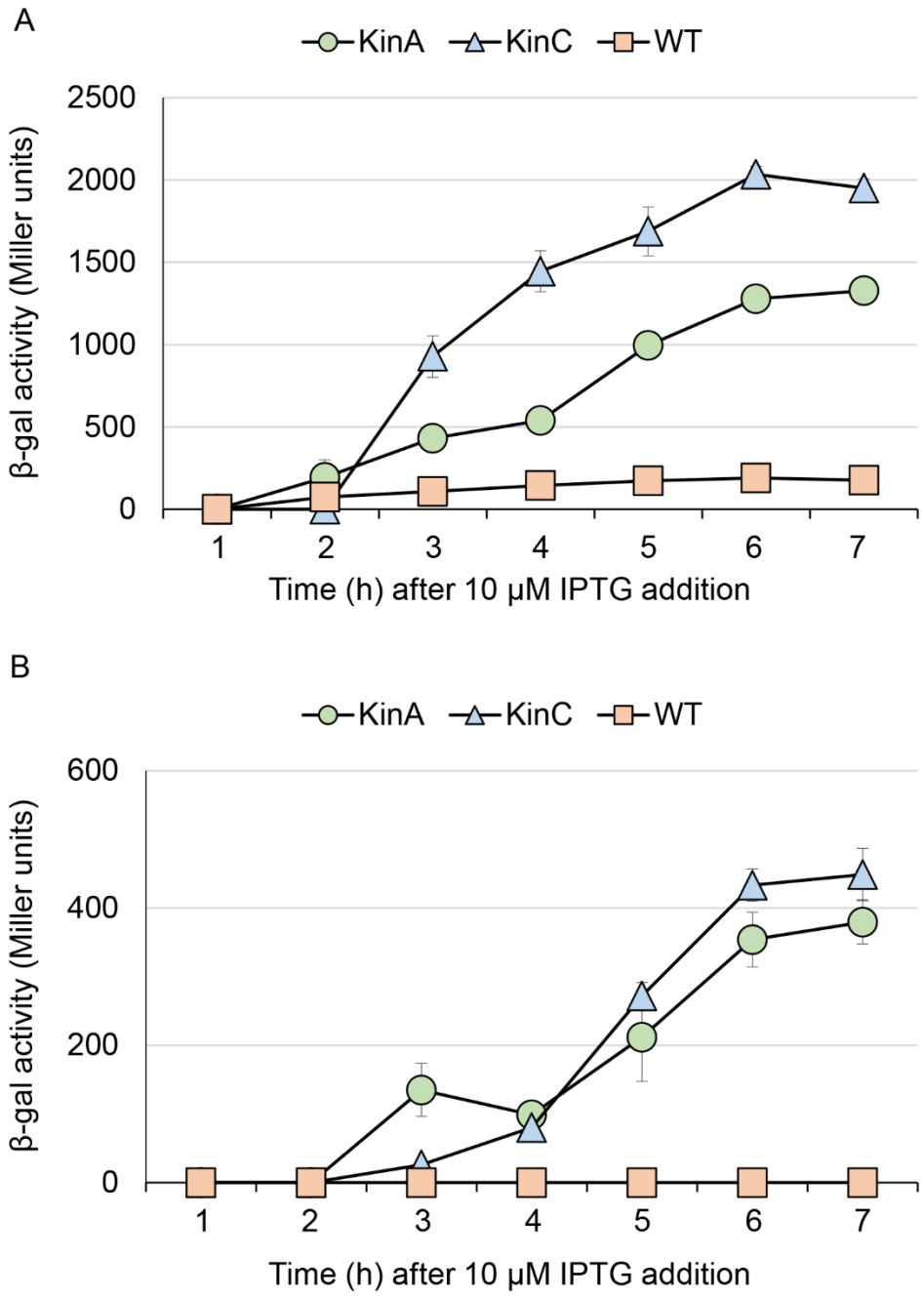


Figure III-5 Comparison of reporter activities for biofilm formation (P*tapA*) and sporulation (P*spoIIG*) among wild type, strains harboring Phy-spank KinA, and Phy-spank KinC.

β-galactosidase activities for (A) biofilm reporter P*tapA-lacZ* and (B) sporulation reporter P*spoIIG-lacZ* were measured in the wild type strain,strains harboring Phy-spank-KinA, and Phy-spank-KinC constructs. Cells were grown in MSgg medium at 30 °C in the presence of 10 μM IPTG. Samples were collected at indicated time points and assayed for β-galactosidase activity.

reporter activities than the wild type in the absence of IPTG. These activities can be attributed to the leakiness of the Phy-spank promoter.

III.iii.4 Sporulation can be triggered by KinC under high IPTG concentration

As seen in Figure III-4, the number of spores ml^{-1} (~10^8 ml^{-1}) in the strain harboring Phy-spank KinA was not found to increase with an increase in IPTG concentration. These results suggested that once the threshold level of KinA is reached, sporulation is initiated. Thus, sporulation requirements can be overruled by the induction of KinA and even the non-starving cells can be forced to sporulate (Eswaramoorthy et al., 2010; Narula, Fujita, et al., 2016). Similar number of spores/ml in the absence of the IPTG in the Phy-spank KinA strain can be attributed to the leakiness of the inducible promoter.

In the case of artificially induced KinC expression system, 10^6 spores ml^{-1} were detected at 0-4 µM IPTG concentration. The number of spores increased to 10^7 spores ml^{-1} at 6 and 8 µM IPTG and further increased to 10^8 spores ml^{-1} at 10 µM IPTG which is similar to wild type levels. At 10 µM IPTG, the number of spores ml^{-1} in the KinC induction strain (~10^8 spores ml^{-1}) were similar to the number of spores ml^{-1} under KinA induction. These results suggested that at higher IPTG concentration, *kinC* can trigger sporulation to similar levels as artificially induced *kinA.* Thus, when KinC protein level is increased in the presence of IPTG, sporulation can also be triggered in the Phy-spank KinC strain. Similar results were reported when KinC levels were artificially induced in a domesticated strain of *B. subtilis*, sporulation was found to be efficiently induced

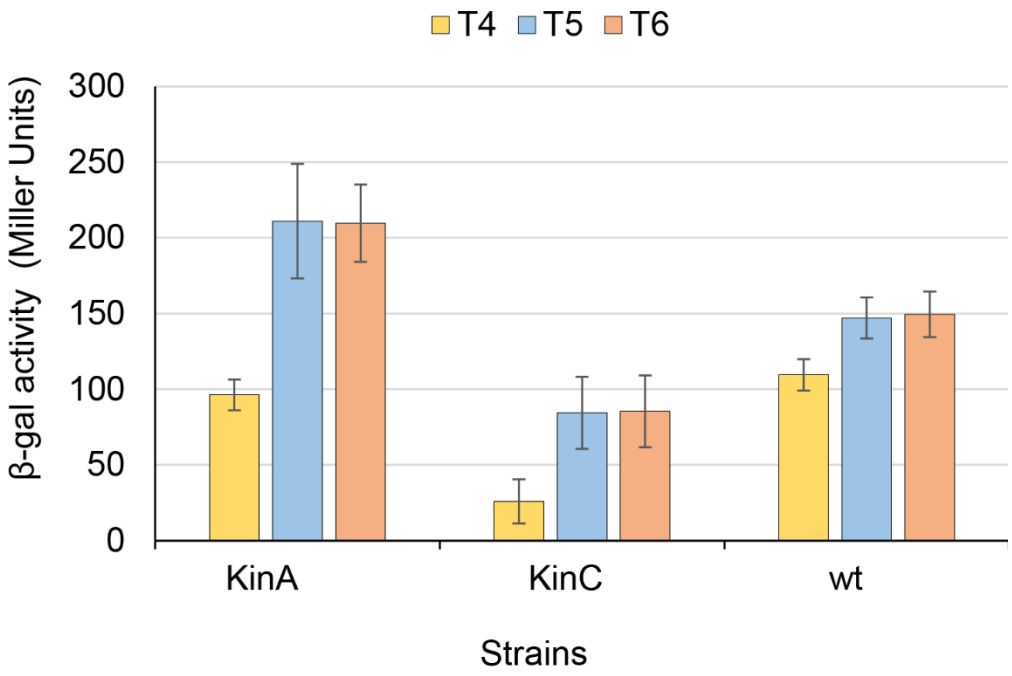


Figure III-6 Comparison of reporter activities for biofilm formation (P*tapA*) among wild type strain, strains harboring Phy-spank KinA, and Phy-spank KinC in the absence of IPTG.
β-galactosidase activities for biofilm reporter P*tapA-lacZ* were measured in the wild type strain and strains harboring Phy-spank-KinA and Phy-spank-KinC constructs. Cells were grown in MSgg medium at 30 °C in the absence of IPTG. T0 is time of IPTG addition. T4, T5, and T6 represent 4, 5, and 6 h respectively after IPTG addition. Samples were collected at indicated time points and assayed for β-galactosidase activity.

(Fujita & Losick, 2005; Vishnoi et al., 2013). This suggested that sporulation can be triggered by an increase in KinC protein levels in both domesticated and undomesticated *B. subtilis* strains.

III.iii.5 Confirmation of expression levels of KinA and KinC by immunoblot analysis

In order to confirm the levels of expression of KinA and KinC, immunoblot analysis was performed using GFP-tagged KinA and KinC under the control of an

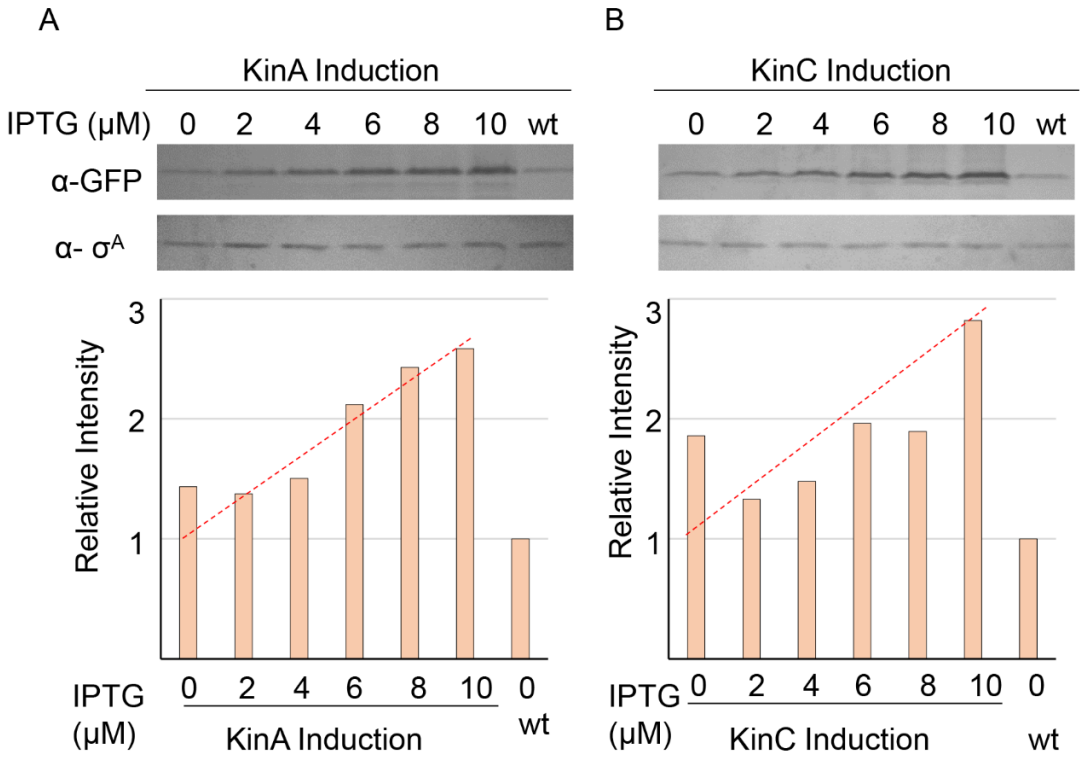


Figure III-7 Protein levels of KinA and KinC increased with the increase in IPTG concentration (0-20 μM).
Cells of the strains harboring the Phy-spank KinA and Phy-spank KinC were cultured in MSgg medium at 30 °C in the presence of indicated concentrations of IPTG. Cell extracts were prepared from the cells harvested at 3 h after IPTg addition. Cell extracts were subjected to SDS-PAGE followed by immunoblot analysis with anti-GFP antibodies. The housekeeping sigma factor, Sigma A (σ^A) was used as a loading control.

artificially induced Phy-spank promoter. Quantitative immunoblot analysis, as seen in Figures III-7A and III-7B, revealed that the levels of KinA and KinC increased gradually with the increase in IPTG concentration (0-10 μM). Under artificially induced synthesis of *kinA*, the protein levels of KinA increased with the increase in IPTG levels (2-6 μM) and saturation levels were achieved at 8-10 μM IPTG. However, no change in spores/ml was observed. In contrast, under *kinC* induction,

the protein levels of KinC as well as number of spores/ml also increased with the increase in IPTG concentration. Wild type strains harboring the KinA-GFP and KinC-GFP under the control of their own promoter at the native locus were used as a control. KinA and KinC levels in the wild type strains (lacking the Phy-spank construct) were found to be lower than the KinA and KinC levels in the IPTG inducible constructs and may not trigger sporulation.

III.iv Discussion

In this Chapter, I found that by artificially inducing KinA and KinC in the undomesticated *B. subtilis* strain, Spo0A low-threshold gene *tapA* as well as SpoA high-threshold gene *spoIIG* are effected. However, much more intense effects on Spo0A low-threshold gene-*tapA* was observed than for Spo0A high-threshold gene, *spoIIG*. Both KinA and KinC inducible strains were found to be “Super Active” than the wild type strain. Reporter activities for biofilm formation (P*tapA*-*lacZ*) at same IPTG concentration (10 µM) were found to be about one and a half times higher under KinC induction than under KinA induction.

The results presented here showed that sporulation can also be triggered in the strain harboring the Phy-spank KinC strain only at high IPTG concentration. In contrast, in the strain harboring Phy-spank KinA, cells enter into sporulation stage once the threshold level of sporulation kinase KinA is achieved. Taken together, the results presented in this chapter indicate that accelerated accumulation of Spo0A~P by increasing the expression levels of KinA and KinC can cause abnormal activation /repression of sporulation and biofilm pathways. Thus, a

gradual increase in the activation of Spo0A is essential for proper cell fate decision making process. As speculated previously (Devi, Vishnoi, Kiehler, Haggett, & Fujita, 2015) and further strengthened by the results mentioned in this chapter, low Spo0A~P and high Spo0A~P levels can be achieved by the distinct pathways regulated by two histidine kinases - KinC and KinA respectively. High levels of activation of KinC are required for sporulation whereas only a threshold level of activation of KinA is sufficient to trigger sporulation.

Cell fate decision making is a stochastic process. In *B. subtilis*, the phosphorylation levels of Spo0A act as an activator as well as repressor for sporulation (Chai et al., 2008). During early stages of starvation, entry into sporulation is prevented by the According to a studfof starvation, when Spo0A~P levels are high, cells enter into sporulation. Thus, bistablilty of Spo0A~P provides an additional control over cell fate decision making process.

According to a study (Vishnoi et al., 2013), the accelerated accumulation of the active form of Spo0A by increasing the KinC expression levels in a domesticated *B. subtilis* strain resulted in the early activation of Spo0A high-threshold genes (*spoIIGB,* required for sporulation). However, no significant effect on Spo0A low-threshold gene (*citG,* fumarate hydratase gene*,* required for TCA cycle) was observed. These results are in contrast with the results presented in this chapter. The differences in these results could be due to the differences in culture conditions or due to the nature of domesticated and undomesticated *B. subtilis* strains.

Another study (Narula, Fujita, et al., 2016), determined how growth rate affected the sporulation process by exploring the role of KinA in a domesticated strain of *B. subtilis.* Their results showed that growth rate is dependent on production levels of KinA and sporulation is triggered by same level of KinA, irrespective of media conditions.

Besides growth rate, other factors such phosphatases and many other proteins can also influence the phosphate flux in the activation of Spo0A. These factors can prevent sporulation under slow growth rate conditions such as low temperature and MSgg medium (Chai et al., 2008).The importance of these other factors cannot be neglected as both low temperature (30 °C) and a slow growth promoting medium such as MSgg are the main culture conditions utilized in this Chapter to determine the role of KinA and KinC in regulating the dynamics of Spo0A~P. However, it is beyond the scope of this dissertation to determine the role of growth rate and other factors in the cell fate decision making process.

Chapter IV: Investigations into the mechanisms of cell fate determination by Spo0A.

IV.i Introduction

When nutrients in the environment are exhausted, the rate of bacterial cell growth slows down and the cell population enters into stationary phase. Under starvation conditions, the soil bacterium, *B. subtilis,* initiates entry into either a unicellular (sporulation) or a multicellular (biofilm) differentiation pathway for survival. These differentiation programs require the activation of a master regulatory transcription factor, Spo0A, by phosphorylation to control hundreds of genes in a coordinated manner. At the top of the differentiation programs, there are at least three histidine kinases (KinA, KinB, and KinC) that autophosphorylate in *cis*. Then, the phosphoryl groups from the phosphorylated kinases are transferred to two intermediate phosphotransferases Spo0F and Spo0B. Finally, Spo0A is phosphorylated by Spo0B in this order with a His-Asp-His-Asp type phospho-transfer cascade, called phosphorelay (Burbulys et al., 1991). When Spo0A is phosphorylated (Spo0A~P), it becomes an active transcriptional regulator that controls more than 500 genes involved in competence, cannibalism, biofilm formation, and sporulation (Fujita et al., 2005; Molle et al., 2003). Among these Spo0A-controlled genes, a set of 121 genes is known to be under the direct control of Spo0A~P (Molle et al., 2003). It is suggested that KinC plays an essential role in triggering biofilm formation while KinA and KinB appear to be primarily responsible for sporulation (Devi, Kiehler, et al., 2015; Yepes et al., 2012).

The phosphorelay is known to be controlled by the transcriptional feedback loops. Initially, increased levels of Spo0A~P as a result of phosphorelay represses the transcription of the gene for the transition state regulatory protein, AbrB, which

is expressed during growth under the control of the house-keeping σ^A-RNA polymerase (RNAP) (M. Strauch et al., 1990). In turn, the transcription of the *sigH* (*spo0H*) gene encoding an alternative σ^H subunit of RNAP is derepressed by the downregulation of the repressor AbrB (Fujita & Sadaie, 1998). Then, the transcription levels of the σ^H-controlled genes, including *kinA*, *spo0F*, and *spo0A* are increased (Fujita & Sadaie, 1998; Predich et al., 1992). Furthermore, Spo0A~P stimulates the transcription of the *spo0F* and *spo0A* genes (Fujita et al., 2005). Therefore, the transcriptional feedback loops contribute to the gradual increase in the Spo0A activity (Fujita & Losick, 2005).

In addition to the transcriptional regulations, the phosphorelay activity is controlled post-translationally by phosphatases. RapA and Spo0E phosphatases remove phosphoryl groups from Spo0F~P and Spo0A~P, respectively (Ohlsen, Grimsley, & Hoch, 1994; Perego et al., 1996). Furthermore, AbrB expressed during growth is inactivated by forming a complex with an anti-repressor AbbA. As a result, AbrB binding to the operator sites is prevented by AbbA (Banse, Chastanet, Rahn-Lee, Hobbs, & Losick, 2008). Transcriptions of genes for RapA and Spo0E are repressed by Spo0A~P and AbrB, respectively (Fujita et al., 2005; M. A. Strauch et al., 1989), while that for AbbA is activated by Spo0A~P (Banse et al., 2008; Fujita et al., 2005).

It has been long debated how cells sense starvation, and respond by entering differentiation pathways. A widely accepted and long standing “Signal sensing” hypothesis proposes that the autophosphorylation activity of the histidine kinases is triggered when the N-terminal “sensor” domain of the kinases receives an as-

yet-unknown starvation signal(s), leading to differentiation pathways (Banse et al., 2008; Hoch, 1993; Stephenson & Hoch, 2001; Szurmant, White, & Hoch, 2007; L. Wang et al., 2001). However, no such starvation signaling molecules have been found. Therefore, the hypothesis has not been proven. An alternative “Threshold” hypothesis proposes that differentiation pathways are triggered by a threshold level of the constitutively active histidine kinases (Eswaramoorthy et al., 2010). In the “Threshold” hypothesis, cell growth slows down when nutrients are depleted, leading to an increase in the cellular concentrations of the kinases through growth-dependent changes in DNA replication, cell volume, and transcription/translation rates (Bipatnath, Dennis, & Bremer, 1998; Klumpp, Zhang, & Hwa, 2009).

The recent experimental and theoretical studies reveal that the slow-down of cell growth increases the cellular concentrations of kinases, initially even slightly, leading to the gradual increase in Spo0A level and activity through the phosphorelay that is amplified by the positive feedback (Eswaramoorthy et al., 2011; Eswaramoorthy et al., 2010; Eswaramoorthy et al., 2009; Narula, Fujita, et al., 2016; Narula, Kuchina, et al., 2016). Although the debates on the mechanism(s) of nutrient or starvation sensing by cells have been inconclusive, using the cell growth rate as an indirect measure of the sensory system is particularly appealing since it bypasses the need for any dedicated metabolite sensing systems (Devi, Vishnoi, et al., 2015; Narula, Fujita, et al., 2016; Narula, Kuchina, et al., 2016).

Nevertheless, the levels of Spo0A protein and activity increase gradually upon starvation and the progressive increase in the activated Spo0A (Spo0A~P) allows

the temporal expression pattern of hundreds of the low- and high-threshold Spo0A-regulated genes (Fujita et al., 2005).

Currently available data suggests that, in early stages of starvation, the cellular concentrations of Spo0A~P gradually increase and reach certain but relatively low levels, at which the expression of SinI is on which sequesters the expressed transcriptional regulator SinR to be inactivated (Chai et al., 2008). Inactivation of SinR results in the derepression of an *eps* operon encoding genes involved in matrix exopolysaccharide synthesis. In the meantime, the relatively low levels of Spo0A~P repress transcription of the *abrB* gene, resulting in the derepression of the *tapA-sipW-tasA* operon encoding genes involved in amyloid protein synthesis (Stover & Driks, 1999; M. A. Strauch et al., 2007). As a result of these events, biofilm formation is turned on.

During extended periods of starvation, Spo0A~P concentrations further increase and turn off the expression of SinI, leading to the repression of exopolysaccharide synthesis. As a result, biofilm formation is turned off (Chai et al., 2008). In turn, the expression of a set of high threshold Spo0A-controlled genes directly involved in sporulation, including *spoIIA*, *spoIIE*, and *spoIIG* are turned on (Fujita et al., 2005). Therefore, biofilm formation in *B. subtilis* is viewed as an alternative developmental process that is independent of sporulation but is controlled by the same master regulator Spo0A~P (D. Lopez, Fischbach, et al., 2009).

To date, more than 50 genes have been identified as contributors to biofilm formation (Stanley & Lazazzera, 2005). Furthermore, diverse arrays of signaling

molecules, including self-produced surfactin and small molecules produced by other organisms have been reported to trigger biofilm formation (Chai, Beauregard, Vlamakis, Losick, & Kolter, 2012; Chen et al., 2012; Kalamara, Spacapan, Mandic-Mulec, & Stanley-Wall, 2018; D. Lopez, Fischbach, et al., 2009; D. Lopez, Vlamakis, Losick, & Kolter, 2009; Shank et al., 2011; Shemesh, Kolter, & Losick, 2010). However, the cellular targets of these molecules are unknown and thus the exact action mechanisms are elusive. As a result, our understanding of the gene networks involved in biofilm formation remains incomplete.

While the previous studies demonstrate that the high threshold levels of Spo0A are required for sporulation (Fujita et al., 2005), there is no direct experimental evidence that the low-threshold levels of Spo0A are sufficient to induce biofilm formation. Here, we constructed an engineered *B. subtilis* strain that produces reduced levels of Spo0A and provided direct evidence that the low-threshold levels of Spo0A are sufficient to trigger biofilm formation, but not for sporulation. We further found that Spo0E, a phosphatase specific for Spo0A~P plays an important role in regulating biofilm formation, but not sporulation.

IV.ii Materials and Methods

IV.ii.1 Strains

All strains used in this study are listed in Tables IV-1, IV-2, and IV-3, parent strain for all experiments was the undomesticated and naturally competent DK1042 (NCIB 3610 background, BGSC3A38 as the *Bacillus* Genetic Stock

Center strain number) (Konkol et al., 2013). *B. subtilis* strains were constructed by transformation with either chromosomal DNA or plasmid DNA as described by Harwood & Cutting (Harwood & Cutting, 1990). Standard recombinant DNA techniques including plasmid DNA construction and isolation using *Escherichia coli* DH5α were performed as described by Sambrook & Russell (Sambrook & Russell, 2001). Tables IV-4 and IV-5 contains the list of plasmids and oligonucleotides used in the study.

IV.ii.2 Culture conditions

Luria-Bertani medium (LB) was used for normal growth of *B. subtilis* (Sambrook & Russell, 2001). Biofilm formation was observed in MSgg medium (Branda et al., 2001) and sporulation was observed in Difco Sporulation Medium (DSM) as described by Harwood and Cutting (Harwood & Cutting, 1990). 1.5% agar was used to make solid media plates.

To assess the colony morphology and wrinkle formation in *B. subtilis* undomesticated strains and various mutants, 2 µl culture of the *B. subtilis* cells exponentially grown in LB was spotted on an MSgg agar plate. The culture drop was air dried for 5 min, after that lid of the agar plate was closed and the plate was incubated at 30 °C for 2-3 d or until colony morphologies are well established. Images were taken with a Canon camera.

To determine pellicle formation, 10 µl culture of the *B. subtilis* cells exponentially grown in LB was inoculated into 2 ml of MSgg in a glass test tube of 15 mm diameter. The test tube was incubated at 30 °C without agitation. Images

of the pellicle were taken at 24 h. For sporulation, *B. subtilis* undomesticated strain were grown in DSM medium overnight at 37 °C. Sporulating colonies were examined on solid DSM supplemented with 1.5% agar as described by Harwood & Cutting (Harwood & Cutting, 1990). Unless otherwise mentioned, all sporulation assays were done in DSM. In some cases (when mentioned in figure legends), sporulation assay was also done in MSgg medium in a manner similar to the DSM. Threonine (1 mg ml^{-1}, final concentration) was added to MSgg medium whenever threonine auxotroph strains were used. When appropriate, antibiotics were included at the following concentrations: 10 µg ml^{-1} of tetracycline, 100 µg ml^{-1} of spectinomycin, 20 µg ml^{-1} of kanamycin, 5 µg ml^{-1} chloramphenicol and 1 µg ml^{-1} of erythromycin.

IV.ii.3 Immunoblot analysis

B. subtilis undomesticated strains and various mutants were grown in DSM and MSgg medium at 37 °C. Cells were harvested at indicated time points. Whole cell lysates were prepared by sonication. Protein concentration was measured by the Bradford method. Total proteins were fractionated by SDS-PAGE (16% acrylamide) and transferred to a nitrocellulose filter. Immunoblot analysis was carried out using polyclonal antibodies against Spo0A (0A) and σ^A. The primary antibodies were detected using an alkaline phosphatase coupled secondary antibody (Anti-rabbit IgG). A colorimetric detection system using nitro blue tetrazolium-5bromo-4-chloro-3-indoyl phosphate (NBT/BCIP) as a substrate was utilized to detect the proteins of interest.

IV.ii.4 β-galactosidase assay

β-galactosidase activity were performed as described by Harwood & Cutting (Harwood & Cutting, 1990) from the samples collected for *B. subtilis* undomesticated strains grown under MSgg and DSM media as required at 37 °C.

IV.ii.5 Microscopy analysis

Cells expressing GFP were analyzed by fluorescence microscopy. Briefly, a 2 µl culture drop from an exponentially grown culture in LB was spotted on a solid MSgg medium supplemented with 1.5% agar. The solid plate was incubated at 30 °C for 24-48 h as indicated in figure legends. Cells were taken from three different zones, designated as the peripheral, medial and central zones from the outside to the inside of the colony, resuspended in T-base [15 mM $(NH_4)_2SO_4$, K_2HPO_4] (Harwood & Cutting, 1990). The cell suspension was placed on an agarose pad containing T-base and then observed under a 100X microscope objective (Olympus, model BX61). The microscope system control and image processing were performed using SlideBook image analysis software (Intelligent Imaging Innovations, Inc.).

Table IV-1. List of *B. subtilis* strains used for microscopy

Strain #	Genotype	Reference
MF8187	ΔkinA::tet comIQ12L thrC::Phag-mCherry erm amyE::PtapA-cfp spc spoIIAΩPspoIIA-iyfp cm	This study
MF8188	ΔkinB comIQ12L thrC::Phag-mCherry erm amyE::PtapA-cfp spc spoIIAΩPspoIIA-iyfp cm	This study
MF8189	ΔkinC comIQ12L thrC::Phag-mCherry erm amyE::PtapA-cfp spc spoIIAΩPspoIIA-iyfp cm	This study
MF8196	omIQ12L thrC::Phag-mCherry erm amyE::PtapA-cfp spc spoIIAΩPspoIIA-iyfp cm	This study
MF8730	amyE::PtapA-cfp spc thrC::Phag-mCherry erm rpoCΩrpoC-yfp kan comIQ12L	This study
MF8740	Δspo0A::kan thrC::PtapA-gfp erm amyE::PΔv-spo0A cm comIQ12L	This study
MF8741	Δspo0A::kan thrC::PspoIIG-gfp erm amyE::PΔv-spo0A cm comIQ12L	This study
MF8743	Δspo0A::kan thrC::PspoIIQ-gfp erm amyE::PΔv-spo0A cm comIQ12L	This study
MF8771	thrC::PtapA-gfp erm comIQ12L	This study
MF8772	thrC::PspoIIG-gfp erm comIQ12L	This study
MF8774	thrC::PspoIIQ-gfp erm comIQ12L	This study
MF8946	Δspo0A::kan ΔkinA amyE::PΔv-spo0A cm thrC::PtapA-gfp erm comIQ12L	This study
MF8947	Δspo0A::kan ΔkinA amyE::PΔv-spo0A cm thrC::PspoIIG-gfp erm comIQ12L	This study
MF8948	Δspo0A::kan ΔkinA amyE::PΔv-spo0A cm thrC::PspoIIQ-gfp erm comIQ12L	This study
MF8949	Δspo0A::kan ΔkinB amyE::PΔv-spo0A cm thrC::PtapA-gfp erm comIQ12L	This study
MF8950	Δspo0A::kan ΔkinB amyE::PΔv-spo0A cm thrC::PspoIIG-gfp erm comIQ12L	This study
MF8951	Δspo0A::kan ΔkinB amyE::PΔv-spo0A cm thrC::PspoIIQ-gfp erm comIQ12L	This study
MF8952	Δspo0A::kan ΔkinC amyE::PΔv-spo0A cm thrC::PtapA-gfp erm comIQ12L	This study
MF8953	Δspo0A::kan ΔkinC amyE::PΔv-spo0A cm thrC::PspoIIG-gfp erm comIQ12L	This study
MF8954	Δspo0A::kan ΔkinC amyE::PΔv-spo0A cm thrC::PspoIIQ-gfp erm comIQ12L	This study
MF8955	ΔkinA thrC::PtapA-gfp erm comIQ12L	This study
MF8956	ΔkinB thrC::PtapA-gfp erm comIQ12L	This study
MF8957	ΔkinC thrC::PtapA-gfp erm comIQ12L	This study
MF8958	ΔkinA thrC::PspoIIG-gfp erm comIQ12L	This study
MF8959	ΔkinB thrC::PspoIIG-gfp erm comIQ12L	This study
MF8960	ΔkinC thrC::PspoIIG-gfp erm comIQ12L	This study
MF8961	ΔkinA thrC::PspoIIQ-gfp erm comIQ12L	This study
MF8962	ΔkinB thrC::PspoIIQ-gfp erm comIQ12L	This study
MF8963	ΔkinC thrC::PspoIIQ-gfp erm comIQ12L	This study

Table IV-2. List of *B. subtilis* strains used for immunoblot assay, colony morphology, pellicle formation and spore counts

Strain #	Genotype	Reference
MF5609	DK1042 comI^{Q12L} (competent 3610) Bs Undomesticated Wild type strain	Gift from Daniel Kearns
MF5751	Δspo0A::kan comI^{Q12L}	Devi et al., 2015
MF6675	Δspo0A::kan amyE::P$_{\Delta}$v-spo0A cm comI^{Q12L}	This study
MF8067	ΔkinB comI^{Q12L}	This study
MF8068	ΔkinC comI^{Q12L}	This study
MF8816	ΔkinA comI^{Q12L}	This study
MF8985	Δspo0A::kan ΔkinA amyE::P$_{\Delta}$v-spo0A cm comI^{Q12L}	This study
MF8986	Δspo0A::kan ΔkinB amyE::P$_{\Delta}$v-spo0A cm comI^{Q12L}	This study
MF8987	Δspo0A::kan ΔkinC amyE::P$_{\Delta}$v-spo0A cm comI^{Q12L}	This study
MF9196	ΔrapA::tet comIQ12L	This study
MF9197	Δspo0E::erm comIQ12L	This study
MF9204	Δspo0E::erm ΔrapA::tet comIQ12L	This study

Table IV-3. List of *B. subtilis* strains used for β-galactosidase assay

Strain	Genotype	Reference
MF5906	Δspo0A::kan thrC::PtapA-lacZ erm comI^{Q12L}	This study
MF5907	Δspo0A::kan thrC::PspoIIG-lacZ erm comI^{Q12L}	This study
MF5616	thrC::PspoIIG-lacZ erm comI^{Q12L}	This study
MF5763	thrC::PtapA-lacZ erm comI^{Q12L}	This study
MF6676	Δspo0A::kan amyE::P$_{\Delta}$v-spo0A cm comI^{Q12L} thrC::PtapA-lacZ	This study
MF6685	Δspo0A::kan amyE::P$_{\Delta}$v-spo0A cm thrC::PspoIIG-lacZ erm comI^{Q12L}	This study
MF7459	amyE::Pspo0A-lacZ spc comI^{Q12L}	This study
MF7460	amyE::Pspo0A-Pv-lacZ spc comI^{Q12L}	This study
MF7461	amyE::Pspo0A-Ps-lacZ spc comI^{Q12L}	This study
MF7463	amyE::PtapA-lacZ cm comI^{Q12L}	This study
MF7464	amyE::PspoIIG-lacZ spc comI^{Q12L}	This study
MF9162	ΔkinA thrC::PtapA-lacZ erm comI^{Q12L}	This study
MF9163	ΔkinB thrC::PtapA-lacZ erm comI^{Q12L}	This study
MF9164	ΔkinC thrC::PtapA-lacZ erm comI^{Q12L}	This study
MF9168	ΔkinA thrC::PspoIIG-lacZ erm comI^{Q12L}	This study
MF9169	ΔkinB thrC::PspoIIG-lacZ erm comI^{Q12L}	This study
MF9170	ΔkinC thrC::PspoIIG-lacZ erm comI^{Q12L}	This study
MF9178	Δspo0A::kan ΔkinA thrC::PtapA-lacZ erm amyE::PΔv-spo0A cm comI^{Q12L}	This study
MF9179	Δspo0A::kan ΔkinB thrC::PtapA-lacZ erm amyE::PΔv-spo0A cm comI^{Q12L}	This study

Table IV-3. List of *B. subtilis* strains used for β-galactosidase assay continued

Strain	Genotype	Reference
MF9180	Δspo0A::kan ΔkinC thrC::PtapA-lacZ erm amyE::PΔv-spo0A cm comIQ12L	This study
MF9181	Δspo0A::kan ΔkinA thrC::PspoIIG-lacZ erm amyE::PΔv-spo0A cm comIQ12L	This study
MF9182	Δspo0A::kan ΔkinB thrC::PspoIIG-lacZ erm amyE::PΔv-spo0A cm comIQ12L	This study
MF9183	Δspo0A::kan ΔkinA thrC::PspoIIG-lacZ erm amyE::PΔv-spo0A cm comIQ12L	This study
MF9198	ΔrapA::tet amyE::PtapA-lacZ cm comIQ12L	This study
MF9199	ΔrapA::tet amyE::PspoIIG-lacZ spc comIQ12L	This study
MF9200	Δspo0E::erm amyE::PtapA-lacZ cm comIQ12L	This study
MF9201	Δspo0E::erm amyE::PspoIIG-lacZ spc comIQ12L	This study
MF9205	ΔrapA::tet Δspo0E::erm amyE::PtapA-lacZ cm comIQ12L	This study
MF9206	ΔrapA::tet Δspo0E::erm amyE::PspoIIG-lacZ spc comIQ12L	This study

Table IV-4. List of plasmids

Plasmid	Description	References
pDR244	temperature-sensitive plasmid with constitutively expressed Cre recombinase	Koo et al., 2017
pMF100	thrC::PspoIIG-lacZ erm	Eswaramoorthy et al., 2010
pMF506	rpoC-yfp spc	This study
pMF642	rpoC-yfp	This study
pMF712	thrC::PtapA-lacZ erm	Devi et al., 2015
pMF829	amyE::PtapA-cfp spc	This study
pMF875	thrC::Phag-mcherry	This study
pMF997	amyE::Pspo0E-lacZ spc	This study
pMF31	amyE::Pspo0A-lacZ spc	This study
pMF173	amyE::Pspo0A-PV-lacZ spc	This study
pMF174	amyE::Pspo0A-PS-lacZ spc	This study

Table IV-5. List of oligonucleotide primers

Primers	Sequence
om40	5’-gccgaattcgaggccaagtactgacaggaggttc-3’
om41	5’-cggctcgagttcaaccgggaccatatcgtcagtcggc-3’
om50	5’-gccaagcttacataaggaggaactact atggtttcaaaaggcgaagaactg-3’
om52	5’-gcgggatccttacttataaagttcgtccatgccaag-3’
om87	5’-gccaagcttacataaggaggaactact atggtcagcaagggagaggaagat-3’
om88	5’-gccggatccttatttgtataattcgtccattccacctgt-3’
om212	5’-cgcgaattcatgctgtcacccttttcttt-3’
om213	5’-cgcaagcttcttgatatgacaatcgttctctttaaagaac-3’
om381	5’-ggcgaattcggaattgacgccccaaagcatattg-3’
om382	5’-ggcaagcttgttgttaaggcacgtccttgtgccc-3’
omf18	5’-gccgaattcatcgatatttatggaaaaga-3’
omf19	5’-gggtgaatcctgttaactacaagcttggc-3’
omf62	5’-gccgaattccggcatgacgatatacaggat-3’
omf63	5’-cggaagcttcgctaagaaataggaaacaag-3’
omf116	5’-gccgaattcctgtcagaagcaggaatcgatatt-3’
omf117	5’-gatatgccactaatattggtgattaagcttggc-3’
omf118	5’-gccgaattcggtaaaatatacaaaagaagatttttcgaca-3’
omf119	5’-ggtgaatcctgttaactacatttggaagcttggc-3’

IV.iii Results

IV.iii.1 Construction of an undomesticated *B. subtilis* strain that produced reduced levels of Spo0A

To experimentally and directly determine whether biofilm are formed in the cells expressing the reduced levels of Spo0A~P, an engineered undomesticated biofilm forming strain expressing the reduced levels of Spo0A was constructed. We have demonstrated that, at early times of starvation, domesticated strains of *B. subtilis* cells produced relatively low cellular levels of the activated Spo0A (Spo0A~P). However, during extended times of starvation, cellular levels of Spo0A~P further increased gradually and reached a threshold level to initiate sporulation (Eichenberger et al., 2003; Fujita et al., 2005; Fujita & Losick, 2005).

Furthermore, our microarray experiments suggested that, at relatively low levels of Spo0A~P during early times of starvation, genes involved in biofilm formation and cannibalism are switched on or off. In contrast, during later times of starvation, at relatively high levels of Spo0A~P, genes involved in sporulation are switched on or off (Fujita et al., 2005). Based on these data, it was hypothesized that a gradual increase in the cellular levels of Spo0A~P was important to switch on or off the low- and high-threshold Spo0A-controlled genes, which lead to cell fate decision, whether to grow or sporulate (Fujita et al., 2005). However, there is no direct experimental evidence that the low-threshold Spo0A~P is sufficient for biofilm formation, since the previous studies used the domesticated non-biofilm forming strain. The *spo0A* gene is transcribed from two distinct promoters named

P_V (promoter for vegetative phase) and P_S (promoter for sporulation phase) under the control of the house-keeping σ^A- and the stationary phase σ^H-RNAP in this order from the upstream, respectively (Figure IV-1) (Chibazakura, Kawamura, & Takahashi, 1991). We found that a domesticated, non-biofilm forming strain which harbored modified *spo0A* promoter , which lacked P_V but retained P_S, produced reduced levels of Spo0A, and had reduced sporulation rates (Fujita et al., 2005). To reproduce these results in the undomesticated strain, we first investigated the promoter activities of P_V, P_S, and the wild type *spo0A* promoter containing both P_V and P_S in the undomesticated biofilm forming *B. subtilis* strain. Each of the promoter DNA fragments was fused to a *lacZ* reporter gene and the resulting construct was introduced into the non-essential *amyE* locus of the chromosomal DNA as a single copy. Then, we assayed the reporter activities for the resulting strains cultured in MSgg (biofilm promoting) and DSM (sporulation promoting) liquid media with shaking (Figures IV-2A and IV-2B). The results of these activities showed that P_V promoter (*P0A-Pv-lacZ*) is activated at relatively low levels only during growth (around T0) while P_S promoter (*P0A-Ps-lacZ*) is activated to higher levels than P_V promoter but only after the activation of P_V promoter (after T0) as shown in Figure IV-2A.

As expected, the wild type promoter (*P0A-lacZ*) showed the highest activities. These results were consistent with the facts that P_V and P_S promoters are differentially transcribed with σ^A- and σ^H-RNAPs, respectively during growth and starvation conditions. Notably, the P_S promoter construct contained three 0A boxes that were originally present in the wild type promoter (Figure IV-1). Therefore, it

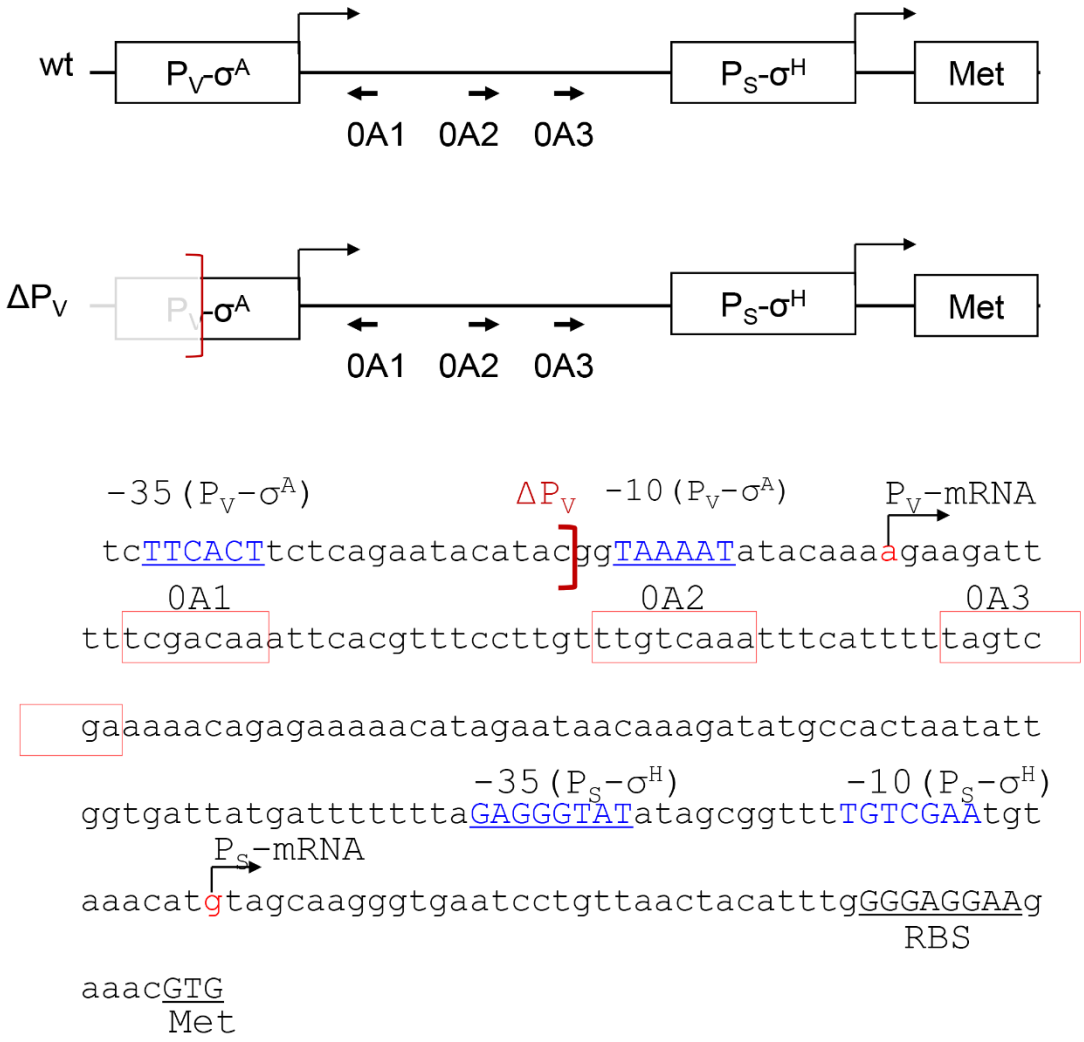


Figure IV-1 Schematic representation of Spo0A promoter region in *B. subtilis*.
The diagram and DNA sequence represent the wild type and ΔP_V (Ps-spo0A) promoter regions, respectively. Three 0A boxes, the -35 and -10 regions, mRNA start sites, RBS (ribosome binding site), and the start codon are indicated.

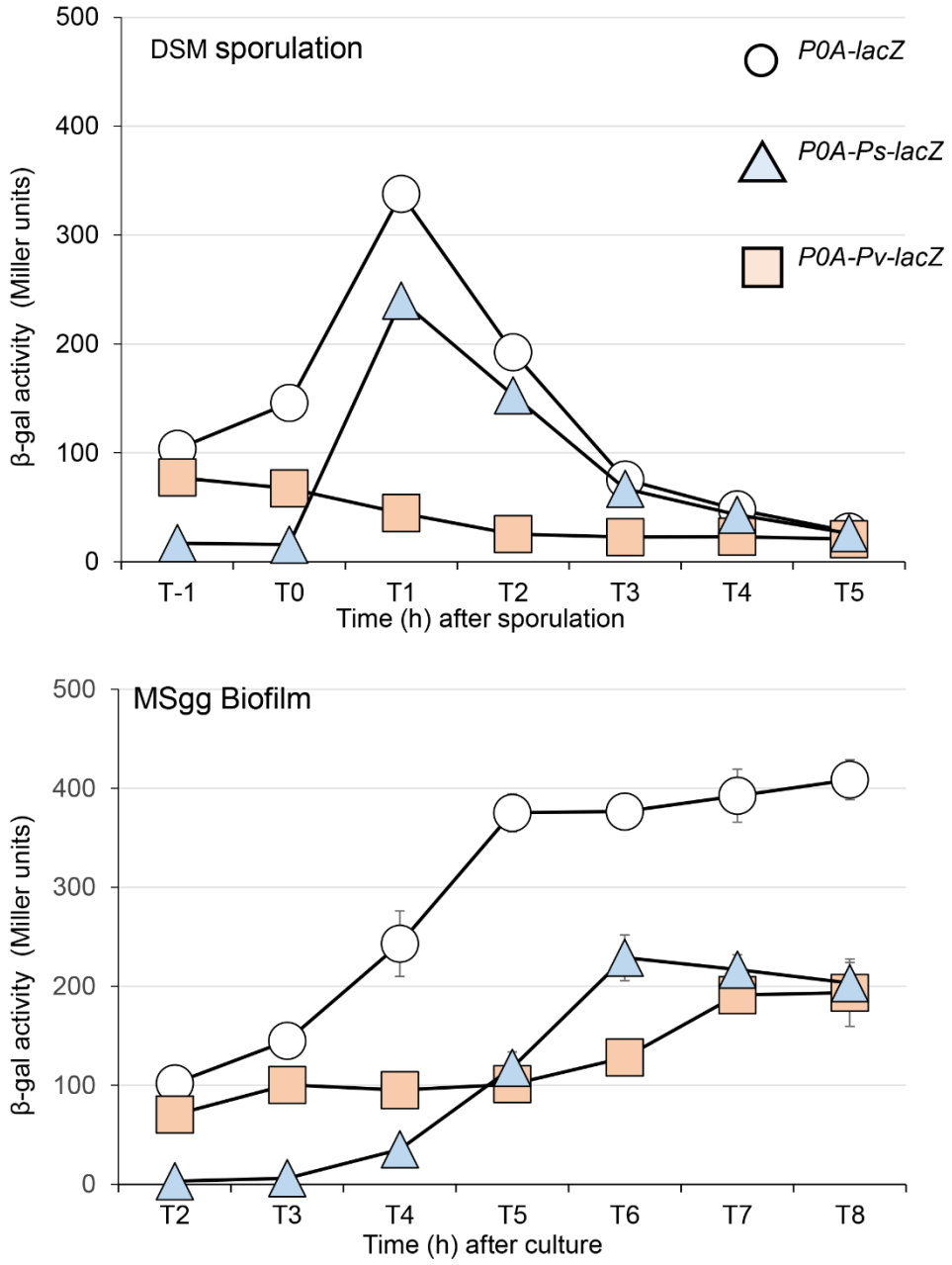


Figure IV-2 Promoter activity of Spo0A under the sporulation and biofilm conditions.

β-Galactosidase activities were measured in strains harboring the lacZ fusion to the wild type *spo0A* promoter Pspo0A (P0A-lacZ), *PΔv-0A* (P0A-Ps-lacZ), and *PΔs-0A* (P0A-Pv-lacZ) in DSM (A) and MSgg (B) media. Culture samples were collected at the indicated times and assayed for β-galactosidase activity. T-1 to T5 in DSM indicates -1 to 5 h after sporulation. T2-T8 in MSgg medium indicates 2-8 h after culture.

was expected that the P_V deleted P_S promoter was positively and negatively controlled by Spo0A~P (Chastanet & Losick, 2011) under starvation conditions. Next, we introduced the P_V deleted *spo0A* gene construct that expressed Spo0A under the control of P_S promoter (named *P_S-spo0A*, containing σ^H-dependent promoter and three 0A boxes) (Figure IV-1) at the *amyE* locus in the undomesticated biofilm forming strain (DK1042) by transformation (Konkol et al., 2013). Then, we disrupted the *spo0A* gene at the native locus by inserting a kanamycin resistance gene (Devi, Vishnoi, et al., 2015). Thus, in the resulting strain harboring the *P_S-spo0A* construct, Spo0A was expressed solely from the P_S promoter.

Next, we determined the cellular levels of Spo0A in the wild type and the *P_S-spo0A* strains cultured under the sporulation (DSM) conditions (Harwood & Cutting, 1990) by immunoblot analysis with anti-Spo0A antibodies (Figure IV-3A). We found that Spo0A expression levels in the *P_S-spo0A* strain were lower than those in the wild type strain in DSM. These results were consistent with the observations that the equivalent strain in the domesticated non-biofilm forming background did not form spores efficiently (Fujita & Losick, 2005). Thus, we established that the engineered strain, expressed reduced levels of Spo0A (referred to as 0A*) in the biofilm forming strain background. In order to test our hypothesis whether biofilm is formed with the reduced levels of 0A~P, the 0A* strain was utilized for the following experiments.

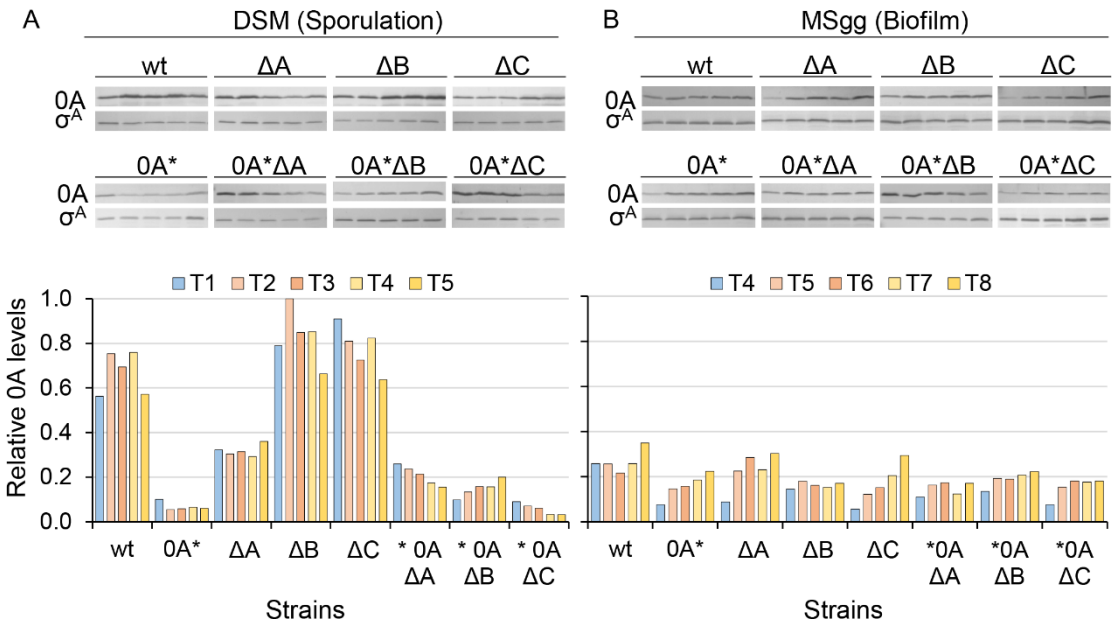


Figure IV-3 Level of accumulation of Spo0A is lower in cells producing Spo0A from the ΔP_V promoter than in cells of the wild-type strain.
Immunoblot analysis of cell extracts from wild type (wt), cells expressing reduced levels of 0A and various kinase mutants under (A) sporulation conditions, DSM) and (B) biofilm promoting conditions, MSgg. Cell extracts were prepared at indicated time points. Proteins were fractionated using SDS-PAGE containing 16% polyacrylamide. 2.5 µg and 10 µg of total protein were loaded for 0A and σ^A respectively in each of the corresponding wells and analyzed by immunoblot analysis with polyclonal anti-spo0A antibodies and anti-σ^A antibodies. The anti-σ^A immunoblot was used as a loading control. Spo0A levels were normalized to σA levels and then to the maximum level of Spo0A. T1-T5 in DSM indicates 1-5 h after sporulation. T4-T8 in MSgg medium indicates 4-8 h after culture.

IV.iii.2 The strain expressing the reduced levels of Spo0A is able to form biofilms but not spores

To examine sporulation, cells of the P_S-*spo0A* strain were cultured in DSM. As controls, cells of the wild type and the *spo0A* knockout strains were examined. No spores were detected in the *spo0A* knockout strains as reported previously (Devi, Kiehler, et al., 2015). As shown in Figure IV-4A, we found that the P_S-*spo0A* strain exhibited a significantly reduced level of sporulation ($10^{2\text{-}3}$ ml^{-1} culture) as compared with the wild type level (10^8 ml^{-1} culture). Next, we tested biofilm formation of those strains in MSgg (Branda et al., 2001) without agitation. In the wild type strain, biofilm formation at the air-liquid interface (called pellicle formation) was observed (Figure IV-4B, panels 1, 10, and 19). In the P_S-*spo0A* strain producing 0A*, pellicle formation was found to be similar to the wild type strain (Figure IV-4B, panels 6, 15, and 24). As a negative control, we confirmed that the biofilm was not formed in the *spo0A* mutant strain (Figure IV-4B, panels 2, 11, and 20) as reported earlier (Branda et al., 2001). Furthermore, we confirmed that biofilm formation occurred in each of the *ΔkinA* (Figure IV-4B, panels 3, 12, and 21) and *ΔkinB* mutant strains (Figure IV-4B, panels 4, 13, and 22), but did not efficiently occur in the *Δkin*C mutant strain (Figure IV-4B, panels 5, 14, and 23). We also confirmed that sporulation efficiencies were dropped in the *ΔkinA* mutant strain, while the *ΔkinB* and *Δkin*C mutant strains efficiently formed spores (10^8 ml^{-1} culture), similar to the wild type strain (Figure IV-4A). These results suggested that the P_S-*spo0A* strain producing 0A* was able to induce biofilm

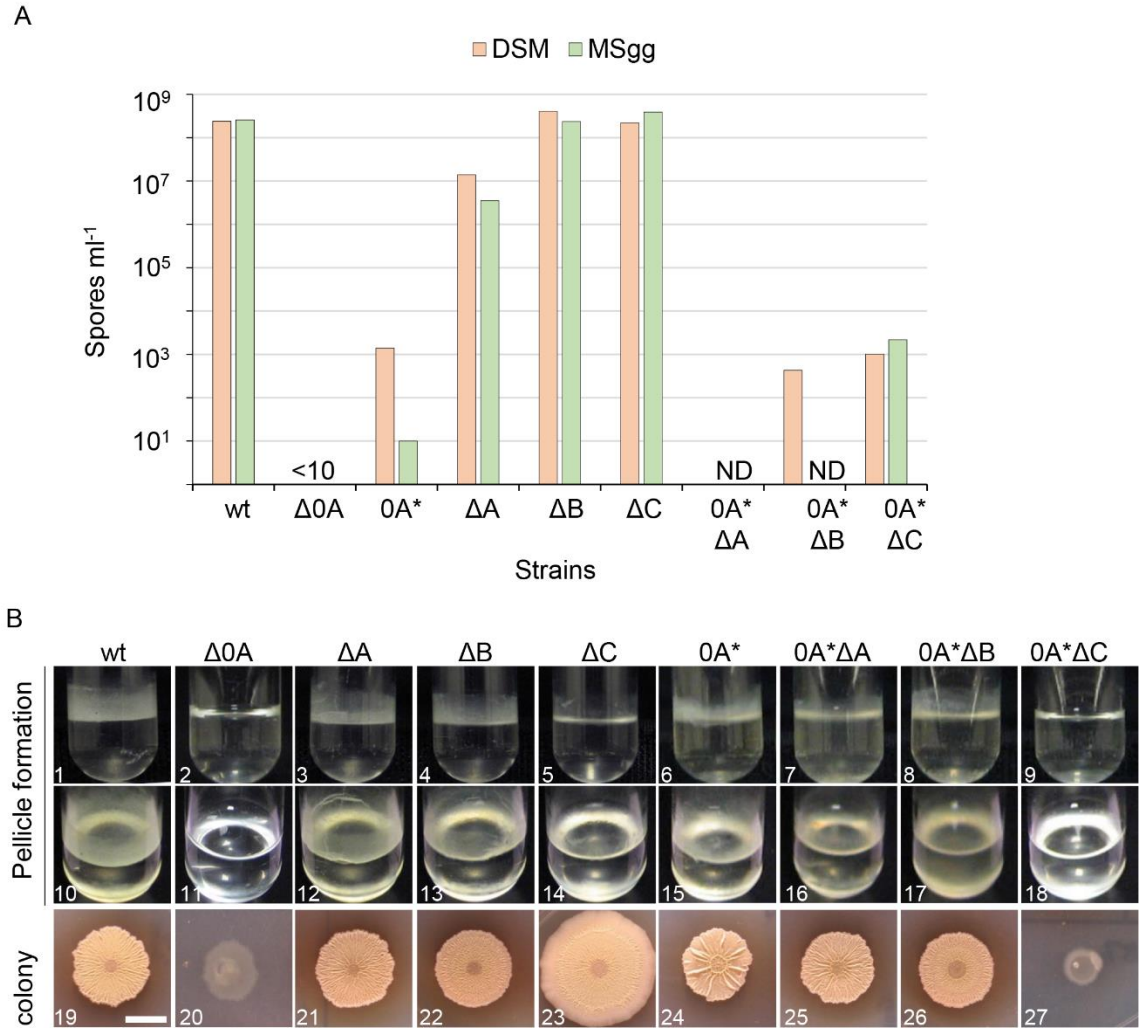


Figure IV-4 Spore counts (A), pellicle formation and colony morphology (B) of wild type (wt), cells expressing reduced level of Spo0A and various kinase mutants under MSgg (biofilm conditions) and DSM (Sporulation conditions).
(A) Number of spores were determined in 24 h cultures in MSgg and DSM media, as CFU ml^{-1} after heat treatment by incubation at 80 °C for 10 min.
(B) Pellicle formation (Side view and Top-down view) and colony morphology on MSgg medium for wild type (wt), strains expressing reduced level of 0A and various kinase mutants were compared. The strains used are MF5609 (panels 1, 9, and 17), MF5751 (panels 2, 11, and 20), MF8816 (panels 3, 12, and 21), MF8067 (panels 4, 13, and 22), MF8068 (panels 5, 14, and 23), MF6675 (panels 6, 15, and 24), MF8985 (panels 7, 16, and 24), MF8986 (panels 8, 17, and 26), and MF8987 (panels 9, 18, and 27). Scale bar is 10 mm.

IV.iii. 3 The strain expressing the reduced levels of Spo0A displays normal expression patterns of genes involved in biofilm formation, but not in sporulation.

In order to determine whether the genes controlling biofilm formation and sporulation were expressed in the P_S-*spo0A* strain, we constructed a series of strains harboring *lacZ* reporter fusions to two Spo0A-controlled promoters. Of these Spo0A-controlled promoters, one is *tapA* (*PtapA-lacZ*), which is positively and indirectly controlled by Spo0A~P and is required for biofilm formation (Devi, Kiehler, et al., 2015), and the other is *spoIIG* operon (*PspoIIG-lacZ*), which encodes the mother-cell specific pro-σ^E and its processing enzyme, and is directly controlled by the high-threshold level of Spo0A~P under sporulation conditions (Fujita et al., 2005). Cells of the P_S-*spo0A* strain harboring each of the reporter genes were cultured in liquid MSgg (biofilm forming conditions) and DSM (sporulation conditions), respectively. Then, cells were collected periodically and assayed for the reporter β-galactosidase activities (Figures IV-5A, IV-5B, IV-6A, and IV-6B). Results indicated that the *tapA* promoter activity measured by *PtapA-lacZ* in the P_S-*spo0A* strain was similar to that in the wild type strain at early times (3-5 h) in MSgg (Figure IV-5B). In contrast, the *spoIIG* promoter activity measured by *PspoIIG-lacZ* in the P_S-*spo0A* strain was significantly lower than that in the wild type strain in DSM (Figure IV-6A). These results indicated that, in the P_S-*spo0A* strain, genes involved in biofilm formation were properly expressed but the genes involved in sporulation were not expressed properly.

As controls, we measured the reporter activities in each of the kinase mutant strains (*ΔkinA ΔkinB*, and *ΔkinC*). In each of the *ΔkinA* and *ΔkinB* mutant strains,

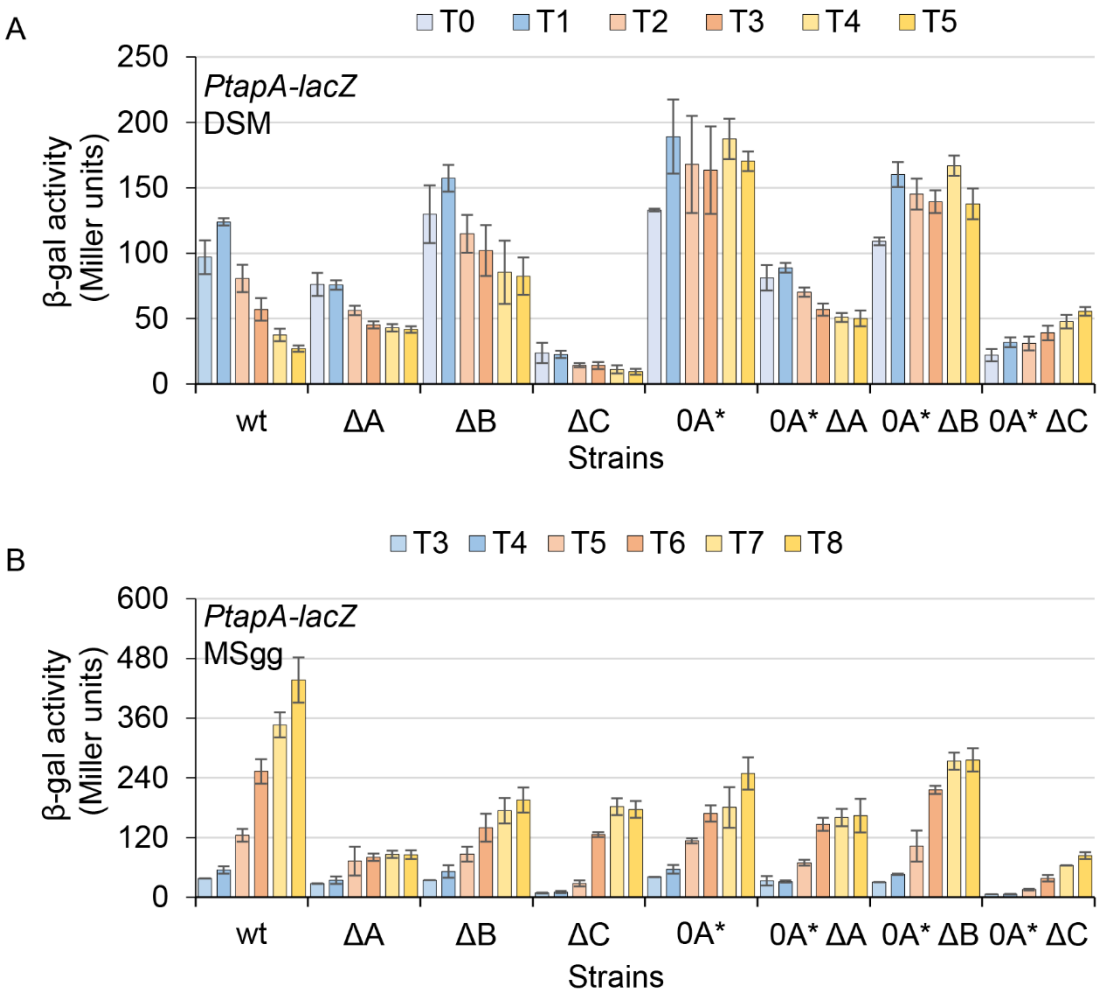


Figure IV-5 Cells producing a reduced level of Spo0A from the ΔP_V promoter are able to form biofilms.

β-galactosidase levels were measured in strains expressing P*tapA-lacZ*, the biofilm promoter P*tapA* fused to *lacZ* in the wild type (wt) and various kinase mutants. β-galactosidase samples were collected from cells grown in (A) DSM and (B) MSgg media at indicated time points. T0-T5 in DSM indicates 0-5 h after sporulation. T3-T8 in MSgg medium indicates 3-8 h after culture.

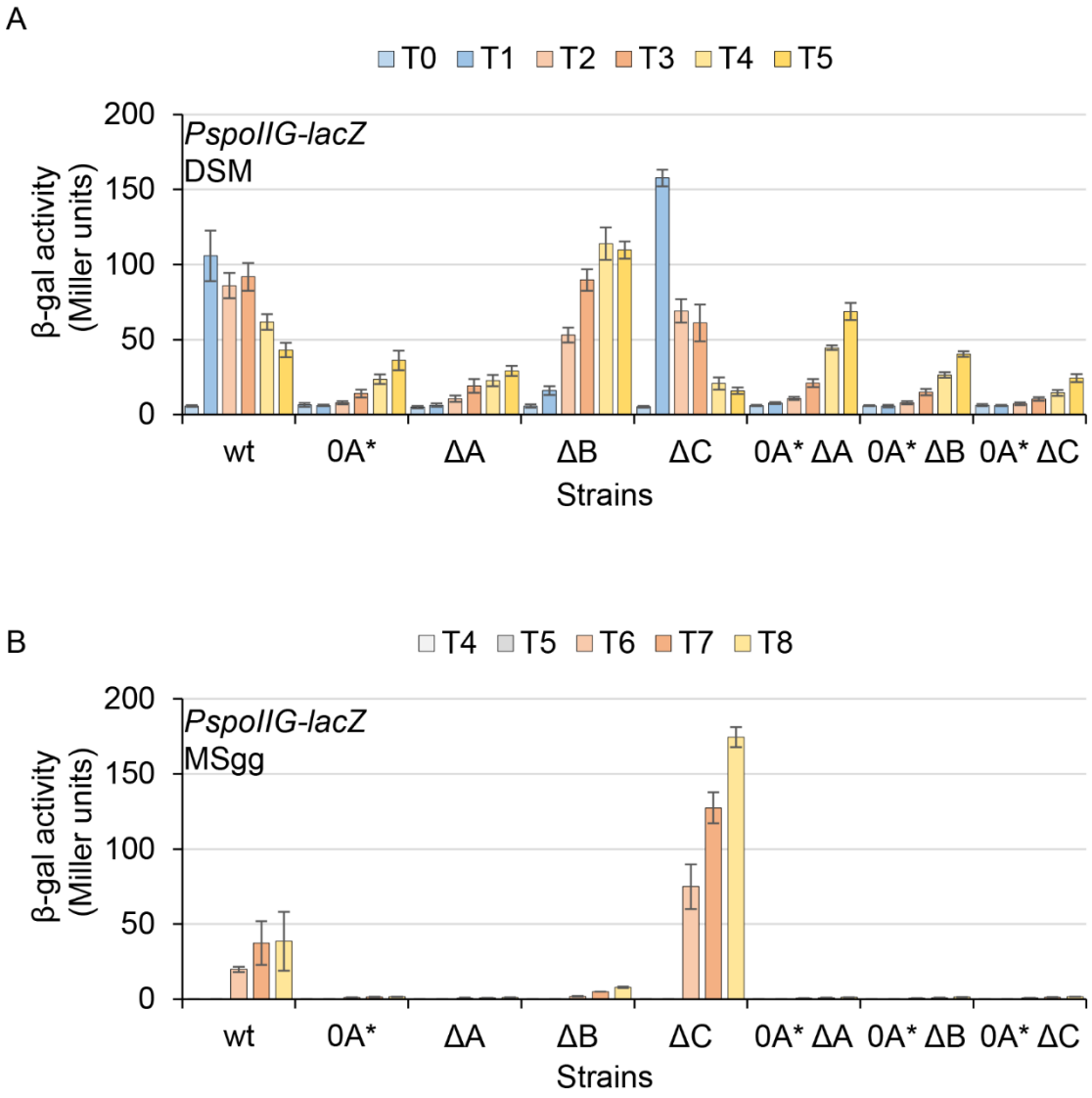


Figure IV-6 Cells producing a reduced level of Spo0A from the ΔP_V promoter are deficient in sporulation.
β-galactosidase levels were measured in strains expressing P*spoIIG-lacZ*, the sporulation promoter P*spoIIG* fused to *lacZ* in the wild type (wt) and various kinase mutants. β-galactosidase samples were collected from cells grown in (A) DSM and (B) MSgg media at indicated time points. T0-T5 in DSM medium indicates 0-5 h after sporulation. T4-T8 in MSgg medium indicates 4-8 h after culture. No activities were detected at 4 and 5 h in MSgg medium.

the *PtapA-lacZ* reporter activities increased gradually at early times in MSgg (Figure IV-5B). In the *ΔkinC* mutant strain, the reporter activities were significantly lower at early times and then increased at later times (Figure IV-5B). These results were consistent with the above observations that biofilm formation occurred in the P_S*-spo0A* strain. We further confirmed that the *PspoIIG-lacZ* reporter activities were detected in the *ΔkinB* and *ΔkinC* mutant strains, similar to those in the wild type. However, *PspoIIG-lacZ* reporter activities in the *ΔkinA* mutant strain were significantly lower than those in the wild type and the other two mutant strains (Figure IV-6A).

Interestingly, we found that the reporter activities showed different profiles depending on the culture conditions. Under biofilm forming conditions in MSgg, the *PtapA-lacZ* reporter activities gradually increased during culture, while under sporulation conditions, the reporter activities reached to peak values at early times (Figures IV-5A and IV-5B). In the wild type strain, the peak values of the *PtapA-lacZ* reporter activities were higher in MSgg than in DSM. In contrast, the *PspoIIG-lacZ* reporter activities increased at early times in DSM (~2 h after onset of sporulation), while these increases were delayed in MSgg (Figures IV-6A and IV-6B). In the wild type strain, the peak values of the *PspoIIG-lacZ* reporter activities were higher in DSM than in MSgg (Figures IV-6A and IV-6B).

These results suggested that in order to make a cell fate decision to form biofilm or spores, proper timing of the Spo0A-controlled gene expression is essential.

We determined the cellular levels of Spo0A in the wild type and the P_S*-spo0A* strains in MSgg using immunoblot analysis with anti-Spo0A antibodies (Figure IV-

3B). We found that Spo0A levels were significantly lower in the *P_S-spo0A* strain than in the wild type strain at early times after entry into stationary phase in MSgg medium (Figure IV-3B). However, these levels gradually increased during culture. These results indicated that the Spo0A protein levels were not well correlated with the *PtapA-lacZ* reporter activities as similar *PtapA-lacZ* reporter activities were detected in both the wild type and *P_S-spo0A* strains (Figure IV-5B). Furthermore, in the biofilm-deficient *ΔkinC* mutant strain, the Spo0A protein levels showed similar trends to those in the biofilm-proficient *P_S-spo0A* strain.

In contrast, the *PspoIIG-lacZ* activities and the Spo0A protein levels were well correlated in DSM as the reporter activities and protein levels were lower in the sporulation-defective *ΔkinA* and the *P_S-spo0A* strains than those in the sporulation-proficient strains (*ΔkinB* and *ΔkinC*) (Figures IV-3A and IV-6A). We further found that the wild type cells cultured in DSM produced higher levels of Spo0A than those cultured in MSgg. These results suggested that, (1) when cells were grown in MSgg (biofilm forming conditions), Spo0A protein increased to an intermediate level, and (2) when cells were grown in DSM (sporulation conditions), Spo0A protein further increased and reached the maximum level

Based on these observations, we speculated that, in MSgg, Spo0A activities (Spo0A~P levels) might be lower in the biofilm deficient *ΔkinC* mutant than in the biofilm proficient strains, while the cellular Spo0A levels were indistinguishable among all tested strains.

IV.iii.4 Spo0E phosphatase plays a significant role in biofilm formation

We wondered why the *PtapA-lacZ* reporter activities were lower in the *ΔkinC* strain than in the wild type strain, while similar levels of Spo0A protein were produced in both strains (Figure IV-5B, and Figure IV-3B). To answer this question, we sought to examine the mechanisms by which cells regulate Spo0A synthesis and activity in response to two different culture conditions, biofilm formation (MSgg) and sporulation (DSM). It is known that among clonal starving *B. subtilis* cells, Spo0A activity is largely heterogeneous between individual cells, possibly due to phosphorelay heterogeneity (noise in a phosphorelay) (Chastanet et al., 2010). Among the regulators of phosphorelay, Spo0E, a phosphatase for Spo0A~P plays a role in delaying Spo0A~P accumulation during starvation (Ohlsen et al., 1994). The other phosphatase is RapA, a phosphatase for Spo0F~P (Perego et al., 1996). These phosphatases allow the majority of the cells to proceed sporulation only when starvation persists (Bischofs, Hug, Liu, Wolf, & Arkin, 2009; Chastanet et al., 2010). However, cells which are deficient in phosphatases (*ΔrapA* and *Δspo0E*) exhibited only a modest effect on sporulation (Perego et al., 1996; Perego & Hoch, 1991) (mainly due to the mechanism by which sporulation decision is made downstream at the activation of the mother cell-specific sigma factor σ^E (Narula et al., 2012). These findings and the published observations prompted us to hypothesize that the phosphorylation level, but possibly not the protein level of Spo0A plays an important and specific role in regulating biofilm formation during the early stages of starvation. Mechanistically, based on our previous study (Fujita & Losick, 2005) we hypothesized that a small

increase in the level of Spo0A~P is more effective to stimulate the genes involved in biofilm formation than those involved in sporulation. To test this hypothesis, we constructed a series of strains harboring deletions of *rapA* (*ΔrapA*), *spo0E* (*Δspo0E*), and both *rapA* and *spo0E* (*ΔrapA Δspo0E*) in the undomesticated biofilm forming strain (DK1042). To monitor Spo0A~P activity, *PtapA-lacZ* and *PspoIIG-lacZ* reporters (for biofilm formation and sporulation, respectively, as described above) were introduced into each of the wild type and the phosphatase mutant strains. First, we examined biofilm formation in the wild type, *ΔrapA*, *Δspo0E*, and *ΔrapA Δspo0E* strains. As shown in Figure IV-7C, we found that more wrinkles and pellicles were formed in the *Δspo0E* and *ΔrapA Δspo0E* double mutant strains (Figure IV-7C, panels 3, 4, 7, 8, 11, and 12) than in the wild type and the *ΔrapA* strains (Figure IV-7C, panels 1, 2, 5, 6, 9, and 10).

Second, similar numbers of spores were produced in all four tested strains both under biofilm and sporulation conditions (Figure IV-8C). Third, the *PtapA-lacZ* reporter activities were more than 2-fold higher in the *Δspo0E* and *ΔrapA Δspo0E* double mutant strains than in the wild type and the *ΔrapA* strains both under biofilm and sporulation conditions (Figures IV-7A and IV-7B). By contrast, the *PspoIIG-lacZ* reporter activities showed minor but not significant variations among four tested strains both under biofilm (MSgg) and sporulation (DSM) conditions (Figures IV-8A and IV-8B). We confirmed that sporulation efficiencies were similar among the four tested strains both under biofilm and sporulation conditions (Figure IV-8C). These results indicated that Spo0E plays an important role in controlling Spo0A~P levels for biofilm formation, but not for sporulation.

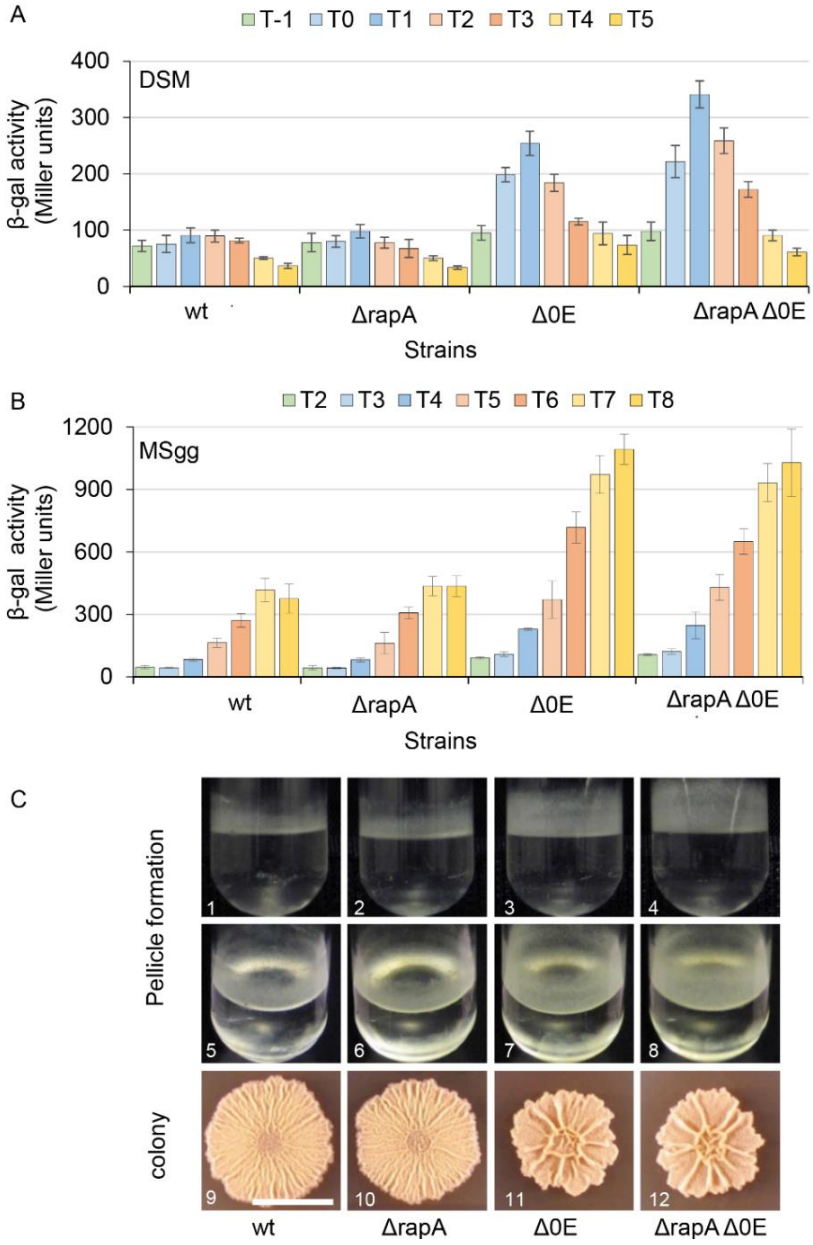


Figure IV-7 Cells harboring the Spo0E phosphatase gene mutation stimulate biofilm formation.

β-galactosidase levels were measured in strains expressing P*tapA-lacZ*, the biofilm promoter P*tapA* fused to *lacZ* in the wild type (wt) and various phosphatase mutants. β-galactosidase samples were collected from cells grown in (A) DSM and (B) MSgg media at indicated time points. T-1 to T5 in DSM indicates -1 to 5 h after sporulation. T2-T8 in MSgg medium indicates 2-8 h after culture. (C) Pellicle formation and colony morphology on MSgg medium for wild type (wt) and phosphatase mutant strains were compared. Scale bar, 10 mm.

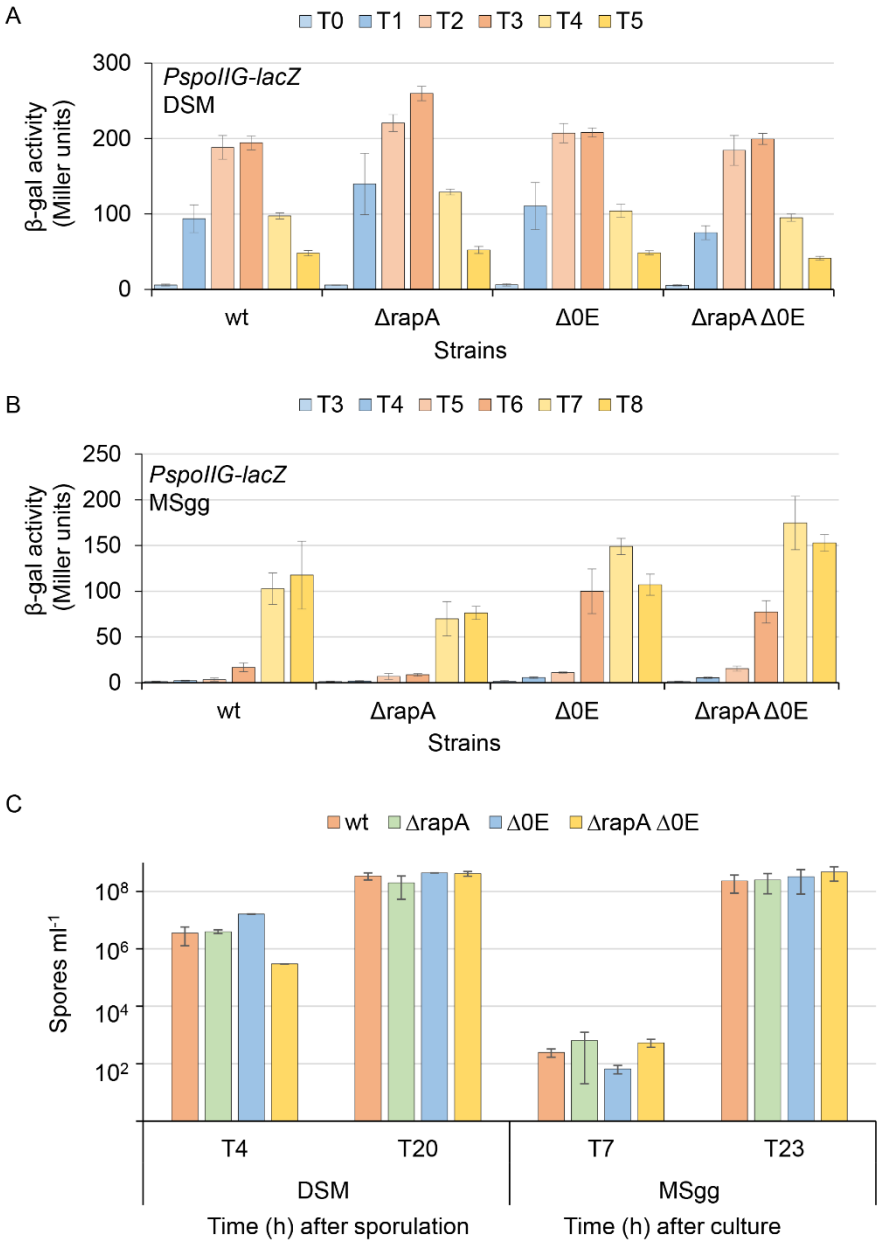


Figure IV-8 Cells harboring the Spo0E phosphatase gene mutation exhibit a modest increase in sporulation under DSM conditions.
β-galactosidase levels were measured in strains expressing P*spoIIG-lacZ*, the sporulation promoter P*spoIIG* fused to *lacZ* in the wild type (wt) and various phosphatase mutants. β-galactosidase samples were collected from cells grown in (A) DSM and (B) MSgg media. T0 to T5 in DSM medium indicates 0 to 5 h after sporulation. T3-T8 in MSgg medium indicates 3-8 h after culture. (C) Spores ml^{-1} reported at T4 and T20 (4 and 20 h after sporulation) in DSM. Spores ml^{-1} reported at T7and T23 (7 and 23 h after inoculation) in MSgg medium. Spores ml^{-1} in the overnight cultures in DSM and MSgg media did not vary (10^8 spores ml^{-1} found for each strain).

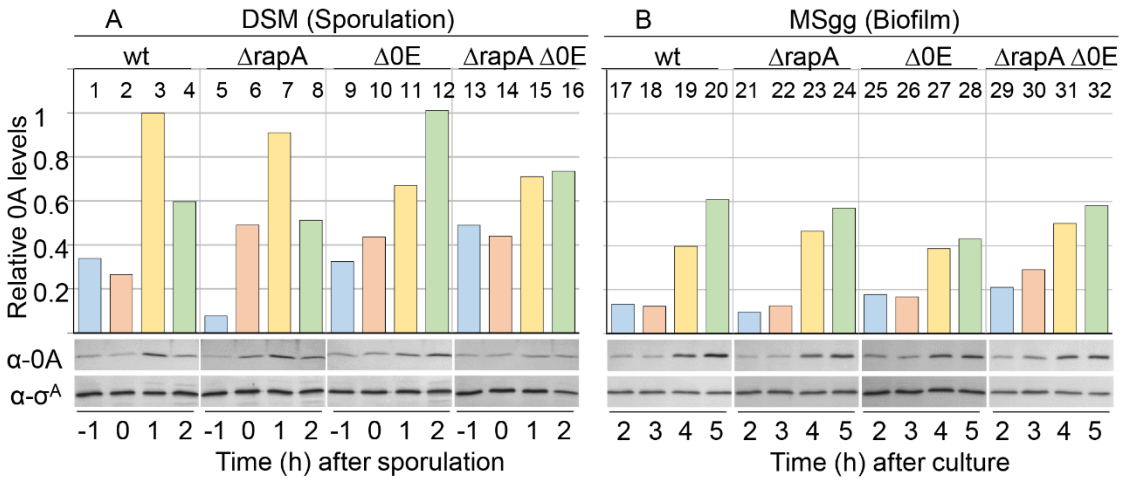


Figure IV-9 Quantification of Spo0A levels in the phosphatase mutants by immunoblot analysis.
Immunoblot analysis of cells cultured under (A) DSM and (b) MSgg medium. Cells extracts were prepared at indicated time points. Proteins were fractionated using SDS-PAGE containing 16% polyacrylamide. Proteins were analyzed by immunoblot analysis with Polyclonal anti-spo0A antibodies and anti-σ^A antibodies. The anti-σ^A immunoblot was used as a loading control. Spo0A levels were normalized to σA levels and then to the maximum level of Spo0A.

We further determined the cellular levels of Spo0A in the above four strains (wild type and phosphatase mutant strains) using immunoblot analysis with anti-Spo0A antibodies. In the *Δspo0E* and *ΔrapA Δspo0E* strains, Spo0A levels were slightly increased at early times (1 h before sporulation to 1 h after sporulation in DSM and 2-4 h after inoculation in MSgg) as compared with the wild type and *ΔrapA* strains as shown in Figure IV-9A (lanes 9, 10, 11, 13, 14, and 15) and Figure IV-9B (lanes 25, 26, 27, 29, 30, and 31). These data suggested that, in the absence of Spo0E, Spo0A~P levels increased during the early times of the transition phase between nutrient-rich and starvation conditions and thus the increased Spo0A~P enhanced the positive transcriptional feedback regulation of phosphorelay genes, including

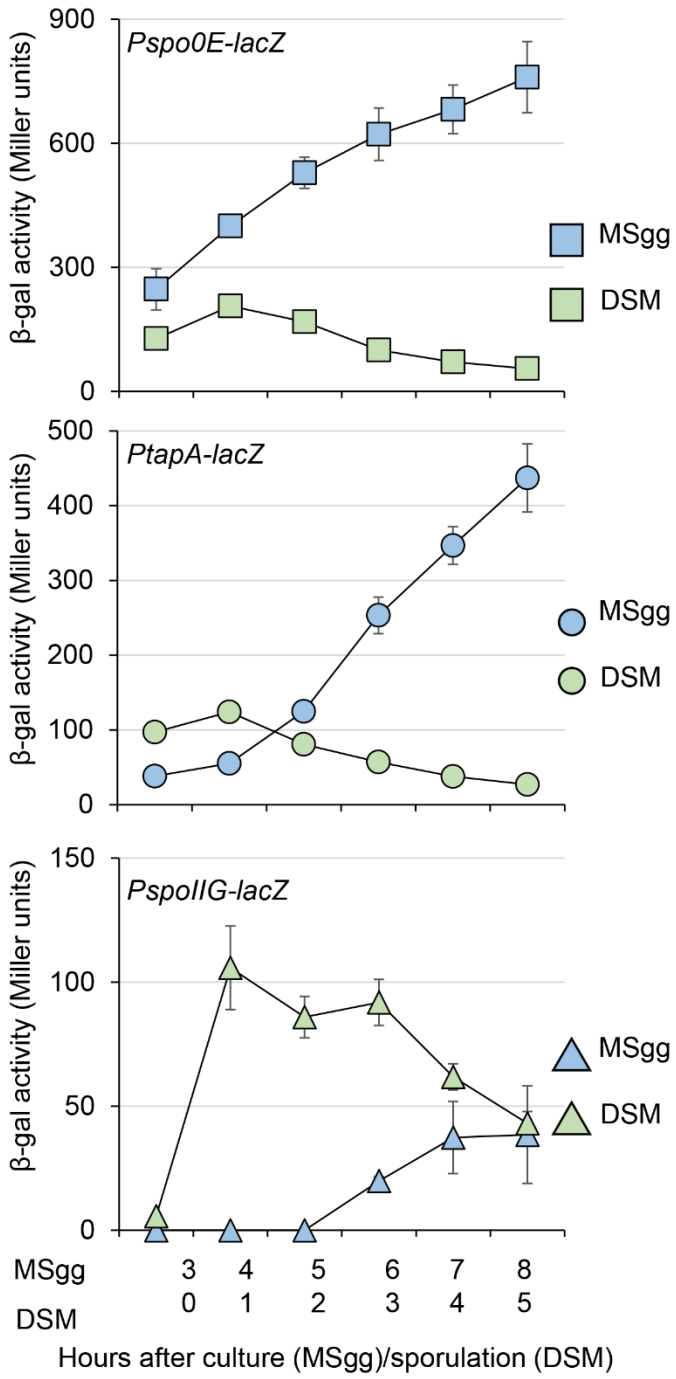


Figure IV-10 Promoter activity of *spo0E*, as compared with *tapA* and *spoIIG* under the sporulation and biofilm conditions.
β-galactosidase activities were measured in a strain harboring the *lacZ* fusions to the *spo0E* promoter. Culture samples were collected at the indicated times in DSM and MSgg media and assayed for β-galactosidase activity The data for *PtapA-lacZ* and *PspoIIG-lacZ* were adapted from the wild type strains used in Figures IV-5A, IV-5B, IV-6A, and IV-6B.

σ^H-regulated *kinA*, *spo0F*, and *spo0A*. As a result, Spo0A protein levels increased through the positive feedback regulation.

However, at later times of culture, the levels of Spo0A protein became similar in all four strains as shown in Figure IV-9A (lanes 4, 8, 12, and 16) and Figure IV-9B (lanes 20, 24, 28, and 32). At later times, increased levels of Spo0A~P appeared to repress the *spo0A* gene transcription in an autorepression manner and thus the Spo0A levels become constant and saturated (Fujita & Sadaie, 1998; M. A. Strauch, Trach, Day, & Hoch, 1992). These results suggested that the autostimulation for Spo0A synthesis by Spo0A~P was more effective in the early times of starvation than at the later times.

IV.iii.5 Sporulation becomes more severely impaired in the strain expressing the reduced levels of Spo0A in the *kinA* null mutation background than either one of them alone

As previously demonstrated and confirmed in the present study, the strain lacking the major sporulation kinase KinA (*ΔkinA*) produced spores at the frequency of 5% to 30% of the wild type level (10^{6-7} ml^{-1}) (Jiang et al., 2000). The P_S-*spo0A* strain, exhibited low sporulation frequencies (10^{2-3} ml^{-1}) than the wild type (10^8 ml^{-1}), and the strain lacking *kinA* (10^{6-7} ml^{-1}), respectively. Based on these findings and previous data, we hypothesized that, among three histidine kinases (KinA, KinB, and KinC), KinA plays a predominant role in regulating Spo0A~P levels required specifically for sporulation. If this hypothesis is correct, we would

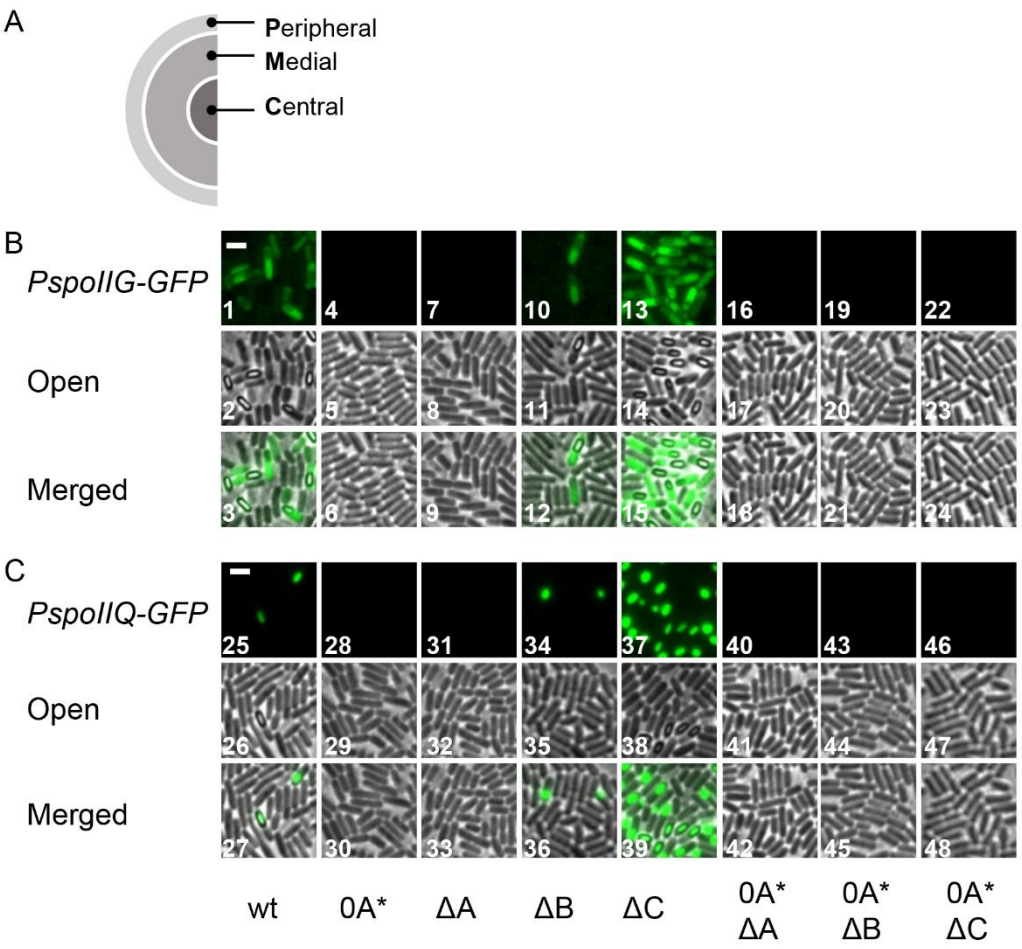


Figure IV-11 Fluorescent microscope analysis of sporulation in the wild type (wt), cells expressing reduced level of Spo0A, and various kinase mutants.
B. subtilis cells expressing (A) *PspoIIG*-GFP, the sporulation promoter *PspoIIG* fused to the GFP reporter gene and (B) *PspoIIQ*-GFP, the sporulation promoter *PspoIIQ* fused to the GFP reporter gene were grown on MSgg agar for 37 °C for 48 h. Cells were taken from the peripheral (P), medial (M), and central (C) zones of each colony, as indicated in the schematic drawing of the colony at the top left, and observed under the fluorescent microscope. Scale bar is 2 µm.

observe that sporulation frequency is further decreased when the *ΔkinA* is introduced into the P_S-*spo0A* strain. In fact, we found that spores were not detected in the strain harboring both the *ΔkinA* and P_S-*spo0A* constructs (<10ml^{-1}) as shown in Figure IV-4A. For comparison, we further investigated the KinB and KinC pathways in the P_S-*spo0A* strain. As reported, sporulation frequency in the strain lacking either KinB (*ΔkinB*) or KinC (*ΔkinC*) was indistinguishable from that in the wild type strain (~10^8 ml^{-1}). We found similar sporulation frequencies among the P_S-*spo0A*, *ΔkinB* and P_S-*spo0A*, and *ΔkinC* and P_S-*spo0A* strains (10^{2-3} ml^{-1}) as shown in Figure IV-4A. These results verified that KinA is more important than the other two kinases to maintain the level and activity of Spo0A required for sporulation (Eswaramoorthy et al., 2009).

Furthermore, by the use of fluorescence microscopy, we also analyzed the expression of two sporulation reporters-*PspoIIG* and *PspoIIQ* at a single cell level, among the wild type and strains producing reduced levels of Spo0A. As shown in Figures IV-11A and IV-11B, we found that the strains, P_S-*spo0A*, *ΔkinA, ΔkinA* and P_S-*spo0A, ΔkinB* and P_S-*spo0A, and ΔkinC* and P_S-*spo0A* were strongly impaired in sporulation. We further determined the cellular levels of Spo0A in the above strains using immunoblot analysis with anti-Spo0A antibodies. In DSM, Spo0A levels were lower in the P_S-*spo0A* strain harboring each of the kinase mutations than in the wild type strain (Figure IV-3A). Interestingly, Spo0A levels in the *ΔkinA* and P_S-*spo0A*, and *ΔkinB* and P_S-*spo0A* were slightly higher than in the P_S-*spo0A*, and *ΔkinC* and P_S-*spo0A* strains, but the regulatory mechanisms are unknown.

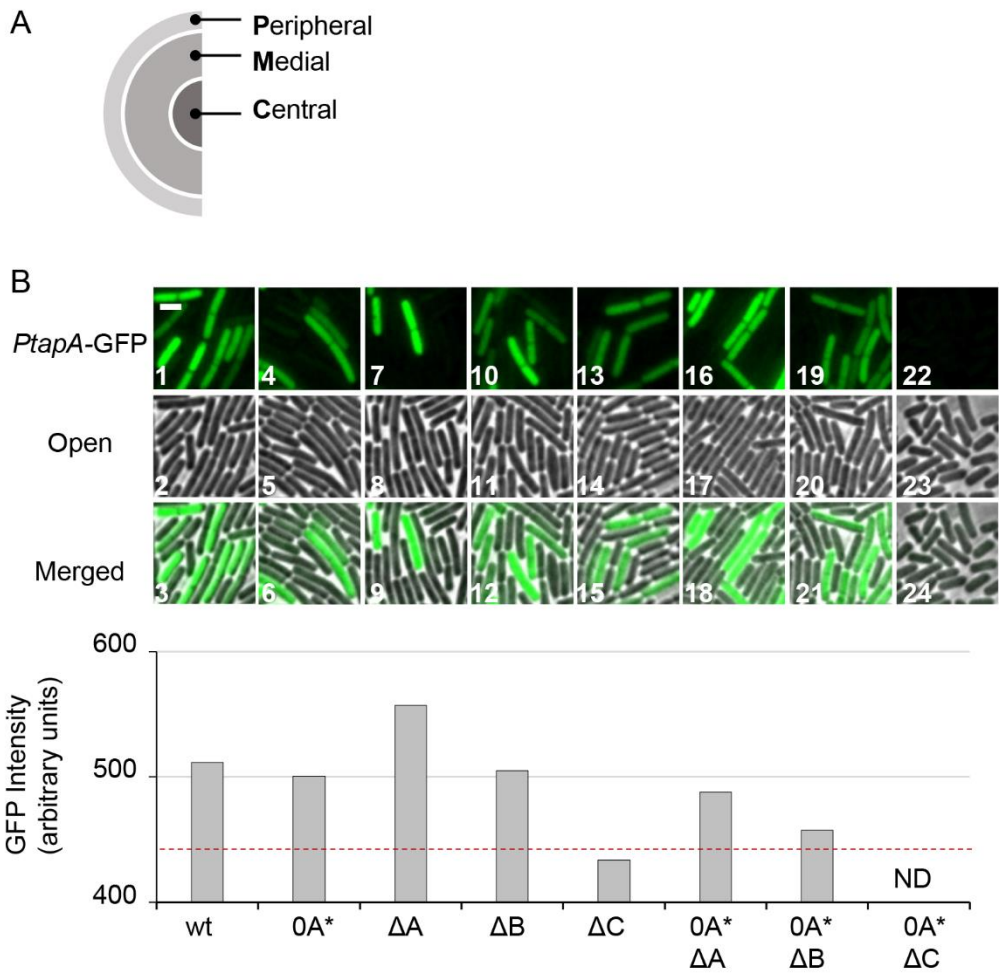


Figure IV-12 Fluorescent microscope analysis of biofilm formation in the wild type (wt), cells expressing reduced level of Spo0A, and various kinase mutants.
B. subtilis cells expressing *PtapA*-GFP, the biofilm promoter *PtapA* fused to the GFP reporter gene were grown on MSgg agar for 37 °C for 24 h. Cells were taken from the peripheral (P), medial (M), and central (C) zones of each colony, as indicated in the schematic drawing of the colony at the top left, and observed under the fluorescent microscope. Scale bar is 2 μm.

IV.iii.6 Cells expressing the reduced levels of Spo0A in the *kinC* null mutation background become defective in growth under the solid biofilm medium conditions

As previously demonstrated and confirmed in the present study, biofilm formation was significantly decreased in the *ΔkinC* strain, but neither in the *ΔkinA* nor *ΔkinB* strain (Devi, Vishnoi, et al., 2015). In contrast, we found that the P_S-*spo0A* strain formed biofilms indistinguishable from the wild type strain (Figure IV-4B, panels 6, 15, and 24). Based on these findings and previous data, we hypothesized that KinA and KinB are dispensable for biofilm formation in the P_S-*spo0A* strain. We found that cells of the P_S-*spo0A* and *ΔkinA*, and P_S-*spo0A* and *ΔkinB* strains exhibited biofilm formation on solid MSgg plate, similar to those of the wild type strain (Figure IV-4B, panels 7, 8, 9, 16, 17, 18, 25, 26, and 27).

Furthermore, by the use of fluorescence microscopy, we also analyzed the expression of biofilm reporter –*tapA* at a single cell level among the wild type and strains producing reduced levels of Spo0A. As shown in Figures IV-11, we found that the strains, P_S-*spo0A* formed biofilm similar to the wild type strain. While, the *ΔkinA* and P_S-*spo0A* and *ΔkinB* and P_S-*spo0A* strains exhibited lower levels of *tapA* expression than the wild type strain. In contrast, *ΔkinC* and P_S-*spo0A* strain showed no *tapA* expression at all. Furthermore, we found that cells of the P_S-*spo0A* and *ΔkinC* strain exhibited severe defects in colony formation on solid MSgg plate (Figure IV-4B, panel 27), while they grew well, similar to the wild type cells in MSgg liquid medium (Figure 13C). We further found that the P_S-*spo0A* and *ΔkinC* strain formed colonies on solid LBGM plate, but did not form biofilm, similar to the *ΔkinC* strain (Figure 13D). These results prompted us to examine whether carbon

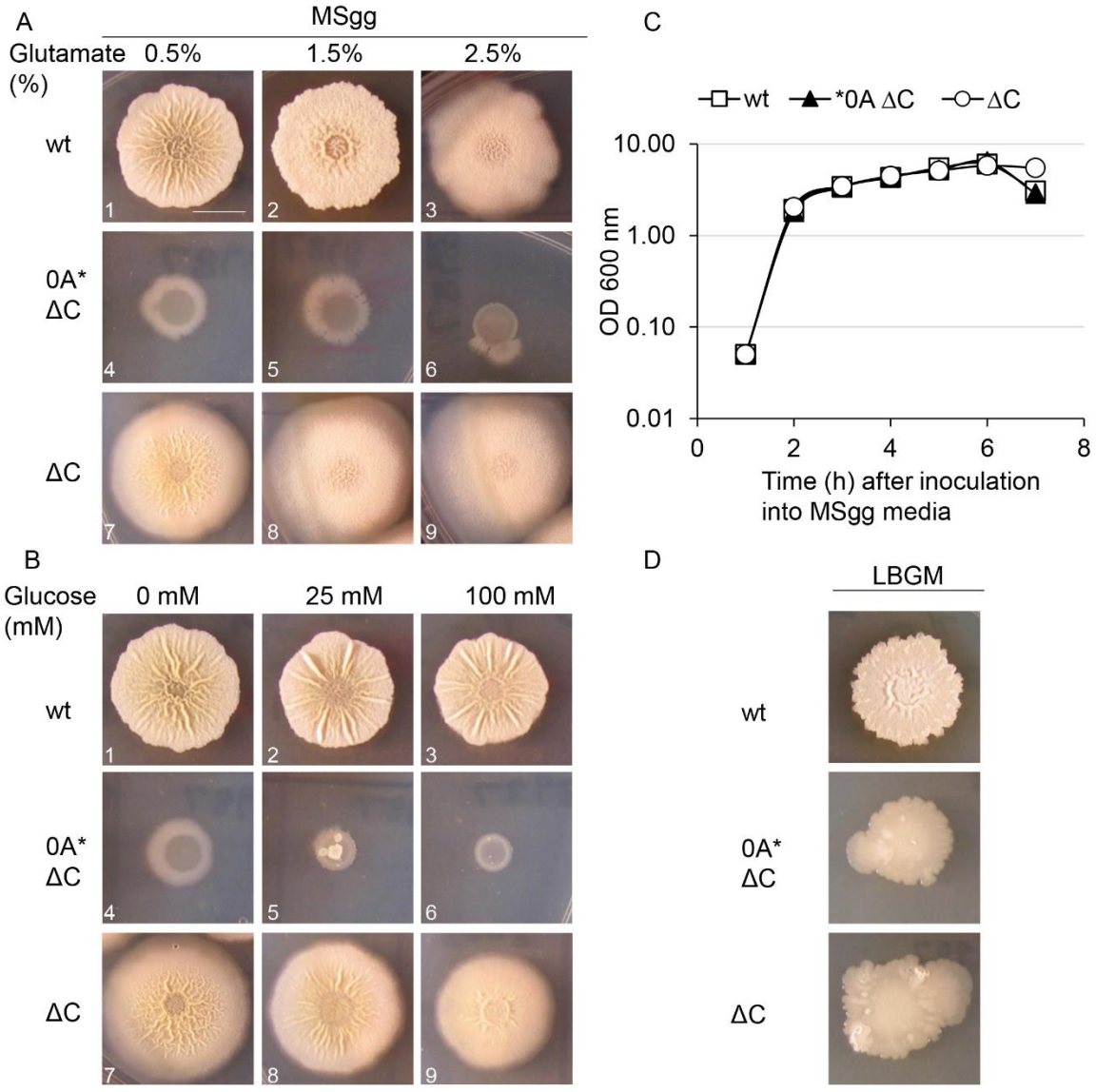


Figure IV-13 Addition of glutamate or glucose could not rescue the growth defect in the strain producing reduced level of 0A and lacking *kinC*.
Colony morphology on MSgg medium for wild type (wt), ΔkinC mutant, and 0A* ΔC in the presence of various concentrations of (A) glutamate and (B) glucose were compared. (C) Growth curve of the strains in MSgg liquid medium with shaking. (D) Colony morphologies of the above strains on LBGM solid medium. Scale bar, 10 mm.

and nitrogen sources have effects on colony formation of the *P_S-spo0A* and *ΔkinC* strain. We found that the colony formation in this strain was not restored in the presence of varying concentrations of glucose and/or glutamine as shown in Figures 13A and 13B. The exact causes of the defective colony formation phenotype in the *P_S-spo0A* and *ΔkinC* strain are currently unknown.

IV.iii.7 A starving *B. subtilis* population contains phenotypically distinct progenitors that individually give rise to biofilm and spore forming cell types

A previous report showed that, within a biofilm, sporulating cells arised from matrix-producing cells, by using fluorescent reporter of the marker genes for biofilm and sporulation pathways (Vlamakis et al., 2008). As shown in Figures II-5A, II-5B, II-6A, and II-6B in this study, we observed that reporter activities for *PtapA-lacZ* (biofilm reporter) and *PspoIIG-lacZ* (sporulation reporter) exhibited distinct profiles, depending on two culture conditions, biofilm formation and sporulation. Our data suggested a possibility that starving cells undergo two distinct cell fates, biofilm formation and sporulation. To test this possibility, cells harboring *Phag*-mCherry (motility reporter), *PtapA-cfp*, and *PspoIIA-yfp* were grown on solid MSgg plate and colony morphologies and profiles of the reporter gene expressions were observed under fluorescent microscope. Colonies were grown on solid MSgg plate and allowed to form biofilms. Then, cells were periodically taken out from three distinct areas of the single colony, designating peripheral, medial, and central from the outside to the inside of the colony radial. As shown in Figure II-9 in this study, at 36 h of incubation, for example, CFP signals derived from the *PtapA-cfp* were

preferentially detected in the chaining cells localized in the peripheral region than the non-chaining cells localized in the other regions (Figure II-9, panels 1, 4, 7, and 10). By contrast, YFP signals derived from the *PspoIIA-yfp* were preferentially detected in the non-chaining cells localized in the medial and central regions than the peripheral region (Figure II-9, panels 2, 5, 8, and 11). In the *ΔkinA* strain, CFP signals derived from the *PtapA-cfp* were detected similarly to the wild type strain, but little or no YFP signals derived from the *PspoIIA-yfp* were detected. In the *ΔkinC* strain, less numbers of chaining cells were detected and CFP signals derived from the *PtapA-cfp* were mainly detected in the non-chaining cells but at lesser frequencies. YFP signals derived from the *PspoIIA-yfp* were preferentially detected in the non-chaining cells localized in the medial and central regions than the peripheral region. mCherry signals derived from *Phag-mcherry* were predominantly expressed in the non-chaining cells, and in some cases YFP signals derived from the *PspoIIA-yfp* were detected in the cells expressing mCherry. In the *ΔkinB* strain, CFP signals derived from the *PtapA-cfp* were similar, but slightly lesser degree with the wild type strain, while more cells expressed mCherry signals derived from *Phag-cfp* and YFP signals derived from the *PspoIIA-yfp* than the wild type.

Taken together, our data suggested that biofilm and spore forming cell types are originated from individual starving cells as independent cell lineage. Therefore, even the genes involved in biofilm formation are expressed within the same cell, the timing and level are important to complete the cell fate determination, whether

biofilm formation or sporulation. Further detailed cell lineage characterizations in a quantitative manner are required to conclude these possibilities.

IV.iv Discussion

We constructed a genetically engineered *B. subtilis* strain that produced reduced cellular concentration of Spo0A than that in the wild type strain. Our engineered cells allowed formation of biofilm but not spores. One of the major technical advances achieved in this study is that the critical cellular threshold levels of Spo0A for cell fate decision, whether to form biofilm or spores, are experimentally determined. The data presented in this paper directly demonstrated that genes involved in biofilm formation are induced by relatively low cellular levels of Spo0A, leading to biofilm formation, but not sporulation. Collectively, the present data demonstrates that the newly developed system is useful to investigate the mechanisms by which Spo0A dynamics regulate cell fate decision making between biofilm formation and sporulation. Thus, these results supported the previously proposed model in which the threshold levels of Spo0A determined cell fate by controlling gene expression programs involved in biofilm formation and sporulation.

IV.iv.1 Control of cell fate decision making by KinA and KinC

It has been demonstrated that KinA preferentially activates the sporulation pathway (Eswaramoorthy et al., 2010), while KinC predominantly activates the biofilm pathway (Devi, Kiehler, et al., 2015). However, the mechanisms by which

each of two similar kinases selectively controls the distinct cell fate decision making through the same phosphorelay pathway have not yet been elucidated. Our data indicated that genes involved in each of the biofilm and sporulation pathways were differentially expressed under each of the favorable conditions. On one hand, under biofilm conditions (MSgg), expression of the biofilm marker gene (*tapA*) increased gradually over the course of incubation (Figure IV-5B). In contrast, under sporulation conditions, the peak of the biofilm marker gene expression shifted to early times, but the peak expression levels were lower than those under biofilm conditions (Figure IV-6B). On the other hand, under sporulation conditions, expression of the sporulation marker gene (*spoIIG*) started at early times of starvation (Figure IV-6A). In contrast, under biofilm conditions, the *spoIIG* expression levels gradually increased over the course of incubation, but the peak expression levels were lower than those under sporulation conditions (Figure IV-6B). KinC is known to be transcribed by σ^A-RNAP at relatively early times of starvation and repressed by Spo0A~P at later times (Fujita & Sadaie, 1998; Kobayashi et al., 1995; LeDeaux & Grossman, 1995). In contrast, *kinA* is transcribed by σ^H-RNAP and repressed by Spo0A~P (Fujita & Losick, 2005; Fujita & Sadaie, 1998; Molle et al., 2003; Predich et al., 1992).

Based on these observations and facts, the peak levels and timings of these two kinases appeared to be dynamically regulated, leading to kinetically distinct Spo0A~P activation levels in response to culture conditions. More specifically, we speculated that KinC is predominantly active to produce relatively low levels of

Spo0A~P under biofilm conditions and, under sporulation conditions, KinA becomes more active than KinC to produce relatively high levels of Spo0A~P.

IV.iv.2 Control of biofilm formation by the Spo0A-specific phosphatase, Spo0E

The results of this study add new evidence showing that Spo0E plays an important role in biofilm formation by modulating Spo0A~P levels at early times of starvation. The Spo0A~P specific phosphatase Spo0E has been identified and characterized in great detail in controlling sporulation. The *spo0E* gene is transcribed by σ^A-RNAP and repressed by AbrB (Perego & Hoch, 1991; M. A. Strauch et al., 1989). Therefore, during growth, under nutrient rich conditions, Spo0E and its target Spo0A~P levels are not high. In turn, during transition from nutrient rich to starvation conditions, both of these two levels increase through phosphorelay (Fujita & Sadaie, 1998; Predich et al., 1992). Based on these regulatory schemes, it is proposed that Spo0A~P levels gradually increase, but do not become too high, leading to proper gene expression programs for biofilm formation and sporulation (Devi, Kiehler, et al., 2015; Fujita et al., 2005).

However, the disruption of the *spo0E* gene shows little or no effect on sporulation efficiency (Perego, 2001), and inhibitory effects on sporulation are observed only when Spo0E is artificially overexpressed (Perego & Hoch, 1991) or the hyper active mutant form is expressed (Ohlsen et al., 1994). Therefore, it has been thought that Spo0E phosphatase acts as an auxiliary fine-tuning device rather than as a primary regulator to control entry into sporulation (Veening, Hamoen, & Kuipers, 2005). We observed that biofilm formation was stimulated in the absence

of Spo0E as compared with the wild type strain (in the presence of Spo0E) (Figure IV-7C). Spo0E expression levels were significantly higher under biofilm conditions than under sporulation conditions (Figures IV-7A and IV-7B). In contrast, Spo0A protein levels in cells cultured under biofilm conditions were lower than those under sporulation conditions (Figures IV-3A and IV-3B). Among three kinases, biofilm formation and the reporter activities were lower in the *kinC* mutant strain (*ΔkinC*) than in the wild type and other two mutant (*ΔkinA* and *ΔkinB*) strains.

These observations can be explained by the assumption that, at early stages of nutrient starvation in MSgg, Spo0A~P (phosphorylated form, not Spo0A) levels increase to an intermediate level, stimulating specifically the expression of genes involved in biofilm formation, but not the genes involved in sporulation. In the meantime, the intermediate Spo0A~P represses *abrB* gene expression, leading to derepression of *spo0E* gene expression. As a result, Spo0E dephosphorylates Spo0A~P, playing a role in keeping the Spo0A~P levels not too high under biofilm conditions. These results suggest that the low-threshold Spo0A-controlled biofilm genes are more sensitively modulated than the high-threshold Spo0A-controlled sporulation genes by Spo0A~P. Our data also suggests that RapA phosphatase neither significantly affects biofilm formation nor sporulation under our tested conditions, although the *spoIIG* reporter activities are slightly changed as compared to those in the wild type strain.

IV.iv.3 Biofilm-forming and sporulating cell lineages appear to arise independently upon starvation

A prior study demonstrated that different cell types, motile, matrix-producing and sporulating, coexist within the biofilms (Vlamakis et al., 2008). Interestingly, it was shown that most sporulating cells arise from matrix-producing cells using fluorescent reporters of the marker genes for these two pathways (Vlamakis et al., 2008). We note that their fluorescent microscopy assays are qualitative rather than quantitative (Vlamakis et al., 2008). As shown here, our data indicated that genes involved in biofilm formation and sporulation expressed at different levels and time points each other (Figure II-9). Furthermore, we found that little or no cells expressed both of these two genes (Figure II-9). These results might suggest an alternative possibility that, even the genes involved in biofilm formation or sporulation are expressed in a cell, the timings and levels of these gene expressions are important to direct appropriate cell fate decision making.

Therefore, we propose a new model in which (1) gene expressions for biofilm formation and sporulation take place in distinct cells at different timings during starvations; (2) biofilm formation occurs only in a certain number of cells with relatively low levels of Spo0A~P under modest starvation conditions at early times of culture; (3) sporulation occurs in a portion of the remaining cells with relatively high levels of Spo0A~P under prolonged starvation conditions; (4) when cells are immediately exposed to harsh starvation conditions, the majority of cells in a population directly undergo sporulation, bypassing the biofilm formation pathway altogether.

IV.iv.4 Model for regulatory pathways and perspectives

Based on our findings and previous data, our proposed model explains three different cell phases depending on the concentration of Spo0A~P (Figure IV-14A). (1) Low Spo0A~P: Under rich medium conditions, cells grow rapidly and the housekeeping σ^A-RNAP transcribes *abrB* gene, leading to an increase in AbrB. Under such conditions, KinA and KinC do not reach sufficient levels to allow the generation of Spo0A~P (Eswaramoorthy et al., 2010; Narula, Fujita, et al., 2016). The resulting high levels of AbrB repress genes involved in biofilm formation and sporulation, including *tapA* (Stover & Driks, 1999) and *sigH* (gene for σ^H) (Chumsakul et al., 2011; M. A. Strauch, 1995)Thus, cells keep growing. (2) Intermediate Spo0A~P: Under biofilm forming conditions, increasing *kinC* gene expression under the control of σ^A-RNAP leads to a rise in cellular KinC levels (Fujita & Sadaie, 1998; Kobayashi et al., 1995; LeDeaux & Grossman, 1995), while the level of KinA remains below threshold and its activity is inhibited by Spo0F (Eswaramoorthy et al., 2010; Narula, Fujita, et al., 2016; Narula et al., 2015).

Under these conditions, Spo0A~P levels increase slowly, leading to the slow decrease and increase of AbrB and SinI (antagonist of SinR) levels, respectively (Perego, Spiegelman, & Hoch, 1988). As a result, the repressive effects of AbrB and SinR are removed and the expression of genes controlling biofilm formation increases (Chai, Kolter, & Losick, 2009; Chai et al., 2010; Chai, Norman, Kolter, & Losick, 2011; Norman, Lord, Paulsson, & Losick, 2013; Winkelman et al., 2013). In the meantime, cell motility is reduced due to decreased levels of σ^D (Fujita et

al., 2005; Molle et al., 2003) and SinI·SlrR, which are required for expression of motility genes (Chai et al., 2010; Cozy et al., 2012; Norman et al., 2013). With the intermediate levels of Spo0A~P, sporulation genes are not expressed at a sufficient level to trigger sporulation (Fujita et al., 2005). (3) High Spo0A~P: Under sporulation conditions, KinA levels rise to lead increased levels of Spo0A~P required for expression of genes involved in sporulation (Eswaramoorthy et al., 2010; Narula, Fujita, et al., 2016; Narula et al., 2015). In this regulatory network, the increased levels of KinA stimulate the phosphorelay to produce high levels of Spo0A~P. The high level of Spo0A~P represses the expression of genes encoding σ^D (Fujita et al., 2005; Molle et al., 2003), and KinC (Fujita & Sadaie, 1998), leading to a decline in cell motility and the loss of KinC input to the phosphorelay pathway.

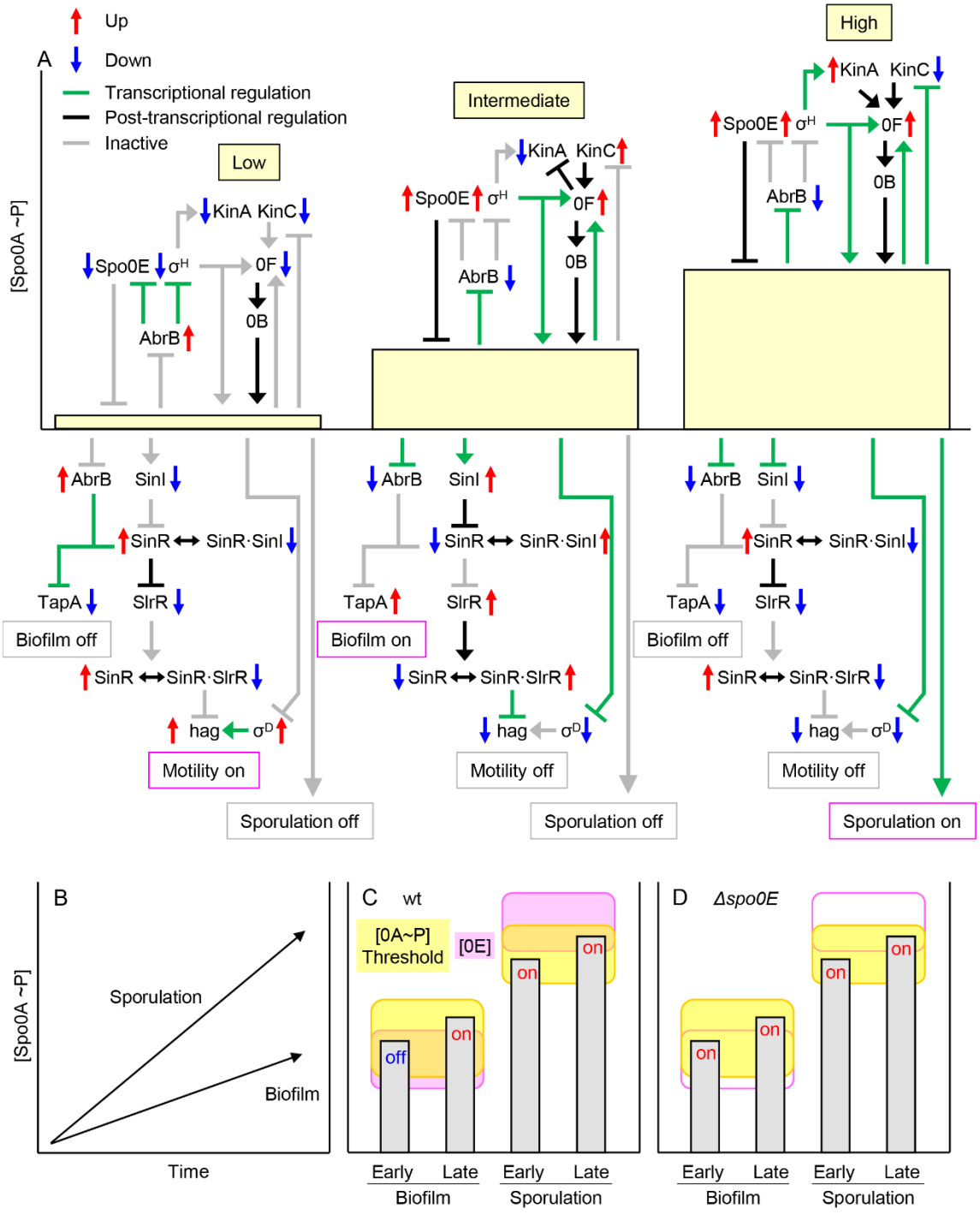
Finally, the expression of genes controlling sporulation is triggered under these conditions, resulting in sporulation (Fujita et al., 2005). At this stage, *sinI* expression is reduced even with high levels of Spo0A~P, leading to repression of biofilm gene expression by SinR (Chai et al., 2008). As a result, the sporulation pathway becomes favored over biofilm formation (Figure IV-14A). Our data indicated that Spo0A~P levels increase more rapidly under sporulation conditions than under biofilm conditions (Figure IV-10 and IV-14B). Taken together, our data suggest that, under biofilm conditions, genes involved in biofilm formation are expressed slowly and gradually over the course of culture, while genes involved in sporulation are expressed only at later times of culture. However, spores originate from non-biofilm forming cell lineages. Furthermore, we speculate on the mechanisms of Spo0E controlling biofilm formation; at early times of biofilm

conditions, Spo0E limits Spo0A~P levels insufficient for biofilm formation (Figure IV-14C). Without Spo0E, Spo0A~P achieves high enough levels to trigger biofilm formation even early in the culture period (Figure IV-14D).

Our data suggest that, contrary to popular assumptions (Lemon et al., 2008; D. Lopez, Fischbach, et al., 2009), cells expressing genes for biofilm formation (matrix-formation) and sporulation arise from two distinct populations of precursor cells. Under biofilm conditions, cells forming chains produce relatively low levels of Spo0A~P slowly and gradually over the course of culture, leading to the expression of genes involved in biofilm formation. In a culture population under biofilm conditions, some of the non-chaining cells produce relatively high levels of Spo0A~P, leading to expression of genes involved in sporulation. Under sporulation conditions, many cells in the population are non-chaining, leading to gradual increase in Spo0A~P. However, this gradual increase in Spo0A~P is faster under sporulation conditions than under biofilm conditions (Figure IV-10 and IV-14B). Thus, under sporulation conditions, genes involved in biofilm formation are expressed at intermediate levels of Spo0A~P during the course of sporulation culture, but the timings are shifted earlier than under biofilm conditions, leading to insufficient biofilm formation. Thus our data suggest that timing of Spo0A-regulated gene expression is important for correct progression of cell differentiation.

Figure IV-14 Schematic model for gene networks controlling biofilm and spore formation.

(A) Low Spo0A~P: under nutrient rich conditions, cells rapidly grow and Spo0A synthesis is off, leading to cell motility. Intermediate Spo0A~P: during early times of starvation, cells produce Spo0A~P at intermediate level through KinC phosphorelay. Under such conditions, biofilm formation is stimulated by up-regulated SinI, and down-regulated AbrB and σ^D, while motility and sporulation are off. High Spo0A~P: Under prolonged starvation conditions, cells produce Spo0A~P at high level through KinA phosphorelay. Under such conditions, genes involved in sporulation are stimulated, leading to sporulation, while motility and biofilm formation are off with low levels of AbrB, SinI, and σ^D. (B) Spo0A~P increases more rapidly under sporulation conditions than under biofilm conditions. (CD) Hypothetical control mechanisms of biofilm formation and sporulation by threshold levels of Spo0A~P. In the wild type strain (C), at early times of biofilm conditions, Spo0A levels are at around the threshold for biofilm formation, but Spo0E is also high and acts on Spo0A~P, leading to the reduced Spo0A~P to the insufficient levels for biofilm formation. At later times of biofilm conditions, Spo0A~P levels become sufficiently high to trigger biofilm formation. Under sporulation conditions, Spo0A~P levels are sufficient to trigger sporulation, but are limited to not too high levels by Spo0E phosphatase. In the *spo0E* mutant strain (D), at early times of biofilm conditions, Spo0A~P increases to sufficient levels to trigger biofilm formation.


A
Up
Down
Transcriptional regulation
Post-transcriptional regulation
Inactive
[Spo0A ~P]
Low
Intermediate
High
KinA KinC
Spo0E σH
0F
0B
AbrB
SinI
SinR ↔ SinR·SinI
TapA
SlrR
SinR ↔ SinR·SlrR
hag
σD
Biofilm off
Biofilm on
Motility on
Motility off
Sporulation off
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Chapter V: Summary and Future Perspectives

In the current study, described in this dissertation, I established microscopic methods to provide insights into spatiotemporal regulation of cell fate decision making process in a developing biofilm colony (Chapter II). I also identified the tentative roles of histidine kinases, KinA and KinC, in regulating the dynamics of activation of master transcription regulator Spo0A (Chapter III). Besides this, I also investigated how Spo0A regulates the mechanisms of cell-fate determination (Chapter IV).

In Chapter II, to address the issue of spatiotemporal regulation of cell fate decision, we constructed a three color fluorescent reporter strain in which transcription reporters of cell motility (*hag*), sporulation (*spoIIA*), and biofilm formation (*tapA*) were fused to mcherry, YFP, and CFP fluorescent reporters respectively. To determine the roles of different kinases on cell motility, sporulation, and biofilm formation, we also constructed kinase deletion mutant strains, in which one of the kinases, either *kinA, kinB,* or *kinC* was deleted and transcriptional reporters of cell motility, sporulation, and biofilm formation were fused to mcherry, YFP, and CFP as described above for the wild type strain. I analyzed and compared the expression profiles of cell motility, sporulation, and biofilm formation in the colonies developed from the cells expressing the above fluorescent reporters at 2X and 100X microscope objective lenses. The 2X microscopic analysis revealed that the genes for cell motility are the first to be expressed during the growth of a biofilm colony, followed by genes for biofilm formation and sporulation. The 100X analysis of the colony at the peripheral region of the colony revealed that cell motility and biofilm formation are mutually exclusive

processes. Genes for cell motility and biofilm formation were not found to be co-expressed. We also established that cells expressing genes for biofilm formation are found in long chains of cells while the cells expressing the gene for cell motility do not require long chains of cells. As *kinC* is proposed to have an important role in biofilm formation, our results strengthened this notion, as the *ΔkinC* deletion strain exhibited less expression of biofilm forming gene, *tapA* and showed least chaining among wild type, *kinA*, *kinB*, and *kinC* strains.

Biofilm colonies comprise of many multilayered structures. To gain an in-depth knowledge of cell fate decision making process, we needed to examine these multilayered structures on a single-cell level. By the use of a novel technique, in which cells were scratched from peripheral (P), medial (M), and central (C) parts of the colony, we were successfully able to examine the multilayered structures at a single-cell level. Results from these studies provided a time-line of events taking place in the development of a biofilm colony. Our results implied that during the first 24 h of colony development, reporters for motility and biofilm formation were expressed throughout the colony while sporulation reporter was found in the medial and central parts of colony. We also found that the sporulation reporter was not expressed in the *ΔkinA* strain which provides support to previously published studies in our lab that KinA has a major role in sporulation process.

Our results also revealed that the reporter for biofilm formation was least expressed in the *ΔkinC* strain, which further reinforces the importance of KinC in the biofilm formation. We also demonstrated that the *ΔkinC* strain, which lacked colony architecture, had more number of spores than the wild type. These results

are contradictory to the previously published study that colony architecture is required for sporulation (Lemon et al., 2008). As the colony size increased, the expression of sporulation reporter increased while the expression of biofilm reporter decreased. At later stages of development of a biofilm colony, reporters for cell motility can be visualized throughout the colony while the sporulation reporter was localized to the peripheral and medial parts of colony. The biofilm reporter was only localized at the peripheral regions of the colony suggesting an important role of matrix production in order to adapt to changes in environment around the colony.

Confocal microscopy analysis of the wild type and the colonies of various kinase mutants in future can provide insight into colony architecture, thickness and biovolume of the colonies. Time-lapse studies focused on cell differentiation processes can also provide more information regarding the lineage of heterogeneity in biofilm formation. Future time-lapse studies can determine how the biofilm forming cells and sporulating cells originate within the biofilm. Furthermore, these studies can help understand and answer the questions whether inhibition of cell motility and switching from motile cells to matrix producing cells is required for biofilm formation.

In Chapter III, I analyzed the roles of histidine kinases, KinA and KinC in regulating the activation of Spo0A to affect the downstream processes of biofilm formation and sporulation. A gradual increase in the level of activation of Spo0A is essential for proper expression of biofilm and sporulation related genes. KinA is mainly involved in sporulation while KinC has a major role in biofilm formation.

Previous studies on domesticated *B. subtilis* strains revealed that upon artificial induction of KinA (Eswaramoorthy et al., 2010; Eswaramoorthy et al., 2009), and KinC (Devi, Kiehler, et al., 2015), sporulation was achieved even under nutrient rich conditions. However, how biofilm formation and sporulation are affected and regulated in an undomesticated *B. subtilis* strain was unknown. In this study, our findings have bridged the gap of knowledge between sporulation and biofilm processes in domesticated and undomesticated strains. In this study, we utilized genetically engineered strains in which synthesis of KinA and KinC was artificially induced under an IPTG-inducible promoter. We investigated the effect of artificially induced KinA and KinC on biofilm and sporulation process. Our results indicated that artificial induction of KinA and KinC had a greater impact on Spo0A low-threshold gene *tapA* (biofilm formation) than on Spo0A high-threshold gene *spoIIG* for sporulation. We also demonstrated that at higher IPTG concentration, KinC can also induce sporulation similar to wild-type levels.

To further increase our knowledge as how cells are affected at a single-cell level by artificial induction of KinA and KinC, we would like to construct some genetically engineered strains which can be used for microscopic analysis. In these engineered strains, under the background of artificial induction of KinA and KinC, transcriptional reporters for biofilm formation (*tapA*) and sporulation (*spoIIG*) will be fused to green fluorescent protein, GFP. By the use of these strains we can examine the effect of KinA and KinC on biofilm formation and sporulation at a single cell level.

We analyzed the protein levels of KinA and KinC in the strains expressing artificially induced KinA and KinC. Future analyses in determining the Spo0A levels in these strains by the use of anti-spo0A antibodies can provide insights into how KinA and KinC affect the levels of Spo0A.

As mentioned earlier, genes required for sporulation are Spo0A high-threshold genes. It has been speculated that genes required for biofilm formation are Spo0A low-threshold genes. Until now, there has been no direct evidence to demonstrate this. In Chapter IV, we provide direct evidence that the genes required for biofilm formation are Spo0A low-threshold genes. In this Chapter, by the use of a genetically engineered strain which produces a reduced level of Spo0A, we found that biofilm formation in this strain is indistinguishable to the wild type while it is strongly impaired in sporulation. We also constructed a series of strains which expressed reduced levels of Spo0A and in which either *kinA*, *kinB*, or *kinC* is deleted. Our results demonstrated that the strains which produced reduced level of Spo0A under either *kinA* or *kinC* null mutations were found to be severely impaired in sporulation and biofilm formation respectively. These results further confirmed the importance of KinA and KinC in the sporulation and biofilm formation respectively. The strain that produced reduced level of Spo0A under *kinC* null mutation was severely impaired in growth on an MSgg agar plate. However, it did not exhibit any growth defect on another biofilm promoting media, LBGM or when grown under liquid culture conditions. Besides this, our data also uncovered the role of Spo0E, a Spo0A specific phosphatase in biofilm formation as a *spo0E* deletion mutant exhibited stimulated biofilm formation than the wild type strain.

However, the sporulation process was found to be unaffected. Our results might indicate that Spo0E plays an important role in fine tuning the levels of Spo0A to favor biofilm formation.

Future analysis into the mechanisms of how Spo0E fine tunes the levels of Spo0A might shed some light on how one cell differentiation process is favored over another. Biofilm formation is a complex process and is under the control of many genetic networks. Future studies into other factors such as AbrB, which is also one of the regulators for matrix production can also provide insights as how biofilm formation is regulated in *B. subtilis*.

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