Study of histidine kinases involved in *Bacillus substilis* differentiation

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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

Seram Nganbiton Devi

December 2014

Study of histidine kinases involved in *Bacillus substilis* differentiation

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Dedicated to my parents, brothers, sisters and my beloved husband for their support and encouragement.

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to my advisor, Dr. Masaya Fujita, for giving me the opportunity and his relentless guidance, encouragement, support, and valuable inputs throughout this endeavor. I would also like to thank him especially for the valuable inputs towards my dissertation. His readiness to discuss the progress of the work and expert suggestions helped me to complete the studies. In many of the conversations I have had over the last four and half years, he generously shared with me his deep insight about molecular and microbiology techniques to understand biological problems. Without his time and effort, completion of all this work would have been impossible.

I am also immensely thankful to our collaborator Dr. Oleg Igoshin and Mr. Jatin Nurula for their various suggestions and inputs in many of my critical experiments. In many of the conversations we have had over the last three and half years, he generously shared with me his deep insight about mathematical modeling to understand biological problems.

I would also like to sincerely thank my committee members Dr. George Fox, Dr. Jacinta Conrad, and Dr. Tim Cooper. Their time, suggestions and patience have been very helpful.

I am also immensely thankful to Dr. Prahathees Eswaramoorty and Ms. Ashley Eswaramoorty for their relentless suggestions and help in many of my experiments. Dr. Eswaramoorty's suggestions in my initial days at Dr. Fujita's lab were instrumental in guiding my career as molecular microbiologist.

The friendly and co-operative working environment in the Department of Biology and Biochemistry was a great help, during all the years, I have spent here. I would like to thank Drs. William Widger, and Anne Delcour who never hesitated in sharing their expertise on biochemistry and gave extremely valuable suggestions whenever needed. I also want to extend my thanks to my friends in the lab, Brittany Kiehler, Monika Vishnoi, Bina dao, Aditi Nagar, Savini, Lindsey, Rachna, Jane, Joanna. Thanks to other lab members Fotis, Efi, Selvaraj, Geeta, Gayani, Alicia, Igor, and Lakshmi, who have helped me with my dissertation.

Special thanks to all my friends in Houston, Arun, Monal, Bhoom, Shravani, Raj, Meenu, Rocky, Rao, Subhadra, and Ritu for being there always. It is because of them that I never felt I was thousands of miles away from home.

This journey would have been impossible without the support from my family. I am indebted to my grandparents, for the love they showered on me. My dad inspired me with his optimism towards everything and helped me in realizing my aspirations by constant encouragement. My mom has been the most important influence in my life. It is impossible to describe her contribution in a few words. Her excellent vision and deeply thought suggestions have helped me in

becoming what I am now. Thanks also to my sisters for making my life happy and cheerful and my brothers for there constant support and guidance. I would also like to thank my in laws Jayanta(da), Aditi Soma(di) and Surajit(da) for being part of our family. I would also like to thank my parents-in-law for being so friendly and understanding.

Lastly, thanks to my dear husband Prasenjit Dey, who has always extended his helping hands. His love, comfort, and support throughout these years helped me to achieve my goals. I would like to thank him for being my closest friend and my deepest love.

This work was supported by grant from National Science Foudation (NSF), NH-AdvResProg (Texas) and Welch Foundation.

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ABSTRACT

In response to nutrient deprivation, *Bacillus subtilis* cells undergo differentiation to form biofilm and spore. This process requires the involvement of a multiple phosphorelay component, wherein phosphate is transferred to a master transcription regulator Spo0A from five histidine kinases (KinA-KinE) via two intermediate phosphotransferases, Spo0F and Spo0B. It has been proposed that the autokinase activity of one or a combination of five kinases is stimulated when the amino (N) terminal 'sensor' domain of kinase receives an yet unidentified starvation signal(s), leading to the increase of the phosphorylated (active) form of Spo0A (Spo0A~P) via phosphorelay and resulting in differentiation into biofilm formation and sporulation. However, the underlying mechanisms by which the putative starvation signal(s) stimulate the kinase and the subsequent control of Spo0A activity remain elusive. In order to test, we conducted, domain-swap experiments were performed by fusing a tetramer-forming protein derived from *Escherichia coli* to the KinA C-terminal domain. Despite the introduction of a foreign domain, the resulting chimeric protein, in a concentration-dependent manner, triggered sporulation by activating Spo0A through phosphorelay, irrespective of nutrient availability. A simultaneous induction system, in which KinC, a kinase that can directly phosphorylate Spo0A, and Spo0A itself are separately controlled by inducible promoters, were constructed. This artificial two-component system can efficiently trigger

sporulation even under nutrient rich conditions. However, the sporulation efficiency was significantly impaired when KinC and/or Spo0A induction was too high. Lastly, the role of KinC during the course of starvation was studied. Conventional genetic approaches showed that KinC preferentially and positively controls the expression of the cannibalism (a mechanism to delay sporulation) genes during early stage of starvation in a manner dependent on phosphorelay, resulting in the delay of sporulation. Evidence suggests that the N-terminal domain is essential for forming a stable tetramer as a functional kinase, but possibly not for sensing an as-yet unknown starvation signal. Furthermore, the data suggest that, upon starvation, two different levels (low and high) of Spo0A activity are achieved through two distinct pathways regulated by KinC and KinA, which are essential for the proper cell fate decision to differentiate into biofilm or sporulation.

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CHAPTER I: GENERAL INTRODUCTION

I.1 Background of *Bacillus subtilis*

We now stand at the crossroad of a fascinating new field of research upon identification of the function of the genes discovered by the bacterial genome sequencing effort. The genome sequence of *B. subtilis* was completed and published in November 1997 (Kunst, Ogasawara et al. 1997)

B. subtilis is a gram-positive bacterium commonly found in soil. Its superb genetic amenability and relatively large size have provided powerful tools to investigate the dynamic structure and function of single cell organism (Sonenshein 2000). It is an obligate aerobe, but can also survive in anaerobic condition in the presence of nitrates or glucose (Nakano and Zuber 1998). It is motile with flagella, but is not considered pathogenic, toxic, or a disease causing agent (McKenney, Driks et al. 2013).

B. subtilis respond to nutrient limitation by forming biofilm and an endospore, a morphologically distinct cell type (Fig. I.1). Spore development is energy intensive and, once committed, cells may not exit this state for prolonged periods. Thus, this bacterium has evolved mechanisms to delay entry into sporulation as long as possible. It accomplishes this by having the capacity to direct a subpopulation of cells down a differentiation pathway that gives rise to so-called cannibals (Gonzalez-Pastor, Hobbs et al. 2003) Ellermeier, Hobbs et al. 2006) Claverys and Havarstein 2007). Cannibal cells are resistant to two toxins, Skf and Sdp, that are secreted to kill a fraction of their siblings. As a result, cannibals overcome nutritional limitation and delay the onset of sporulation. In

addition to spores and cannibals, *B. subtilis* can undergo several other developmental processes. For instance, within multicellular aggregates known as biofilms a subpopulation of cells differentiates to produce an extracellular matrix that encases the community (Branda, Chu et al. 2006) (Chu et al., 2006) Chai et al., 2008) (Vlamakis et al., 2008). It was recently identified the lipopeptide surfactin as a 'quorum-sensing' molecule produced by *B. subtilis* that appears to activate the membrane histidine kinase KinC with the consequent phosphorylation of Spo0A (Lopez, Vlamakis et al. 2009). Cannibalism and matrix production are both activated by low levels of Spo0A~P (Fujita, Gonzalez-Pastor et al. 2005). The three developmental pathways described above for *B. subtilis*, sporulation, cannibalism and matrix production, are strongly interconnected. They are activated by the same master regulatory protein, Spo0A (Gonzalez-Pastor, Hobbs et al. 2003, Fujita, Gonzalez-Pastor et al. 2005, Kearns and Losick 2005, Branda, Chu et al. 2006). However, Spo0A does not regulate these three pathways identically. For instance, the subpopulation of sporulating cells, which derives from the subpopulation of matrix-producing cells, shows different spatiotemporal distribution within the biofilm when compared with the matrix producers (Vlamakis, Aguilar et al. 2008). Spo0A initially produce the regulatory protein at a low level that is sufficient to activate genes involved in specialized processes, such as cannibalism and biofilm formation, but not spore formation. Cells that have activated Spo0A enter a state in which the transcription factor accumulates to high levels, thereby unleashing the expression of genes critical for the initial stages of spore formation. Finally, at intermediate stages of

sporulation, Spo0A accumulates to very high levels in a cell-specific manner when it promotes gene expression in the mother cell compartment of the sporangium.

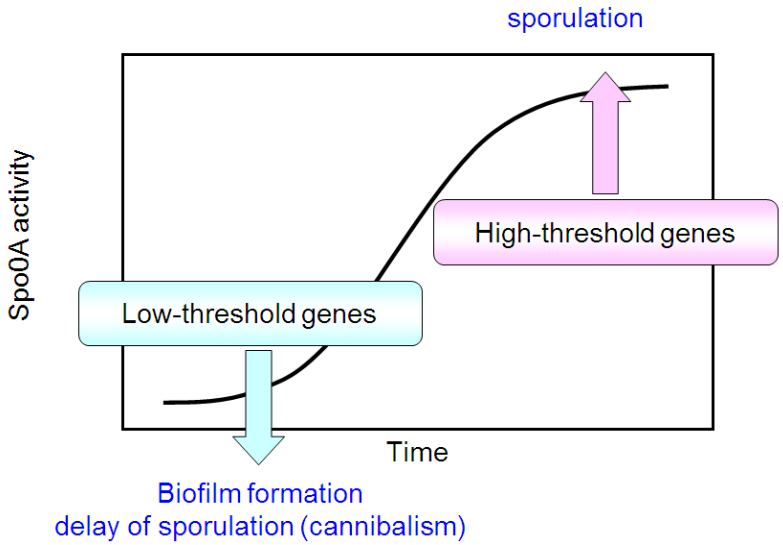

Figure I.1: Low and high threshold gene expression

Different genes are turned on or off at different times as Spo0A levels progressively increase, with genes that do not contribute to sporulation directly, such as genes involved in cannibalism and biofilm formation, being activated earlier and at lower doses of Spo0A than genes that play a direct role in spore formation.

The process of sporulation has been the subject of continuous microbiological investigation since the seminal 1876 reports of Robert Koch and Ferdinand Cohn (Bednarski and Bednarska 2003). Bacteria use multiple strategies to overcome environmental challenges. Some frequently used survival strategies involve alteration in gene expression that temporarily alters the phenotype of a cell. Another eloborate and advanced mechanism of stress response is sporulation, wherein the bacterial species is sequestered into a

spore until environmental conditions become condusive for the spores to germinate and return to vegetative state. The mother cell nurtures the endospores formed within itself, which eventually lyse to release the spore into the environment. The endospore is metabolically dormant and capable of surviving extreme temperature, desiccation and ionizing radiation (Nicholson, Munakata et al. 2000).

I.2 General functions of *B. subtilis*

There are several uses for *B. subtilis* and the enzymes it produces. It can be used to produce the enzymes- proteases and amylase (Ara, Ozaki et al. 2007). Prior to the advent of the cheaper industrial-scale production of antibiotics in early 20th century *B. subtilis* was widely used as a broad-spectrum antibiotic (Stephens 1998). In certain parts of Western Europe and the Middle East it is still used in alternative medicine. The proton-binding properties of *B. subtilis* can be exploited for decontamination of hazardous chemical such as degradation of radioactive waste (Ara, Ozaki et al. 2007). Other commercial applications of *B. subtilis* include cleaning agents in detergents, in de-haring and batting in the leather industry, in the production of special Japanese and Korean food, starch modification, the de-sizing of textiles, and other specialized chemicals (Feng, Liu et al. 2007). *B. subtilis* (natto) is also used in the production of Natto, a traditional Japanese dish of fermented soya beans. *B. subtilis* also produces some fungicidal compounds, which are being investigated as control agents of fungal

pathogens. It is currently being used as a fungicide for plant and ornamental seeds as well as various agricultural seeds (Feng, Liu et al. 2007).

I.3 Sporulation in *B. substilis*

Sporulation in *B. subtilis* is primarily induced by starvation, although other minor factors can trigger initiation of sporulation. The sporulation developmental program is not initiated immediately when growth slows due to nutrient limitation. This is because of the irreversible nature of sporulation and demand for great deal of time and energy. The cell monitors its surroundings efficiently and ensures that sporulation is embarked upon at only the most appropriate times. An early stage in the process is an asymmetrically located division, which divides the bacterium into two unequal cells: the larger mother cell and the smaller prespore (also called the forespore) (Figure I.2). The electron micrograph of a *B. substilis* vegetative and sporulating cell is shown in (Figure I.3). The initiation of sporulation is controlled by a network of regulatory proteins. The major protein involved in the initiation of sporulation is Spo0A, which act as a transcription factor to carry out spore formation.

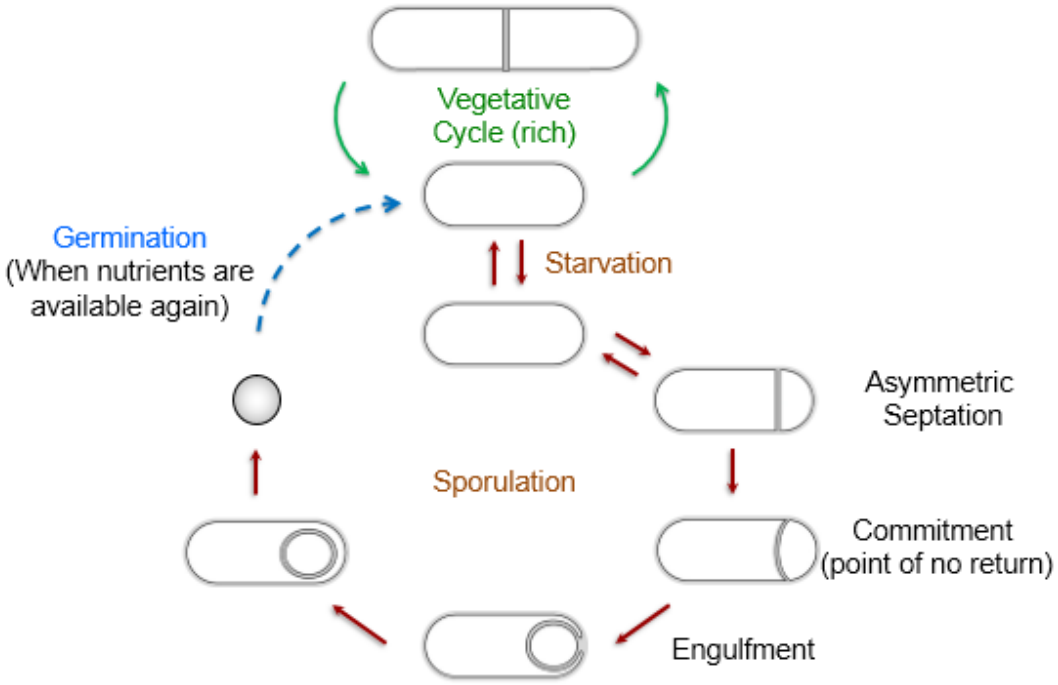

Figure I.2: Cell cycle of *B. substilis*

Cells divide by binary fission during nutrient rich condition. Upon starvation, sporulation begins with an asymmetric cell division to produce smaller forespore and larger mother cell compartments. Next, the mother cell engulfs the forespore, and following membrane fission at the opposite pole of the sporangium, a double-membrane bound forespore is formed. In the final step, the mother cell lyses to release a mature spore into the environment. Spores are capable of quickly germinating and resuming vegetative growth in response to nutrients.

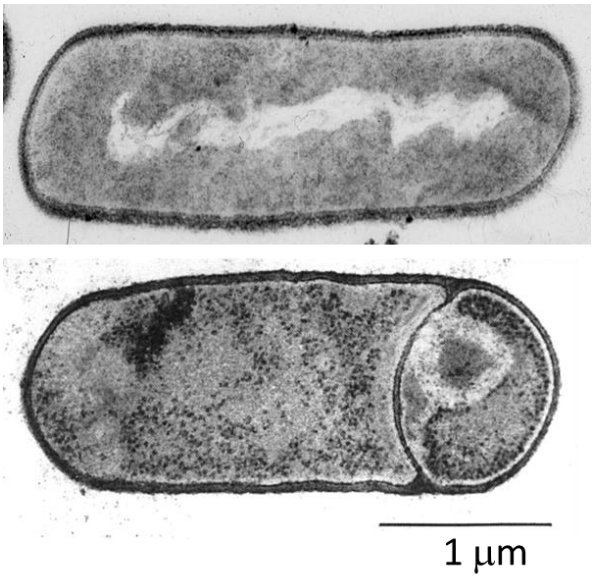

Figure I.3: Electron micrograph of *Bacillus substilis* top-growing cell and bottom- sporulating cell.

I.4 Physical properties of spores

Spores are morphologically different from growing cells. This morphological difference eminates with an asymmetric division near to one pole of the cell, resulting in the formation of a smaller cell, the forespore and a larger cell, the mother cell. In stark contrast to earlier reports, recent study indicates, that this septation can occur with equal probability near the old or new pole (Veening, Stewart et al. 2008). The sporulation septum is unique in physical and chemical properties, and contains a thinner layer of peptidoglycan separating the two compartments but maintain certain similarity to the normal mid-cell division septum. The septum is made of specialized protein and some of the proteins are present on only one side of the septum. Changes in gene expression underlie

morphological differentiation in both the pre-divisional sporangium and later in the two compartments (Figure. I.4).

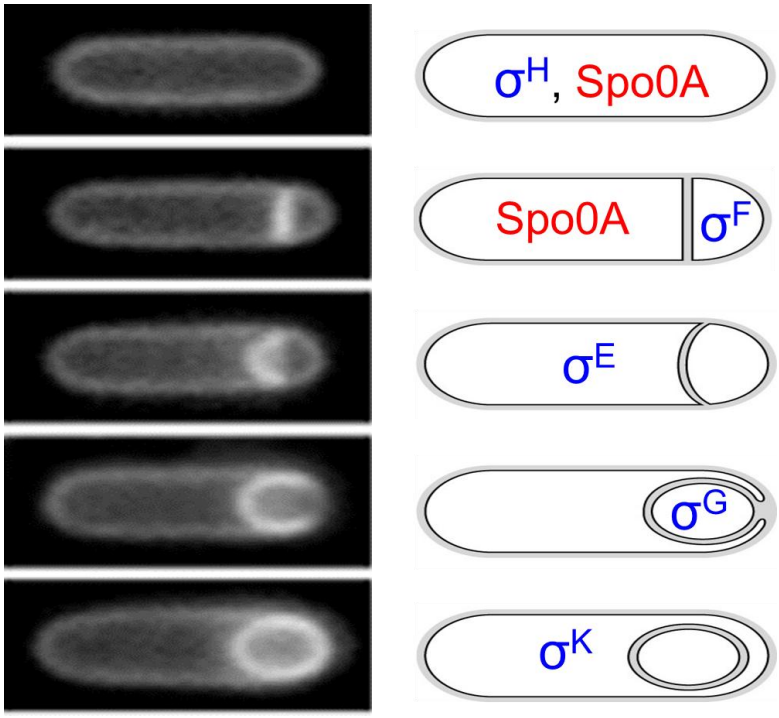

Figure I.4: Coupling of gene transcription and spore morphogenesis in *Bacillus substilis*

Following entry into sporulation, the cells form an asymmetric septum, which then procced to curve out, it eventually becomes distorted and surrounds the smaller cell. This process of engulfment is completed by the loss of attachment between the forespore and the mother cell.. These morphological changes are accompanied by activation of specific transcription factors in each compartment. Initially, σ^F is activated only in the forespore, followed by σ^E in the mother cell, σ^G in the forespore and finally σ^K in the mother cell. The transcription factors involved in regulating the compartment-specific gene expression is indicated. This image was kindly provided by Dr. David Rudner (Harvard Medical School).

I.5 Molecular mechanism of sporulation initiation

Sporulation in *B.substilis* is governed by a multi-component phosphorelay involving two phosphotransferases in addition to histidine kinase and its cognate response regulator. At least three histidine protein kinases KinA, KinB and KinC are involved in the initiation of sporulation (Weinrauch, Penchev et al. 1990). KinA is the major kinase responsible for initiation of sporulation (Hoch 2000). KinA contains three PAS domains: PAS-A, PAS-B, and PAS-C (from amino to carboxyl terminus) as shown in figure (I.4). The PAS domain has been reported as a dimerization domain common to the proteins in which imperfect repeat sequences were first recognized: the Drosophila period clock protein (PER), vertebrate aryl hydrocarbon receptor nuclear translocator (ARNT), and Drosophila single-minded protein (SIM) (Crews, 1998) (Nambu, 1991). Numerous PAS domain-containing two-component systems have been identified in bacteria (Galperin, 2001). PAS domains appear to be used as sensor modules that sense a wide range of stimuli and regulate an associated histidine kinase (Galperin, 2001) (Robinson, 2000) (Taylor, 1999). Presumably, the mechanism relies on the ability of the PAS domain to sense environmental change via a bound ligand (signaling molecule) moiety and to couple that signal to conformational changes of the PAS domain. The PAS domains have the ability to sense a variety of stimuli including oxygen, redox potential, and light (Taylor, 1999). The PAS-A domain can bind ATP, which led to the hypothesis that KinA directly senses the availability of ATP (Taylor and Zhulin 1999, Stephenson and

Hoch 2001). The other histidine kinases KinB and KinC both are transmembrane kinases as shown in (Figure I.5). KinB was identified based on the sequence homology to histidine protein kinases (Hoch 1993). KinC was identified in two different genetic screens. One is involved in identification of genes required for the bypass of the phosphorelay caused by altered function mutations of spo0A the other one involved the isolation of multicopy clones that suppressed the sporulation defect caused by null mutation of spo0K (oligonucleotide permease) (Kobayashi, Shoji et al. 1995, LeDeaux and Grossman 1995). But deletion of either KinB or kinC or both causes little or no decrease in sporulation efficiency. Further, from genome sequence using homology search, two other histidine kinases are found, KinD and KinE. KinD has two transmembrane domains at N-terminus and shows ability to phosphorylate spo0F to a lesser extent in the absence of other kinases in vitro (Fabret, 1999). KinD acts as a repressor or sporulation as its expression leads to delay in onset of sporulation (Jiang, 2000) As *kinD* mutant proceeds to sporulation more quickly than a wild-type cell (Aguilar, Vlamakis et al. 2010). It is still unclear, how KinD affects Spo0A though it has been hypothesized to function as a phosphatase and thereby inhibit the initiation and/or progression of sporulation. Another kinase, KinE is found to be a localized in cytoplasm and shows many obvious role in the phosphorelay. It also shares sequence similarity to other kinases (Fabret, Feher et al. 1999, Jiang, Shao et al. 2000).

The traditional view of sporulation is, upon sensing the starvation signal through sensor domain, the sporulation kinase becomes activated by autophosphorylation and thereby trigger sporulation phosphorelay and activates master regulator spo0A. However, the actual sporulation signal has never been identified. Recetly, our studies show that, (Fujita and Losick 2005) the synthesis of KinA beyond threshold level leads to entry into sporulation regardless of nutrient availability (Eswaramoorthy, Duan et al. 2010). However, recent studies have shown that none of the KinA PAS domain alone is essential to induce sporulation (Eswaramoorthy, Guo et al. 2009, Eswaramoorthy and Fujita 2010). Moreover, the loss of either of the PAS-B and PAS-C domains has a greater impact on sporulation induction while the loss of PAS-A has an essentially negligible effect on sporulation induction (Eswaramoorthy, Dinh et al. 2010, Eswaramoorthy and Fujita 2010). While KinA can clearly initiate sporulation, the specific physiological cues that stimulate KinA remained to be known.

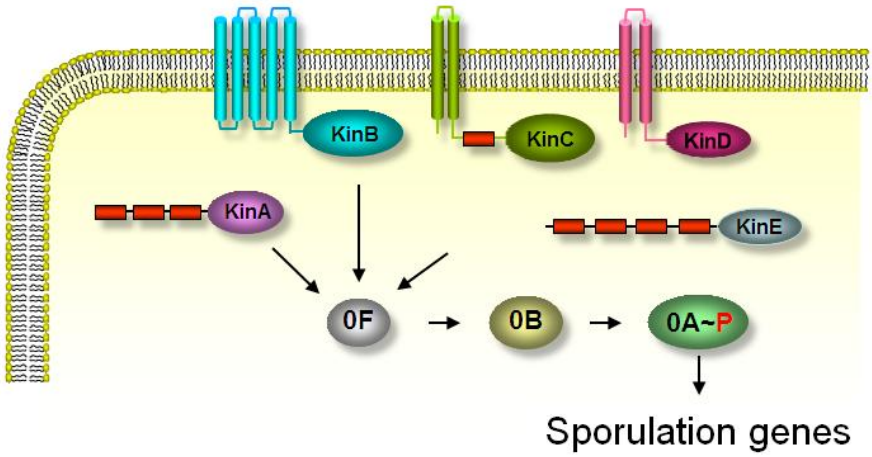

Figure I.5: Sporulation Kinases and multicomponent phosphorelay

The activity of each kinase is regulated by complex signal-transduction pathways that appear to respond to environmental, metabolic, and cell-cell signals. Cylendrical structure in KinB, C and D depicts transmembrane domain. Box at N-terminus of kinA, KinC and KinE indicates PAS domain.

The sporulation is a tightly regulated process. Spo0A, a master transcription factor whose phosphorylation state governs its ability to bind to promoters and thereby regulate gene expression, drives these events (Fawcett, Eichenberger et al. 2000, Molle, Fujita et al. 2003). The phosphorylation of Spo0A is mediated by a phosphorelay composed of a series of phosphatases and kinase-inhibitory proteins, which transfers phosphates from several kinases. The kinases are assumed to respond to several environmental cues (Jiang, Shao et al. 2000). The decision to initiate sporulation is governed by the phosphorylation status of the transcription factor, Spo0A. The extent of Spo0A phosphorylation determines a wide range of physiological outcomes from the development of biofilms and cannibalism (when Spo0A is phosphorylated at lower levels) to sporulation shown in (Figure I.1) (when Spo0A is phosphorylated at higher levels) (Lopez, Vlamakis et al. 2009).

The phosphorelay is comprised of the primary sensor histidine kinase, KinA, two intermediate phosphotransferases, Spo0F and Spo0B, and the ultimate response regulator, Spo0A, as the master transcription factor (Hoch 1993) (Figure I.6). Genes for phosphorelay components are also the subject of Spo0A control both directly and indirectly (Hoch 1993). When the phosphorelay starts to be activated in sporulating cells, the low concentration of Spo0A~P

results in repression of transcription of the *abrB* gene, which encodes a transcription regulator, AbrB. Transcription of the gene (sigH, also known as spo0H) for σ^H, an alternative σ subunit of RNA polymerase (RNAP), is negatively regulated by AbrB, and thus the decrease in the level of AbrB protein leads to derepression of the transcription of the sigH. The subsequent increase in the concentration of σ^H RNAP leads to transcription of genes for KinA, Spo0F, and Spo0A. In addition to σ^H RNAP, Spo0A~P also acts as the positive regulator for the transcription of spo0F and spo0A. Therefore, Spo0A, per se, is subject to control at the level of its synthesis by a self-reinforcing closed cycle in the phosphorelay network (Hoch 1993). The nature of the initial event of this regulatory circuit is not known.

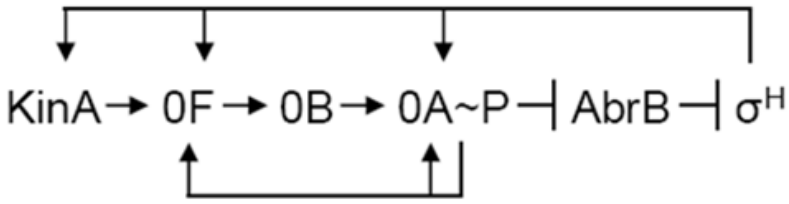

Figure I.6: Schematic diagram for the regulatory networks for the phosphorelay in wild type strain.

The pointed and blunt arrows represent positive and negative regulation. Under nutrient starvation culture conditions, KinA provides the phosphate supply to the master transcriptional regulator, Spo0A, via two phophotransferases Spo0F and Spo0B (Perego and Hoch 1991). Phosphorylated Spo0A (Spo0A~P) becomes a positive or negative regulator for sporulation genes, including those for Spo0A itself, Spo0F and transition state transcription regulator AbrB. AbrB represses the transcription of the sigH (spo0H gene), encoding the alternative sigma factor σ^H, which is also essential for sporulation. Thus, phosphorylated Spo0A represses

Fig I.6 cont.

abrB, thereby stimulating σ^H expression. Consequently, transcription of the genes for KinA, Spo0F and Spo0A is activated in the closed-loop system.

There are reports that many genes in the regulon are differentially responsive to high and low doses of Spo0A, with some genes being activated or repressed at a low dose of the regulatory protein and others requiring a threshold level of Spo0A in order to be turned on or off. Further analysis lead to the proposal that different genes are turned on or off at different times as Spo0A levels progressively increase. The genes that do not contribute to sporulation directly, such as genes involved in cannibalism and biofilm formation, being activated earlier and at lower doses of Spo0A, than genes that play a direct role in spore formation shown in (Figure I.1). Conditions of nutrient limitation, which lead to entry into sporulation, typically cause the appearance of two kinds of cells: those that have activated Spo0A and those that have not (Chung, Stephanopoulos et al. 1994, Gonzalez-Pastor, Hobbs et al. 2003). The skf and sdp operons are responsible for the production of a killing factor and a sporulation-delaying protein that block sibling cells that have not activated Spo0A from entering sporulation. Instead, the sibling cells undergo lysis, thereby providing a source of nutrients for the cells that have activated Spo0A and hence blocking them from progressing further into sporulation. Seen in the light of this cannibalistic process, it makes sense that genes that delay progression into sporulation are activated at a low level of Spo0A as shown in (Figure I.7).

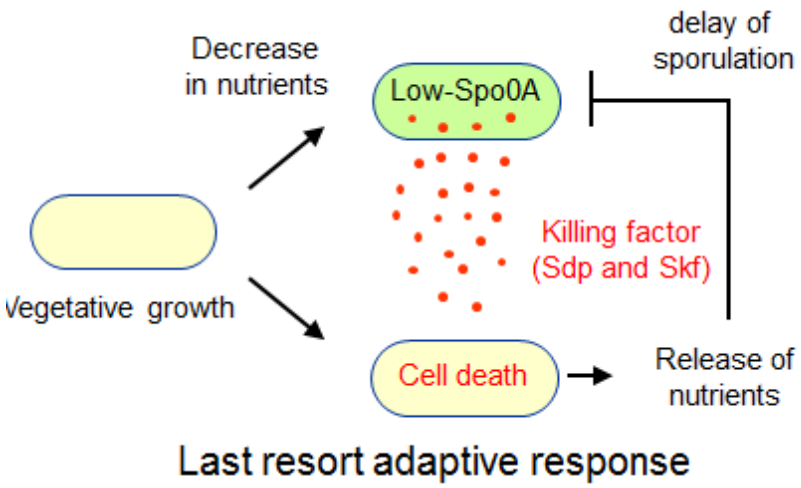

Figure I.7: Delay of sporulation is controlled by low dose of Spo0A~P

Nutrient deprivation leads to the activation of Spo0A in a subpopulation of the cells, The skf and sdp operons are responsible for the production of a killing factor and a sporulation-delaying protein that block sibling cells that have not activated Spo0A from entering sporulation.

I.4 Scope of this dissertation

The mechanism of the cell fate decision-making process is not very clear. Sporulation process requires the involvement of a multiple phosphorelay component, wherein phosphate is transferred to a master transcription regulator Spo0A from five histidine kinases (KinA-KinE) via two intermediate phosphotransferases, Spo0F and Spo0B. It has been proposed that (Grossman, 1995) the autokinase activity of one or a combination of five kinases is stimulated when the amino (N) terminal 'sensor' domain of kinase receives an as yet unidentified starvation signal(s), leading to the increase of the phosphorylated (active) form of Spo0A (Spo0A~P) via phosphorelay and resulting in

differentiation into biofilm formation and sporulation. However, the underlying mechanisms by which the putative starvation signal(s) stimulate the kinase and the subsequent control of Spo0A activity remain elusive. Our previous studies suggest (Eswaramoorthy, 2009) (Eswaramoorthy, 2010) that (1) the activity of the major sporulation kinase KinA is dependent on the tetramer formation, which is mediated by the N-terminal domain, regardless of culture conditions, (2) a threshold level of the kinase is primarily important for entry into sporulation, and (3) as a result of the increased kinase activity, the cellular level of active Spo0A (Spo0A~P) increases gradually over the course of starvation. Based on these studies, we hypothesize that (1) the primary role of the N-terminal domain of kinase is to form a functional tetramer, but not for sensing an unknown starvation signal, (2) the gradual increase of the Spo0A activity is controlled by the integrated signal processing through phosphorelay after nutrient starvation, and (3) Spo0A activity necessary for biofilm formation and sporulation is regulated by the relative activities of KinA and KinC. To test these hypotheses, in this dissertation work, we have used the artificial sporulation induction (ASI) system (Fujita and Losick 2005) in combination with synthetic biology approach to perform the *in vivo* characterization of the sporulation kinase in *B. substilis.* Our ASI offers a unique and appropriate opportunity to analyze *in vivo* physiological functions of the phosphorelay network. It is important to emphasize that our ASI is not an overexpression of the KinA protein, but the expression level of KinA is controlled in a tunable fashion under any culture conditions.

In chapter II, we attempted to check the function of the N-terminal sensor domain of KinA. We replaced the endogenous three-PAS repeat in the N-terminal domain of KinA with a two-PAS repeat derived from *Escherichia coli* and examined the function of the resulting chimeric protein. We performed domain-swap experiments in which the N-terminal domain of KinA was replaced with a tetramer-forming protein derived from *Escherichia coli* and examined the function of the resulting chimeric protein.

In chapter III, we studied KinC histidine kinase (HK) (Hoch 1993, LeDeaux, Yu et al. 1995), which was originally identified as sporulation kinase, but later studies indicate it to be involved in biofilm formation and canibolism (LeDeaux, Yu et al. 1995, Lopez, Fischbach et al. 2009). Sporulation and biofilm formation are regulated by the phosphorylated (active) form of the response regulator (RR) Spo0A (Spo0A~P). Upon starvation, phosphate is transferred to Spo0A from at least five HKs (KinA-KinE) via two intermediate phosphotransferases, Spo0F and Spo0B.(Hoch 1993) Presently, it is still unclear what environmental factor(s) is required for the activation of KinC and how the KinC activity determines the cell fate, whether to sporulate or form biofilm. Using the ASI system described earlier, we tested the idea that synthetic networks to control the temporal dynamics of Spo0A~P accumulation.

In chapter IV, to further the understanding of the biological role of KinC on sporulation in the laboratory strain, here, we utilized the wild-type genetic system in combination with an IPTG-inducible KinC expression system. We investigated

the role of KinC during growth and sporulation in the wild-type strain and found that KinC preferentially and positively controls the expression of the cannibalism genes in a manner dependent on phosphorelay, resulting in the delay of sporulation.

CHAPTER II: EXPRESSION LEVEL OF A CHIMERIC KINASE GOVERNS ENTRY INTO SPORULATION IN *BACILLUS SUBTILIS*

B. subtilis switches from growth to sporulation upon starvation. An unidentified starvation signal(s) to initiate sporulation is believed to be detected by the N-terminal sensor domain of the cytoplasmic histidine kinase, KinA, the major sporulation kinase. The N-terminal sensor domain contains three PAS domains (PAS-ABC), which are known to bind a variety of ligands and protein-protein interactions. Following the sensing of the putative sporulation signal by the N-terminal PAS domains, KinA triggers autophosphorylation of the catalytic histidine residue in the C-terminal domain to transmit the phosphate moiety, via phosphorelay, to the master regulator Spo0A for sporulation. However, until today the specific signal(s) that is hypothesized to initiate sporulation has never been detected. My previous results show that any two of the three PAS domains in the N-terminal of KinA are sufficient to maintain KinA autokinase activity under physiological conditions. KinA forms a tetramer mediated by the N-terminal PAS domain. Based on these results, I hypothesize that if tetramer formation mediated by the N-terminal domain is necessary and sufficient for KinA activity, a chimeric protein composed of the N-terminal domain derived from the unrelated protein families, but involved in tetramer formation and the following C-terminal catalytic domain of KinA would be active as a kinase to trigger sporulation. To test this hypothesis, we replaced the endogenous three-PAS repeat in the N-terminal domain of KinA with a similar two-PAS repeat derived from *Escherichia coli* and examined the function of the resulting chimeric protein. we found that the introduction of two-PAS repeat from *E. coli* triggered porulation by activating Spo0A through phosphorelay, irrespective of culture conditions. Furthermore,

chemical cross-linking study showed that the chimeric protein exists predominantly as a tetramer, mediated by the N-terminal domain, as was found for KinA. These results suggest that a tetramer formation mediated by the N-terminal domain is important and sufficient for autokinase activity catalyzed by the C-terminal domain. Thus, I propose that the primary role of the N-terminal domain of KinA is to form a functional tetramer, but not sensing an unknown signal.

II.1 Introduction

Bacterial cells are directly exposed to the environment. Hence, they must sense and respond rapidly to changes in their local environment in order to survive. One prevailing strategy to overcome this problem is to utilize the two-component system composed of a sensor histidine kinase (HK) and a response regulator (RR) (Parkinson 1993, Stock, Robinson et al. 2000). In the most typical case, the HK is a transmembrane protein with the N-terminal sensor domain often situated in the extracytoplasmic compartment, such as the periplasm, inner or outer membrane, or even extracellular space, and the C-terminal autokinase domain, containing a conserved phospho-accepting histidine residue, normally resides in the cytosol (Parkinson 1993, Dutta, Qin et al. 1999, Stock, Robinson et al. 2000). In contrast, the RR comprises a regulatory domain that includes a phospho-accepting aspartate residue at the N terminus, followed by an associated effector domain typically containing a DNA-binding motif, so that it becomes active as a transcription factor upon phosphorylation. Thus, using these

two components, various environmental signals can be detected by the HK and transmitted as phosphate groups to the RR for cellular adaptation.

The individual sensor domains of HKs are highly variable and thus lack sequence homology with other HKs (Dutta, Qin et al. 1999, Taylor and Zhulin 1999, Szurmant, White et al. 2007). Therefore, it is believed that a variety of environmental signals can be detected, with a high degree of sensitivity and specificity, by the unique amino acid sequence motif localized in the sensor domain. In fact, two-component systems have been found in diverse sensory processes, including sporulation, chemotaxis, osmolarity, metabolism of oxygen, nitrogen, and phosphate, induction of transport systems, and light and temperature sensing (Hoch 1993, Ninfa, Atkinson et al. 1993, Heermann, Altendorf et al. 1998, Christie, Salomon et al. 1999, Hoch 2000, Aguilar, Hernandez-Arriaga et al. 2001, Stock, Levit et al. 2002, Wolanin, Webre et al. 2003, Malpica, Franco et al. 2004, Neiditch, Federle et al. 2006)

Among these sensory systems in bacteria, sporulation in *B. subtilis* is not controlled by a simple two-component system, but an expanded version using four components, termed a phosphorelay (Hoch 1993, Hoch 2000). The phosphorelay is comprised of the primary sensor histidine kinase, KinA, two intermediate phosphotransferases, Spo0F and Spo0B, and the ultimate response regulator, Spo0A, as the master transcription factor(Burbulys, Trach et al. 1991). Under nutrient starvation culture conditions, KinA autophosphorylates followed by a transfer of a phosphate moiety to Spo0A in a His-Asp-His-Asp sequence

through a four-component phosphorelay. When the active phosphorylated form of Spo0A (Spo0A~P) reaches a certain concentration, the cells start to divide asymmetrically to give rise to two distinct cell types, a smaller forespore and a larger mother cell (Chung, Stephanopoulos et al. 1994, Fujita, Gonzalez-Pastor et al. 2005).

During that process, Spo0A~P works by directly or indirectly activating or repressing the transcription of hundreds of genes (Fawcett, Eichenberger et al. 2000, Molle, Fujita et al. 2003). Among them, genes that do not contribute to sporulation directly (e.g., competence, cannibalism, and biofilm) are activated at early times with a low dose of Spo0A~P, while genes that play a direct role in sporulation, such as spoIIG (an operon for the synthesis of σ^E, the mother cell-specific sigma factor) and spoIIA (an operon for the synthesis of σ^F, the forespore-specific sigma factor), are activated at later times with a high dose of Spo0A~P. Thus, the progressive increase in the activated Spo0A (Spo0A~P) explains the temporal and spatial expression patterns of the low- and high-threshold Spo0A-regulated genes (Fujita and Losick 2003, Fujita, Gonzalez-Pastor et al. 2005).

Genes for phosphorelay components are also the subject of Spo0A control both directly and indirectly (Hoch 1993, Strauch, Wu et al. 1993, Fujita and Sadaie 1998). When the phosphorelay starts to be activated in sporulating cells, the low concentration of Spo0A~P results in repression of transcription of the abrB gene, which encodes a transcription regulator, AbrB. Transcription of

the gene (sigH, also known as spo0H) for σ^H, an alternative σ subunit of RNA polymerase (RNAP), is negatively regulated by AbrB, and thus the decrease in the level of AbrB protein leads to derepression of the transcription of the sigH. The subsequent increase in the concentration of σ^H RNAP leads to transcription of genes for KinA, Spo0F, and Spo0A. In addition to σ^H RNAP, Spo0A~P also acts as the positive regulator for the transcription of spo0F and spo0A. Therefore, Spo0A, per se, is subject to control at the level of its synthesis by a self-reinforcing closed cycle in the phosphorelay network (Chibazakura, Kawamura et al. 1991, Fujita and Sadaie 1998). The nature of the initial event of this regulatory circuit is not known.

Although the individual sensor domains of HKs are unique, recent studies have revealed the PAS domain as one of the representative structural motifs found in the sensor domains (Taylor and Zhulin 1999, Galperin, Nikolskaya et al. 2001). The PAS domain, which is highly variable at the sequence level but structurally conserved, was initially identified as a common motif among the Drosophila melanogaster clock protein PER, mammalian ARNT (a dimerization partner of the dioxin receptor), and SIM (the product of the single-minded gene) (Crews 1998, Taylor and Zhulin 1999, Neiditch, Federle et al. 2006). The PAS domain is now known to be involved in the protein-protein interaction and, in some cases, also in the binding of small ligands(Taylor and Zhulin 1999, Szurmant, White et al. 2007). However, how the PAS domains function to activate the HK in response to ligand binding has been largely unknown (Stock,

Robinson et al. 2000) (Szurmant, White et al. 2007). In this regard, it is believed that the autophosphorylation activity of KinA is induced when the N-terminal sensor domain, containing three PAS domains, receives a hypothetical signal(s) that originates only under starvation conditions, and thus this “signal-sensing” step is crucial to trigger the sporulation phosphorelay (Stephenson and Hoch 2002). However, KinA does not have a transmembrane domain and localizes in the cytosol, suggesting that the sensor domain recognizes the signal(s), which might be transported from the nutrient-limited environment to the cytosol or synthesized endogenously in response to starvation (Hoch 1993, Hoch 2000). More importantly, no starvation signal(s) of either an extracellular or intracellular nature acting directly on KinA has been identified. Thus, the molecular mechanisms of the signal sensing by the cytoplasmic KinA and the subsequent signal transduction by the phosphorelay system are not fully understood.

To address these problems, several studies using primarily biochemical approaches have been attempted, but the results are conflicting, perhaps due to the different methodologies employed. Stephenson and Hoch claimed that in an in vitro system, ATP bound to the most-N-terminal PAS-A domain, and it might not serve as a signal but rather as a phosphate source for the phosphorylation at the histidine residue in the C-terminal domain (Stephenson and Hoch 2002). Lee et al. reported that amino acid substitution mutations in the PAS-A domain affected the activity of KinA, suggesting that PAS-A is required for the kinase activity (Lee, Tomchick et al. 2008).

More recently, our in vivo studies showed that (i) the most N-terminal PAS domain of KinA (PAS-A), which was originally believed to be essential for signal sensing (Stephenson and Hoch 2002, Lee, Tomchick et al. 2008), is dispensable (Eswaramoorthy, Guo et al. 2009, Eswaramoorthy and Fujita 2010); (ii) induction of the synthesis of KinA beyond a certain level results in the increase of Spo0A activity above the threshold, thereby allowing sporulation to proceed efficiently, irrespective of nutrient availability (Eswaramoorthy, Duan et al. 2010). These results suggest that the activity of KinA is not regulated in response to the unknown sporulation signal(s), but rather by a threshold level of the kinase, which is primarily important for entry into sporulation. Currently, the control mechanism(s) responsible for the increase in the KinA protein level during sporulation is unclear.

Toward conclusive determination of whether the N-terminal “sensor” domain of KinA indeed acts as the true sensor for an unidentified sporulation-specific signal(s), here we report the replacement of the N-terminal domain of KinA with an unrelated protein segment containing two PAS domains, the N-terminal domain of YdaM derived from Escherichia coli (Wolanin, Webre et al. 2003). We found that the resulting chimeric protein, $YdaM_N$-$KinA_C$, formed a tetrameric homocomplex, as was observed for KinA (Eswaramoorthy, Guo et al. 2009), and triggered a massive entry into sporulation, irrespective of nutrient availability and in a manner dependent on the concentration of the chimeric

kinase, which is consistent with our threshold model (Eswaramoorthy, Duan et al. 2010).

II.2 Materials and methods

II.2.1 Strains, plasmids, and oligonucleotides

All *B. subtilis* strains were derived from the prototrophic strain PY79. Details of the full genotypes of strains are provided in table II-1. All plasmid constructions were performed in *Escherichia coli* DH5α using standard methods. The *E. coli* BL21(DE3) pET vector system (Novagen) was used for protein overexpression. The plasmids used in this study are listed in Table II-2. Oligonucleotides used for plasmid construction are listed in Table II-3.

Table II.1 List of Strains

β-Galactosidase assay		
Strain	Genotype or description	Source or reference
MF4276	*ΔkinB::kan, thrC::P_{spoIIA}-lacZ erm*	This study
MF4253	*ΔkinA::tet, ΔkinB::kan, amyE::$P_{hy\text{-}spank}$-kinA spc, thrC::P_{spoIIA}-lacZ erm*	This study
MF4254	*ΔkinA::tet, ΔkinB::kan, amyE::$P_{hy\text{-}spank}$-ydaM$_N$-kinA$_C$ spc, thrC::P_{spoIIA}-lacZ erm*	This study
MF4244	*amyE::$P_{hy\text{-}spank}$-ydaM-N-kinA-C spc, thrC::P_{spoIIA}-lacZ erm*	This study
MF4300	*Δspo0F::tet, amyE::$P_{hy\text{-}spank}$-ydaM$_N$-kinA$_C$ spc, thrC::P_{spoIIA}-lacZ erm*	This study
MF4301	*Δspo0B::tet, amyE::$P_{hy\text{-}spank}$-ydaM$_N$-kinA$_C$ spc, thrC::P_{spoIIA}-lacZ erm*	This study
MF4302	*Δspo0A::kan, amyE::$P_{hy\text{-}spank}$-ydaM$_N$-kinA$_C$ spc, thrC::P_{spoIIA}-lacZ erm*	This study
MF4328	*kinAΩ$P_{hy\text{-}spank}$-kinA spc, thrC::P_{spoIIA}-lacZ erm*	This study
MF4329	*Δspo0F::tet, kinAΩ$P_{hy\text{-}spank}$-kinA spc, thrC::P_{spoIIA}-lacZ erm*	This study
MF4330	*Δspo0B::tet, kinAΩ$P_{hy\text{-}spank}$-kinA spc, thrC::P_{spoIIA}-lacZ erm*	This study
MF4331	*Δspo0A::kan, kinAΩ$P_{hy\text{-}spank}$-kinA spc, thrC::P_{spoIIA}-lacZ erm*	This study

GFP fusion protein		
Strain	Genotype or description	Source or reference
MF3352	*ΔkinA::tet, ΔkinB::cm, amyE::$P_{hy\text{-}spank}$-kinA-gfp spcR kan*	This study
MF4201	*ΔkinA::tet, ΔkinB::cm, amyE::$P_{hy\text{-}spank}$-ydaM$_N$-kinA$_C$-gfp spc kan*	This study
MF4080	*ΔkinA::tet, ΔkinB::cm, amyE::$P_{hy\text{-}spank}$-ydaM$_N$-gfp spc*	This study
MF2732	*amyE::$P_{hy\text{-}spank}$-gfp spc*	This study
MF3593	*ΔkinB::cm, kinAΩkinA-gfp kan*	This study
MF3360	*ΔkinA::tet, ΔkinB::cm, amyE::$P_{hy\text{-}spank}$-kinA$_N$-gfp spc*	This study

YFP and mCherry		
Strain	Genotype or description	Source or reference
MF4213	*ΔkinA::tet, ΔkinB::kan, amyE::$P_{hy\text{-}spank}$-kinA spc, thrC::P_{spoIIQ}-mCherry erm, spoIIAΩP_{spoIIA}-yfp cm*	This study
MF4203	*ΔkinA::tet, ΔkinB::kan, amyE::$P_{hy\text{-}spank}$-ydaM$_N$-kinA$_C$ spc, thrC::P_{spoIIQ}-mCherry erm, spoIIAΩP_{spoIIA}-yfp cm*	This study

E. coli BL21 (DE3) strain harboring plasmid for protein overexpression		
Strain	Plasmid or description	Source or reference
MF1369	pMF193 (KinA)	(Fujita and Losick 2003)
MF1226	pGK10 (Spo0F)	(Fujita and Losick 2003)
MF1253	pMF184 (Spo0B)	(Fujita and Losick 2003)
MF2488	pMF338 (Spo0A)	(Fujita and Losick 2003)
MF3831	pMF535 (YdaM$_N$-KinA$_C$)	This study

Table II. 2. Plasmids used in this study.

Plasmid	Description	Source
pMF528	*amyE::*$P_{hy\text{-}spank}$*-ydaM*$_N$*-kinA*$_C$ *spc*	This study
pMF547	*amyE::*$P_{hy\text{-}spank}$*-ydaM*$_N$*-gfp spc*	This study
pMF552	*thrC::*$P_{hy\text{-}spank}$*-ydaM*$_N$*-gfp erm*	This study
pMF583	*thrC::*P_{spoIIA}*-lacZ erm*	This study
pMF193	KinA in pET vector	(Fujita and Losick 2003)
pGK10	Spo0F in pET vector	(Fujita and Losick 2003)
pMF184	Spo0B in pET vector	(Fujita and Losick 2003)
pMF338	Spo0A in pET vector	(Fujita and Losick 2003)
pMF535	YdaM$_N$-KinA$_C$ in pET vector	This study

Table II.3. Oligonucleotide primers used in this study.

Primer	Sequence
oP4	cgggcatgcttatttttttggaaatgaaatttaaacgc
oP28	gccctcgagcaggtcattcttcgggacatctca
oP39	cggggatccttagtggtggtggtggtggtg ttttttggaaatgaaatttaaacgc
oP74	gcccatatgatgattacgcacaacttcaataccctg
om48	gccctcgagatgagtaaaggagaagaactt
omf187	gcccaattgatcaaacagtagcaaaagtaaaggtc
omf188	gccaagcttgatatgatcggataatgagtg
omf317	gccgcatgcttatttgtatagttcatccatgcc

II.2.2 Media and culture conditions

To induce the synthesis of KinA, the chimeric protein, or green fluorescent protein (GFP) under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible hyper-spank promoter (Phy-spank) (16) in the engineered *B. subtilis* cells, IPTG was added to Luria-Bertani (LB) cultures during the exponential growth phase (optical density at 600 nm [OD600], 0.5) as the rich

medium conditions. To induce protein synthesis under normal sporulation conditions, the engineered cells were grown in casein hydrolysate (CH) medium at 37°C. At the mid-exponential phase of growth (OD600, 0.5) in CH medium, cells were suspended in Sterlini-Mandelstam (SM) medium (40) supplemented with IPTG.

Sporulation efficiency and β-galactosidase assays. Sporulation efficiency was determined in 16-h cultures as CFU per milliliter after incubation at 80°C for 10 min compared with the CFU of the pre-heat treatment sample. Assays of β-galactosidase activity were performed as described previously (12).

II.2.3 Immunoblot analysis

Whole-cell lysates for immunoblot analysis were prepared by sonication. Immunoblot analysis was performed as described previously (12). Polyclonal rabbit antibodies against GFP (17) were used to detect GFP-tagged proteins. σ^A was detected by a polyclonal rabbit anti-σ^A antibdoy and served as an internal standard control (14).

II.2.4 Protein cross-linking

Protein cross-linking with bis-maleimidohexane (BMH; Pierce) was performed as described previously (Eswaramoorthy, Duan et al. 2010).

II.2.5 Protein purification

All His-tagged proteins were expressed in *E. coli* BL21(DE3). All proteins except $YdaM_N$-$KinA_C$ were soluble, and thus purification steps were carried out at

4°C as described previously (Fujita and Sadaie 1998, Fujita and Losick 2003). Renaturation of $YdaM_N$-$KinA_C$ from the inclusion body and the following purification steps were performed as described previously (Fujita and Sadaie 1998).

II.2.6 In vitro phosphorylation.

Phosphorylation reactions were performed as described previously (Fujita and Losick 2003).

II.2.7 Microscopy.

Cells expressing GFP were examined by fluorescence microscopy as described previously (Eswaramoorthy, Guo et al. 2009). The microscope system control and image processing were performed using SlideBook image analysis software (Intelligent Imaging Innovations, Inc.).

II.3 RESULT

II.3.1 Construction of Chimera and assay system to measure the activity of the construct

Based on our prior results (Eswaramoorthy, Duan et al. 2010, Eswaramoorthy and Fujita 2010), we hypothesized that if the sensor domain of KinA were replaced with a domain that is unrelated to sporulation but involved in tetramer formation, the resulting chimeric protein would be active as a kinase to

trigger sporulation in a concentration-dependent manner, irrespective of nutrient availability.

To test this hypothesis, we constructed a chimeric protein in which the three N-terminal PAS domains of KinA were replaced with an amino acid sequence possibly involved in protein-protein interactions but derived from an unrelated bacterial species. By using the help of an E. coli genome database (E. coli wiki) in combination with the SMART (simple modular architecture research tool) online tool (Ponting, Schultz et al. 1999), we identified YdaM, which carries an N-terminal domain containing two PAS domains. YdaM exhibits diguanylate cyclase (DGC) activity in vitro that produces bis-(3-5)-cyclic-diguanosine monophosphate (c-di-GMP) from GTP and plays an antagonistic role in the expression of the biofilm-associated curli fimbriae (Weber, Pesavento et al. 2006). The catalytic activity resides in the C-terminal GGDEF domain of the protein. Thus, as a first attempt, we constructed a chimeric protein in which the N-terminal domain of KinA was replaced with an N-terminal domain of YdaM, which does not contain the catalytic active site of DGC (Figure II-1). Then, we examined the functionality of the $YdaM_N$-$KinA_C$ chimeric protein in vivo.

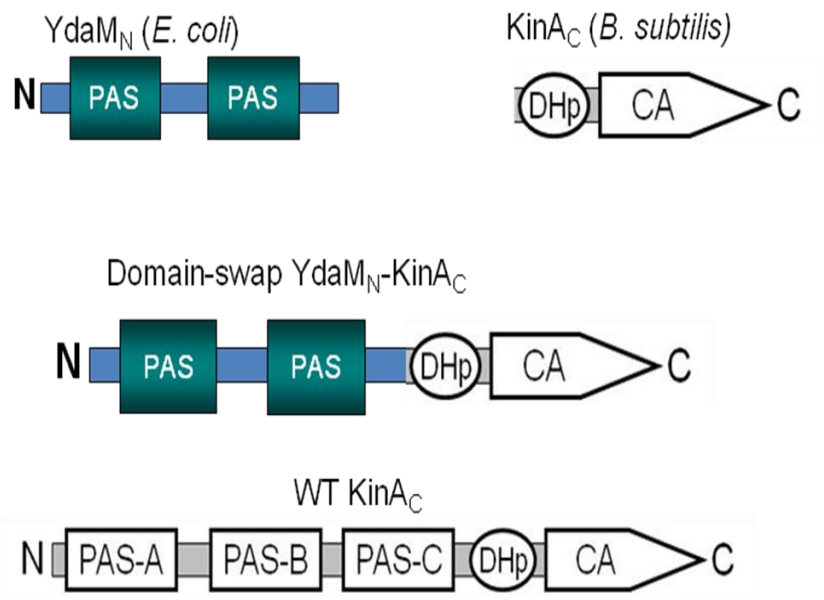

Figure II.1: Domain swap Chimera construction

N-terminal domain of KinA was replaced with an N-terminal domain of YdaM, which does not contain the catalytic active site of DGC. It is fused with the c-terminal domain of KinA to construct domain swap YdaM$_N$-KinA$_C$.

To reduce the possible variation in the level of gene expression among the constructs (e.g., regulation at the transcriptional or translational level) and to analyze the structure-function relationship of the chimeric protein in comparison with KinA, the genes for YdaM$_N$-KinA$_C$ and its derivatives were placed under the control of an IPTG-inducible hyper-spank promoter (Phy-spank), followed by a sequence including the optimal ribosome-binding site in *B. subtilis*, as was reported for KinA (Fujita and Losick 2002, Eswaramoorthy, Guo et al. 2009). Then, each of the resulting genes was introduced into the nonessential amyE locus of the chromosomal DNA as a single copy. To avoid the phosphate transfer from the endogenous KinA and another sporulation kinase, KinB (Trach and

Hoch 1993), these two genes were knocked out in all the strains used for this assay. Thus, in this genetic background (Δ*kinA* and Δ*kinB*), the true activity of the chimeric protein as the sporulation kinase could be examined in comparison with the wild-type (KinA) control. To reduce the possible variation in the level of gene expression among the constructs (e.g., regulation at the transcriptional or translational level) and to analyze the structure-function relationship of the chimeric protein in comparison with KinA, the genes for $YdaM_N$-$KinA_C$ and its derivatives were placed under the control of an IPTG-inducible hyper-spank promoter (Phy-spank), followed by a sequence including the optimal ribosome-binding site in *B. subtilis*, as was reported for KinA (Fujita and Losick 2002, Eswaramoorthy, Guo et al. 2009). Then, each of the resulting genes was introduced into the nonessential amyE locus of the chromosomal DNA as a single copy. To avoid the phosphate transfer from the endogenous KinA and another sporulation kinase, KinB (Trach and Hoch 1993), these two genes were knocked out in all the strains used for this assay. Thus, in this genetic background (ΔkinA and ΔkinB), the true activity of the chimeric protein as the sporulation kinase could be examined in comparison with the wild-type (KinA) control.

First, we examined the influence of the concentration of IPTG on the efficiency of sporulation under nutrient-rich conditions in LB medium, under which the starvation signal is presumably absent (Eswaramoorthy, Duan et al. 2010). Cells expressing the $YdaM_N$-$KinA_C$ protein (here referred to as the $YdaM_N$-$KinA_C$

strain) sporulated with high efficiency (~10^{-1} [spores per viable cells]) when the protein synthesis was induced by increasing the concentration of IPTG (Table II-4). By inducing with at least 200 μM IPTG, the sporulation efficiency was comparable (in a range of 10^{-1}) to that of the sporulating wild-type cells under normal sporulation conditions in SM medium. We noticed that the absolute number of spores in the $YdaM_N$-$KinA_C$ strain was approximately 10% (~10^7/ml) of that in the wild-type strain (which lacked the IPTG-inducible construct), and the number of viable cells of the $YdaM_N$-$KinA_C$ strain decreased in an IPTG concentration-dependent manner, although the reason for the reduced viable number in this strain is not clear. The sporulation efficiency of $YdaM_N$-$KinA_C$ became similar to that of the wild-type strain, as described above (Table II-4). In contrast, without IPTG, the sporulation efficiency was significantly low (~10^{-6}). Under the same culture conditions, a strain expressing KinA under the same expression system as that of the $YdaM_N$-$KinA_C$ strain (here referred to as the KinA strain) was examined as a control (Eswaramoorthy, Guo et al. 2009). The results indicated that, at 10 μM IPTG, sporulation was induced to the same levels as in the sporulating wild-type strain (~10^{-1}) (wt; that is, lacking the IPTG-inducible construct), while sporulation was not efficiently induced without IPTG (~10^{-4}) as reported previously (Table II-4) (Eswaramoorthy, Guo et al. 2009, Eswaramoorthy and Fujita 2010). The wild-type strain, which lacks the IPTG-inducible construct, showed a significantly low level of sporulation efficiency under nutrient-rich conditions in LB medium (~10^{-5}) as observed previously

(Eswaramoorthy and Fujita 2010). Next, we confirmed that inducing the synthesis of the N-terminus of YdaM ($YdaM_N$) showed no significant effect on triggering sporulation under the same culture conditions (~10^{-6} sporulation efficiency) (data not shown). As we already reported (Eswaramoorthy, Guo et al. 2009, Eswaramoorthy and Fujita 2010), a strain expressing the C-terminal autokinase domain of KinA preferentially accepts phosphate from Spo0F~P in a reverse phosphotransfer reaction, resulting in an inability to trigger massive entry into sporulation. These results indicate that the individual N- and C-terminal domains ($YdaM_N$ and $KinA_C$, respectively) are not functional when expressed alone, but sporulation is triggered efficiently only when the fused chimeric protein is produced.

Table II.4 Induction of synthesis of $YdaM_N$-$KinA_C$ triggers sporulation in LB medium.

IPTG (μM)	LB					
	$YdaM_N$-$KinA_C$			KinA		
	CFU/ml		Efficiency	CFU/ml		Efficiency
	Viable cells	Spores		Viable cells	Spores	
0	3.3×10^8	2.1×10^3	6.4×10^{-6}	4.5×10^8	2.6×10^5	5.8×10^{-4}
10	4.5×10^8	4.5×10^4	1.0×10^{-4}	3.2×10^8	1.5×10^8	4.7×10^{-1}
50	4.5×10^8	2.7×10^5	6.0×10^{-4}	1.1×10^8	6.7×10^7	6.1×10^{-1}
100	1.8×10^8	3.4×10^6	1.9×10^{-2}	5.0×10^8	3.2×10^7	6.4×10^{-2}
200	1.7×10^8	2.9×10^7	1.7×10^{-1}	5.0×10^8	3.0×10^7	6.0×10^{-2}
500	1.1×10^8	5.5×10^7	5.0×10^{-1}	4.9×10^8	3.1×10^7	6.3×10^{-2}

Efficiency was determined in 16-h cultures, as CFU/ml after heat treatment by incubation at 80^0C for 10 min, compared with CFU/ml in the pre-heat treatment sample.

Second, we repeated the above experiments, but this time under starvation conditions in SM medium. Data not shown, we obtained essentially the same results as those under nutrient-rich conditions in LB medium. These results indicated that when the $YdaM_N$-$KinA_C$ chimeric protein is expressed to a certain level, it is functional, if not fully but at least partially, to trigger sporulation, irrespective of starvation signal.

Table II-5 Induction of synthesis of $YdaM_N$-$KinA_C$ and effect on sporulation in SM medium. Efficiency was determined in 16-h cultures, as CFU/ml after heat treatment by incubation at 80^0C for 10 min, compared with CFU/ml in the pre-heat treatment sample.

II.3.2 Spo0A, the master regulator for entry into sporulation, is activated by the $YdaM_N$-$KinA_C$ chimeric protein through phosphorelay

We predicted that $YdaM_N$-$KinA_C$ would function as a kinase. Due to technical limitations for direct measurement of the histidine autokinase activity in vivo, a reporter system consisting of the β-galactosidase gene (lacZ) fused to the Spo0A-directed promoter is widely used as an indirect measurement of the activity (Fujita and Losick 2005, Eswaramoorthy, Guo et al. 2009). For this, we constructed a reporter system in which lacZ gene expression is driven by the Spo0A-dependent spoIIA promoter (Hatt and Youngman 1998). We then introduced the reporter system into a strain that carried the IPTG-inducible $YdaM_N$-$KinA_C$ to examine β-galactosidase activity in the presence of various concentrations of IPTG. As a control, the KinA strain was examined under the

same conditions as those for the $YdaM_N$-$KinA_C$ strain. Using these systems, we examined the kinetics of Spo0A activation as an indirect assessment of the kinase activity when the synthesis of $YdaM_N$-$KinA_C$ was induced with IPTG. The wild-type strain, which lacks the IPTG-inducible construct, served as another control.

During the course of experiments, we detected the reporter activity in the $YdaM_N$-$KinA_C$ strain at relatively low levels at early times (~2 h), but the activity became significant at later times (~5 h) after IPTG addition (200 μM) under nutrient-rich conditions in LB medium (Fig. II-2A). In contrast, under normal sporulation conditions in SM medium, we found that the peak of the activity was shifted to around 2 h after IPTG addition (200 μM) (Fig.II-2A).

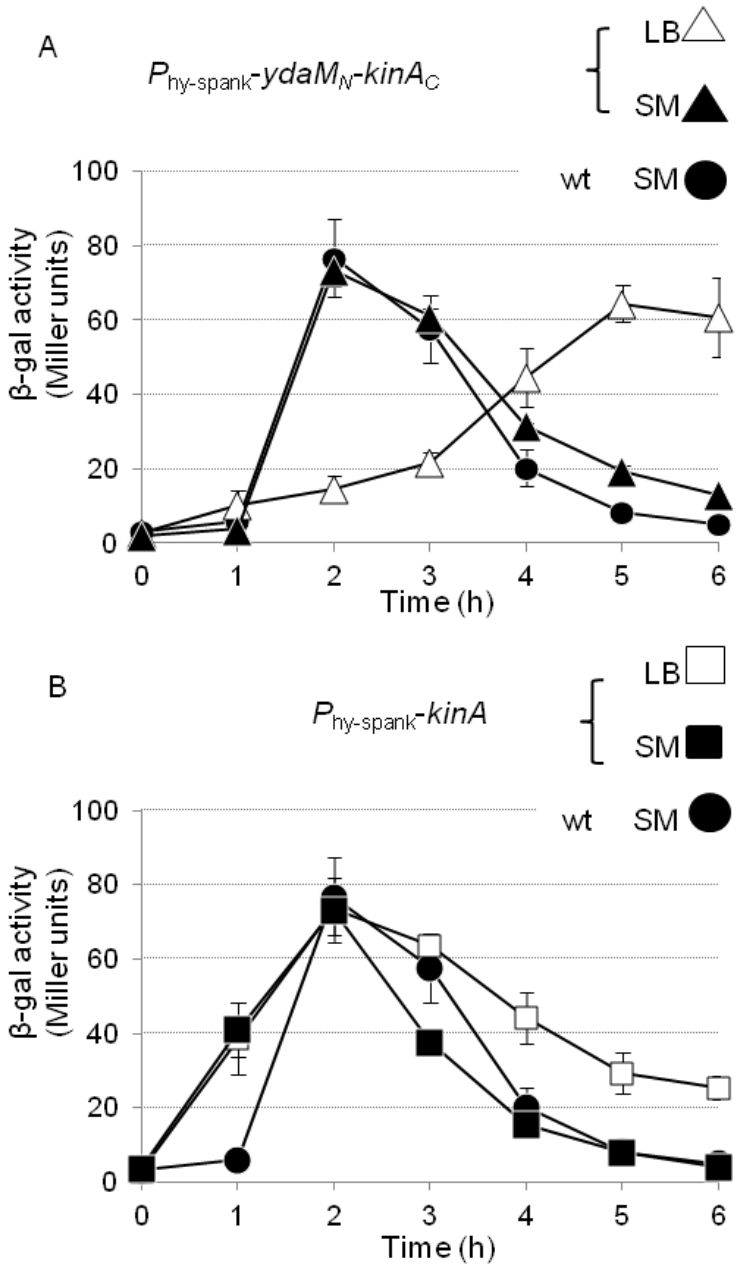

Figure II.2: Activity of $YdaM_N$-$KinA_C$ in cells cultured in LB and SM media. Cells of the IPTG-inducible $YdaM_N$-$KinA_C$ (A) and KinA (B) strains harboring the PspoIIA-lacZ reporter construct were cultured in LB (open symbols) and SM (filled symbols) media and collected at the indicated times after the addition of IPTG (200 μM for $YdaM_N$-$KinA_C$ [MF4254; triangle] and 10 μM for KinA [MF4253; square]). The wild-type strain (wt; MF4238) cultured in SM medium served as a

Fig II.2 cont.
control, and the identical data sets are displayed as filled circles in both panels A and B. The time courses of $YdaM_N$-$KinA_C$ and KinA activities were shown as a measure of β-galactosidase activity of a Spo0A-dependent PspoIIA-lacZ reporter. All experiments were performed at least three times independently, and average values and standard deviations are plotted.

In the KinA strain, the reporter activities reached maximum levels at around 2 h after IPTG addition (10 μM) under either nutrient-rich conditions in LB medium or normal sporulation conditions in SM medium (Fig.II-2B). In the absence of IPTG, little or no reporter activity was detected in either the $YdaM_N$-$KinA_C$ or the KinA strains. In the wild-type strain (which lacks the IPTG-inducible construct), the reporter activities reached maximum levels at around 2 h in SM medium (Fig. II-2A and B), while little or no reporter activity was detected in LB medium (data not shown). I note that, in general, many genes involved in sporulation are controlled to express in a just-in-time manner. Thus, as observed in the experiments shown in Fig. II-3 and II-4 (see below), the reporter activity declined beyond the peak, even when the regulatory proteins were sufficiently present, perhaps due to the transcriptional repression at that point. These results indicate that, in the $YdaM_N$-$KinA_C$ strain, activation of Spo0A is mediated by the chimeric protein but is delayed in LB medium compared with SM medium, suggesting that the chimeric protein is negatively regulated, perhaps through the N-terminal $YdaM_N$ domain, when the cells are grown under rich medium conditions. Alternatively, it remains possible that the PAS domain of $YdaM_N$ may respond to some stimuli, such as redox states (45) generated in SM medium,

resulting in an early activation of the reporter gene. Nevertheless, these results are consistent with our prediction that sporulation is induced as a result of Spo0A activation mediated by induction of synthesis of the $YdaM_N$-$KinA_C$ protein, irrespective of nutrient availability.

To confirm the compartment-specific transcription of the sporulation genes in the individual cells expressing the $YdaM_N$-$KinA_C$ protein, we performed fluorescence microscopy experiments. First, the gene for yellow fluorescent protein (YFP) was placed under the control of the Spo0A-dependent spoIIA promoter to express YFP in the sporulating cells before polar septation. Second, the gene for mCherry was fused to the σ^F-RNAP-controlled spoIIQ promoter to express mCherry in the forespore. Then, each of the two reporter gene constructs was integrated into the chromosome of the same strain as a single copy of each. These two reporter genes were introduced into the KinA strain for the control experiments. As shown in Fig. II-3, each of the fluorescent reporters was expressed in a compartment-specific manner, but essentially displayed a similar time course pattern to that of the β-galactosidase reporter (Fig. II-2A and B); timing of the expression of each sporulation gene in the $YdaM_N$-$KinA_C$ strain was delayed in LB medium compared to SM medium.

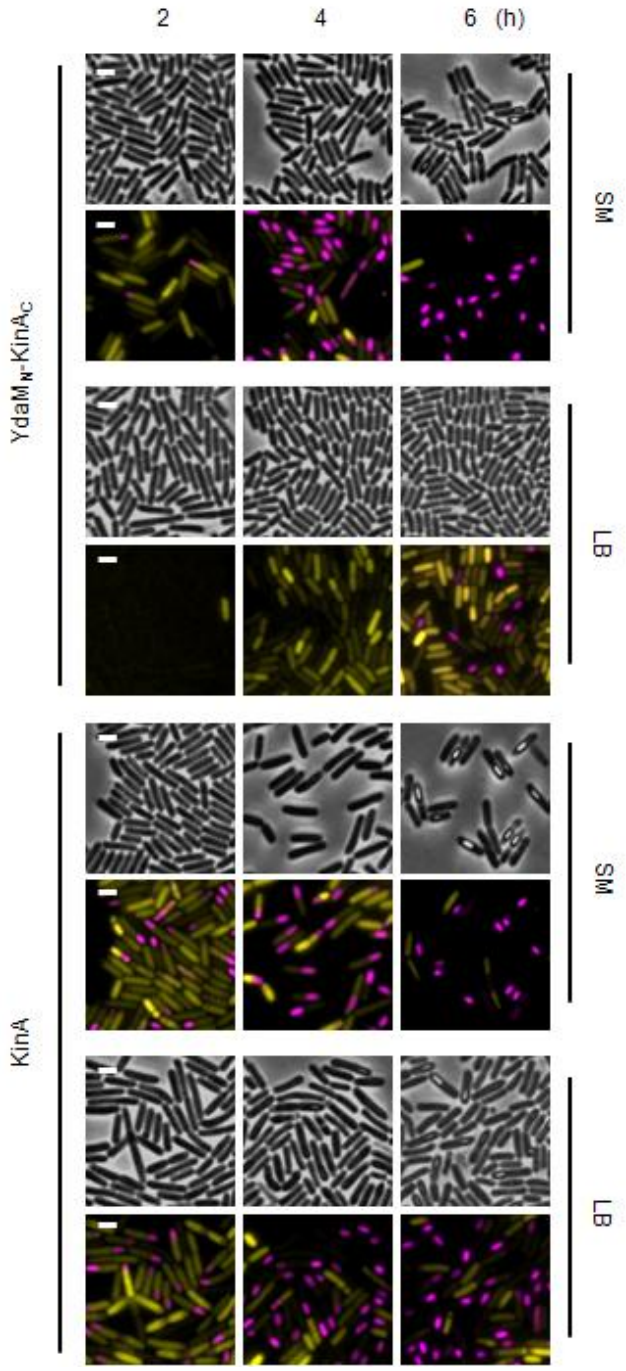

Figure II-3: Fluorescence microscopic analysis of the sporulation process in the YdaM$_N$-KinA$_C$ strain.

Cells of the YdaM$_N$-KinA$_C$ (MF4203) and KinA (MF4213) strains harboring both PspoIIA-yfp and PspoIIQ-mCherry fluorescent reporter genes were cultured in LB

Figure II-3 cont.

and SM media in the presence of IPTG and collected at the indicated time of incubation. The IPTG concentrations used for inducing the synthesis of $YdaM_N$-$KinA_C$ and KinA are 200 µM and 10 µM, respectively. Fluorescence microscopy was carried out as indicated in Materials and Methods. The fluorescence images were false-colored yellow for YFP and magenta for mCherry. The corresponding phase contrast images are shown in black and white. Images shown are representative of more than 500 cells in the whole cell population inspected in three separate fields of view. Bar, 2 µm.

Next, we investigated whether Spo0A is activated by the $YdaM_N$-$KinA_C$ protein via the phosphotransferases Spo0F and Spo0B, as in the case of KinA (Burbulys, Trach et al. 1991). For this, the deletion mutation of the gene for Spo0F or Spo0B was introduced into the $YdaM_N$-$KinA_C$ or KinA strain, each harboring the spoIIA promoter fusion to lacZ as described above. Exponential-phase cells of each strain grown in liquid LB were spotted on solid LB agar with no supplements, 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside (X-Gal), or both X-Gal and IPTG. In the wild-type background, the reporter activity was detected as blue colonies only in the presence of both X-Gal and IPTG, indicating that Spo0A is activated by either $YdaM_N$-$KinA_C$ or KinA (see Fig. II-4). When the deletion mutation of the gene for Spo0F or Spo0B was introduced into the $YdaM_N$-$KinA_C$ strain, the capacity of the cells to activate Spo0A in response to the inducer was reduced significantly, similar to changes observed with the spo0A mutant in the corresponding KinA strain (see Fig. II-4). These results indicated that $YdaM_N$-$KinA_C$ exerts its effect through the phosphorelay, as similarly observed with the KinA strain (Fujita and Losick 2005). I We note that a

relatively small colony was obtained in the KinA strain with the deletion of spo0A, suggesting that cross talk between Spo0B and some unknown downstream factors may mediate this effect (see Fig. II-4). All the above results are consistent with the idea that $YdaM_N$-$KinA_C$ functions as a kinase. Therefore, to verify directly whether $YdaM_N$-$KinA_C$ possesses autokinase activity and transfers phosphate to Spo0A through phosphorelay, We performed in vitro phosphorylation experiments with the purified protein components (see Fig. II-5).

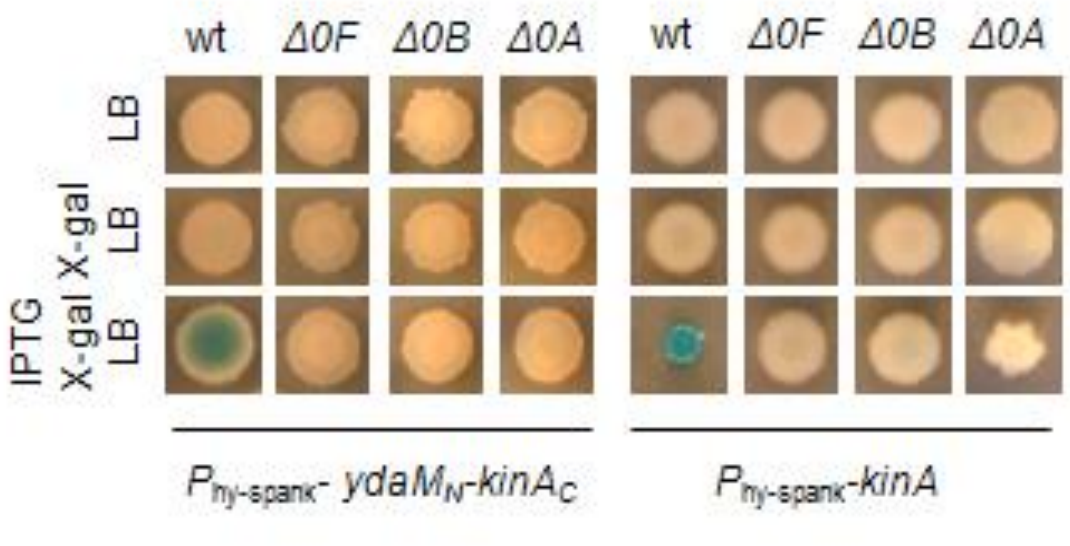

Figure II.4: The $YdaM_N$-$KinA_C$ chimera kinase activates Spo0A in a phosphorelay-dependent manner in vivo.

Cells of the $YdaM_N$-$KinA_C$ strain containing P_{spoIIA}-*lacZ* reporter constructs were grown on LB agar medium, with X-gal (0.2 mg/ml), or with both IPTG (1 mM) and x-gal (0.2 mg/ml). Each of the phosphorelay mutations (Δ0F, Δ0B, and Δ0A) was introduced into the strain independently and grown as described above. As controls, cells of the KinA strains harboring the same constructs as those of the $YdaM_N$-$KinA_C$ strain were examined. The following strains were used in this figure: MF4244 ($P_{hy\text{-}spank}$-$ydaM_N$-$kinA_C$, wt), MF4300 ($P_{hy\text{-}spank}$-$ydaM_N$-$kinA_C$, *Δspo0F*), MF4301 ($P_{hy\text{-}spank}$-$ydaM_N$-$kinA_C$, *Δspo0B*), MF4302 ($P_{hy\text{-}spank}$-$ydaM_N$-$kinA_C$, *Δspo0A*), MF4328 ($P_{hy\text{-}spank}$-*kinA*, wt), MF4329 ($P_{hy\text{-}spank}$-*kinA*, *Δspo0F*), MF4330 ($P_{hy\text{-}spank}$-*kinA*, *Δspo0B*), MF4331 ($P_{hy\text{-}spank}$-*kinA*, *Δspo0A*).

II.3.3: Autophosphorylation and phosphotransfer activities of $YdaM_N$-$KinA_C$

We determined the autophosphorylation activity of purified $YdaM_N$-$KinA_C$ in the presence of radiolabeled ATP. Purified KinA was used as a control. We

found that $YdaM_N$-$KinA_C$ exhibited autophosphorylation activity, but the time course was delayed compared with that of KinA (Fig. II-5A).

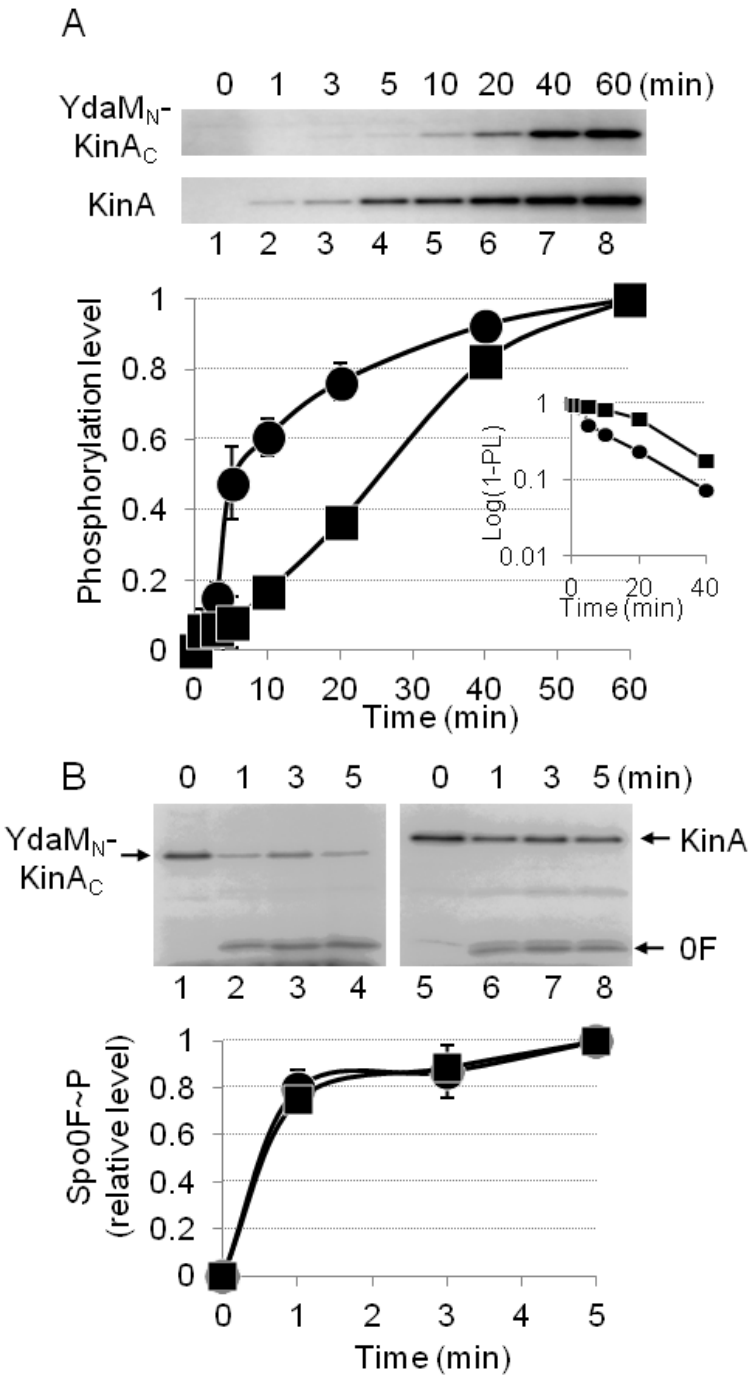

Figure II.5: Autophosphorylation and phosphotransfer activities of $YdaM_N$-$KinA_C$.

(A) Autophosphorylation activities of $YdaM_N$-$KinA_C$ and KinA were measured in an in vitro reaction. The purified $YdaM_N$-$KinA_C$ and KinA were incubated with [γ-32P]ATP as described in Materials and Methods. At the indicated times, aliquots were removed from the reaction mixture, mixed with SDS-PAGE sample buffer, and analyzed by SDS-PAGE (top panels). The fractions of phosphorylation levels plotted on the y axis in the graph were defined as the ratio of each of the labeled proteins at the indicated time point to the maximum level of that present at the end point of the reaction and are expressed as the relative level (RL). $YdaM_N$-$KinA_C$~P, filled squares; KinA~P, filled circles. The inset graph indicates the semilogarithmic plot of the value of (1 − RL) as a function of time. The estimated values of kobs (pseudo-first-order rate constant) for the autophosphorylation of $YdaM_N$-$KinA_C$ and KinA were calculated from the slopes. (B) The phosphotransfer activities from phosphorylated $YdaM_N$-$KinA_C$ or KinA to Spo0F were measured in an in vitro reaction. The purified $YdaM_N$-$KinA_C$ or KinA incubated with [γ-32P]ATP for 1 h was incubated with aliquots of Spo0F as described in Materials and Methods. At the indicated times, aliquots were removed from the reaction mixture, mixed with SDS-PAGE sample buffer, and analyzed by SDS-PAGE (top panels). The fraction of phosphorylation level (Spo0F~P) plotted on the y axis in the graph was defined as the ratio of the labeled Spo0F at the indicated time point to the maximum level of that present at the end point of the reaction. Spo0F~P with $YdaM_N$-$KinA_C$, filled squares; Spo0F~P with KinA, filled circles.

Under our standard reaction conditions, the estimated values of kobs for the autophosphorylation of $YdaM_N$-$KinA_C$ and KinA were calculated from the slopes shown in the inset of Fig. II-5A; the values of (kobs) were approximately 0.046 min^{-1} and 0.066 min^{-1}, respectively. The source of the different kinetic behaviors of these two proteins and how these differences are related to the process of sporulation are unknown. In the following sections, we examine

several possibilities regarding the relationship between enzyme kinetics and cellular response.

Second, by mixing each of the phosphorelay components with $YdaM_N$-$KinA_C$ for the in vitro reaction, we found that Spo0F was preferentially phosphorylated by $YdaM_N$-$KinA_C$, indicating that $YdaM_N$-$KinA_C$ possesses the same substrate specificity as KinA and phosphorylates Spo0A via phosphorelay (see Fig. II-6B and C). Thus, these in vitro results confirmed the in vivo results shown in Fig.II-3.

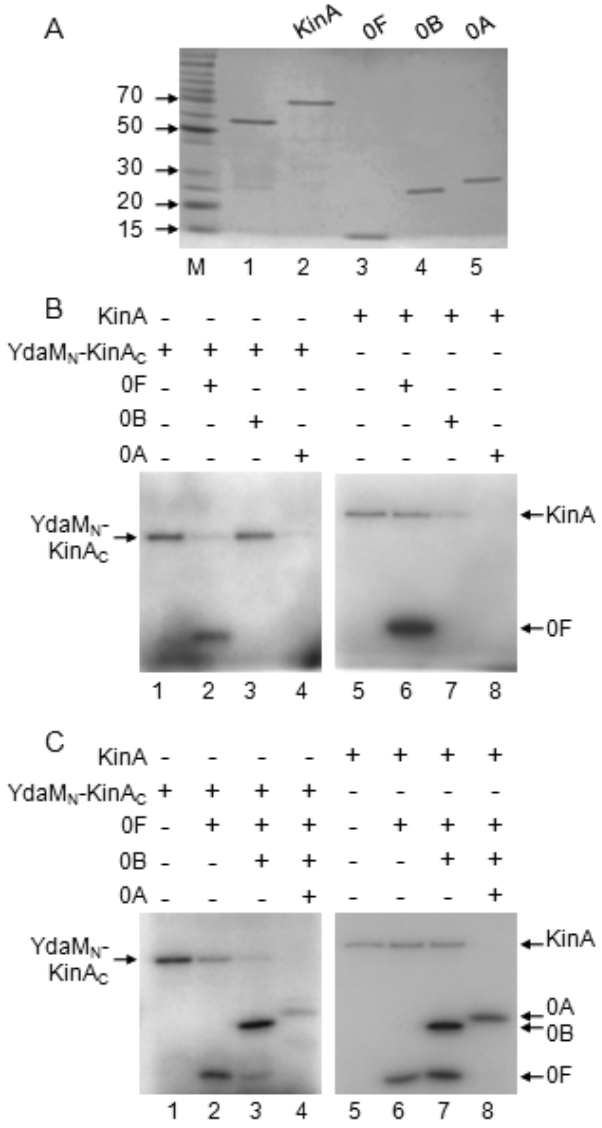

Figure II.6: YdaM$_N$-KinA$_C$ activates Spo0A in a phosphorelay-dependent manner in vitro.

(A) Analysis of purified proteins by SDS-PAGE. Each of the His-tagged YdaM$_N$-KinA$_C$ (lane 1), KinA (lane 2), Spo0F (0F, lane 3), Spo0B (0B, lane 4), and Spo0A (0A, lane 5) proteins were purified as described in Materials and Methods. Equal amounts (0.5 µg) of each purified protein were resolved by SDS-PAGE. Molecular masses (kDa) of the protein size marker (Invitrogen) are indicated to

Fig II-6 cont.

the left (lane M). The proteins were stained by Coomassie brilliant blue. (B) $YdaM_N$-$KinA_C$ transfers phosphate to Spo0F in a specific manner. In vitro phosphorylation was performed with [γ-32P]ATP as described in Materials and Methods using a various combination of the purified proteins as indicated on the top of each panel. Reaction mixtures containing $YdaM_N$-$KinA_C$ (lane 1), $YdaM_N$-$KinA_C$ and Spo0F (lane 2), $YdaM_N$-$KinA_C$ and Spo0B (lane 3), $YdaM_N$-$KinA_C$ and Spo0A (lane 4), KinA (lane 5), KinA and Spo0F (lane 6), KinA and Spo0B (lane 7), and KinA and Spo0A (lane 8) were analyzed by SDS-PAGE and autoradiography. (C) Spo0A phosphorylation mediated by the phosphorelay. In vitro phosphorylation was performed with [γ-32P]ATP as described in Materials and Methods using a various combination of the purified proteins as indicated on the top of each panel. Reaction mixtures containing $YdaM_N$-$KinA_C$ (lane 1), $YdaM_N$-$KinA_C$ and Spo0F (lane 2), $YdaM_N$-$KinA_C$, Spo0F, and Spo0B (lane 3), $YdaM_N$-$KinA_C$, Spo0F, Spo0B, and Spo0A (lane 4), KinA (lane 5), KinA and Spo0F (lane 6), KinA, Spo0F, and Spo0B (lane 7), and KinA, Spo0F, and Spo0B, and Spo0A (lane 8) were analyzed by SDS-PAGE and autoradiography.

We also determined the rate of phosphotransfer from the phosphorylated $YdaM_N$-$KinA_C$ or KinA to Spo0F. As expected from the published results for KinA, within 1 min, the labeled phosphoryl group of KinA was efficiently transferred to Spo0F. Similarly, phosphorylated $YdaM_N$-$KinA_C$ donated the labeled phosphate to Spo0F efficiently, indicating that there was no significant difference between $YdaM_N$-$KinA_C$ and KinA in their phosphotransfer abilities (Fig. II-5B). All the above in vivo and in vitro results indicated that $YdaM_N$-$KinA_C$ functions as a kinase to activate Spo0A in a phosphorelay-dependent manner. Thus, we refer to the chimeric protein as $YdaM_N$-$KinA_C$ chimeric kinase.

As indicated above, our results so far suggest that sensing the redox changes by the PAS domain of $YdaM_N$, or the different kinetic parameters

between KinA and $YdaM_N$-$KinA_C$, may explain, in part, the reason why the $YdaM_N$-$KinA_C$ strain requires a longer time period (approximately 3 h more) to achieve the threshold of Spo0A activity in LB medium than in SM medium. However, due to the difficulties in investigating these problems, the exact mechanism(s) is still unclear. Alternatively, we hypothesized that (i) the protein level of $YdaM_N$-$KinA_C$, (ii) the local subcellular concentration of $YdaM_N$-$KinA_C$, (iii) the tetramer formation of $YdaM_N$-$KinA_C$, or (iv) some combination or all of the above three factors is preferentially reduced in LB medium, leading to the delay in the activation of phosphorelay. To test these hypotheses, the following experiments were performed.

Second, by mixing each of the phosphorelay components with $YdaM_N$-$KinA_C$ for the in vitro reaction, we found that Spo0F was preferentially phosphorylated by $YdaM_N$-$KinA_C$, indicating that $YdaM_N$-$KinA_C$ possesses the same substrate specificity as KinA and phosphorylates Spo0A via phosphorelay (see Fig. II-6B and C). Thus, these in vitro results confirmed the in vivo results shown in Fig. II-3.

Third, we determined the rate of phosphotransfer from the phosphorylated $YdaM_N$-$KinA_C$ or KinA to Spo0F. As expected from the published results for KinA (22), within 1 min, the labeled phosphoryl group of KinA was efficiently transferred to Spo0F. Similarly, phosphorylated $YdaM_N$-$KinA_C$ donated the labeled phosphate to Spo0F efficiently, indicating that there was no significant difference between $YdaM_N$-$KinA_C$ and KinA in their phosphotransfer abilities (Fig. II-6B). All the above in vivo and in vitro results indicated that $YdaM_N$-$KinA_C$

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As indicated above, our results so far suggest that sensing the redox changes by the PAS domain of $YdaM_N$, or the different kinetic parameters between KinA and $YdaM_N$-$KinA_C$, may explain, in part, the reason why the $YdaM_N$-$KinA_C$ strain requires a longer time period (approximately 3 h more) to achieve the threshold of Spo0A activity in LB medium than in SM medium. However, due to the difficulties in investigating these problems, the exact mechanism(s) is still unclear. Alternatively, we hypothesized that (i) the protein level of $YdaM_N$-$KinA_C$, (ii) the local subcellular concentration of $YdaM_N$-$KinA_C$, (iii) the tetramer formation of $YdaM_N$-$KinA_C$, or (iv) some combination or all of the above three factors is preferentially reduced in LB medium, leading to the delay in the activation of phosphorelay. To test these hypotheses, the following experiments were performed.

II.3.4 Similar levels of the chimeric kinase protein are detected in cells cultured in either LB or SM

First, to determine whether $YdaM_N$-$KinA_C$ is produced at different levels in LB and SM media, we performed quantitative immunoblot analyses using a C-terminal GFP-tagged $YdaM_N$-$KinA_C$ under the control of Phy-spank. We confirmed that the $YdaM_N$-$KinA_C$-GFP construct showed essentially similar levels of sporulation efficiency as the strain expressing the untagged protein, indicating that $YdaM_N$-$KinA_C$-GFP is functional. As controls, we used strains harboring the

gene for KinA-GFP under the control either of its own promoter at the endogenous locus or Phy-spank, as reported previously (Fujita and Losick 2005).

Cells harboring the gene for $YdaM_N$-$KinA_C$-GFP under the control of Phy-spank were grown in the presence of various IPTG concentrations in LB or SM medium. Cell extracts from these two strains were prepared at 2 h and 5 h after IPTG addition. Then, we measured the levels of the GFP-fusion protein with immunoblot analysis using anti-GFP antibodies. For the control, cell extracts were prepared from the wild-type strain (harboring KinA-GFP at the endogenous locus) cultured for 2 h under normal sporulation conditions in SM medium and used for the standard sample. A constitutively expressed sigma factor, σ^A, was used as a loading and normalization control. In response to the concentration of added IPTG, the protein levels of $YdaM_N$-$KinA_C$-GFP increased similarly in both LB and SM media (Fig. II-7A and B). At the effective concentration of IPTG (200 μM) to trigger sporulation, levels of $YdaM_N$-$KinA_C$-GFP protein in both LB and SM media reached approximately 2-fold above the endogenous KinA-GFP levels in the wild-type sporulating cells (Fig. II-7A and B). These protein levels were approximately constant from 2 to 5 h during the culture period, although the protein levels in the presence of low concentrations of IPTG (10 and 20 μM) were slightly decreased at 5 h. For comparison of the activities of the chimeric kinase with the protein levels, the activity data were plotted as a function of IPTG concentration (Fig. II-7C). We predicted that if the signal to be sensed by $YdaM_N$ was produced only under starvation conditions in SM medium, the $YdaM_N$-$KinA_C$ kinase, even at relatively lower protein levels, would be more active in SM

medium than in LB medium. However, the results indicated that there was no significant medium-dependent effect on the activity itself (Fig. II-7C), essentially as observed for KinA (data not shown), except the delay of the peak activity was observed only in the $YdaM_N$-$KinA_C$ strain in LB medium (Fig. II-A). As reported previously, I noted that little or no Spo0A activity could be detected in the wild-type strain (which lacks the IPTG-inducible construct) under the LB conditions, even at a later growth stage at which Spo0A activity was detectable in the $YdaM_N$-$KinA_C$ strain. These results suggest that the $YdaM_N$-$KinA_C$ kinase triggers sporulation efficiently in a concentration-dependent manner, irrespective of nutrient availability. As discussed in the above section, the chimeric protein might be positively or negatively regulated, depending on nutrient availability, and resulted in the delay of peak activity in LB medium compared with SM medium.

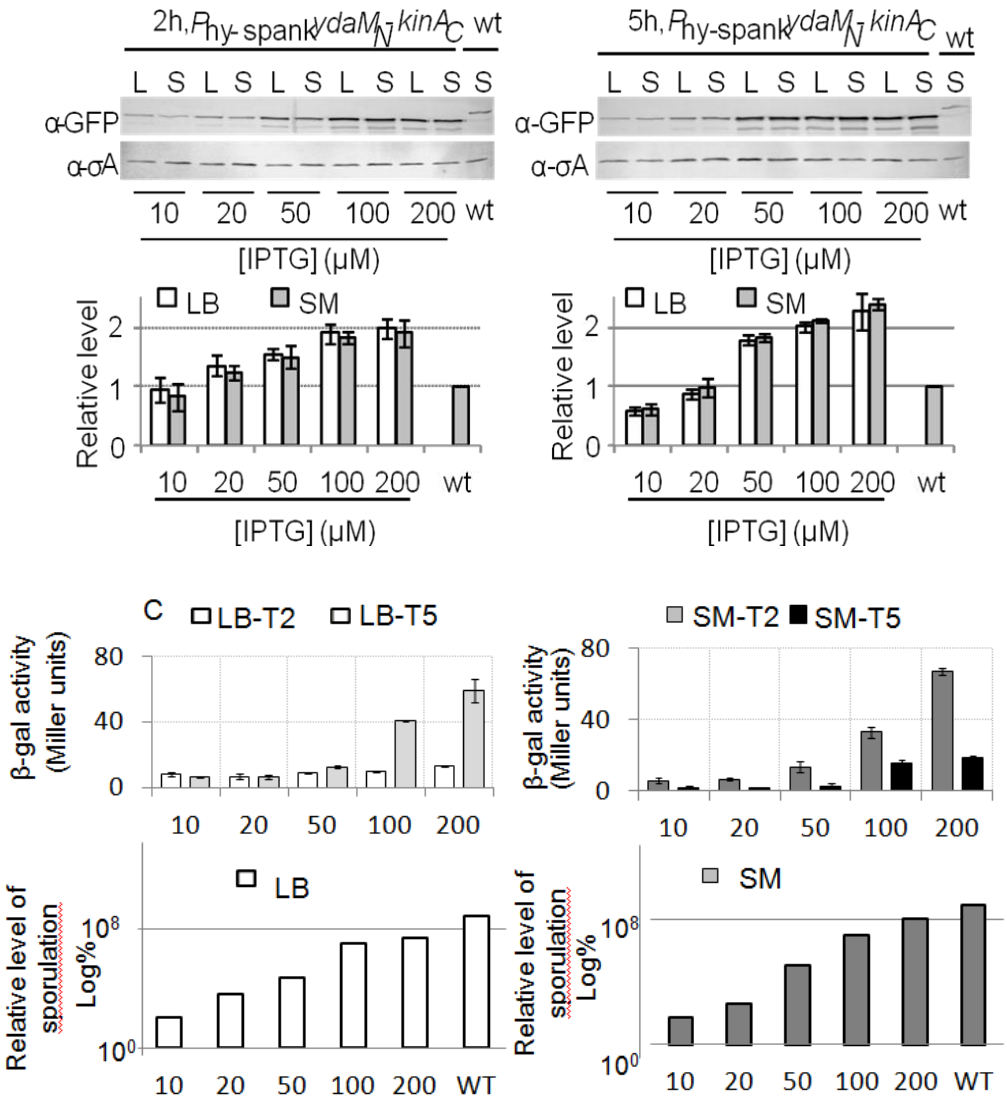


Figure II-7: Protein levels and activities of $YdaM_N$-$KinA_C$ in cells cultured in LB and SM media.

Cells of the strain harboring the gene for $YdaM_N$-$KinA_C$-GFP under the control of the Phy-spank promoter (MF4201) were cultured in LB and SM media in the presence of the indicated concentrations of IPTG. Cell extracts were prepared from cells harvested at 2 h (A) and 5 h (B) after the addition of IPTG. Cell extracts were subjected to SDS-PAGE followed by immunoblot analysis with anti-GFP antibodies. The constitutively expressed σ^A protein was detected with anti-σ^A antibodies and used as a loading control as reported previously (14). The

Fig II-7 cont.

intensities of each band were quantified with an image analyzer (FluorChem; Alpha Innotech). Each of the protein levels was normalized to σ^A levels and then to the endogenous KinA-GFP level produced in the wild-type strain (wt; MF3593) cultured for 2 h in SM medium. Relative levels shown in the graph are after the normalization of the intensities. (C) Cells of the $YdaM_N$-$KinA_C$ strain harboring the PspoIIA-lacZ reporter construct (MF4254) were cultured in LB and SM media and collected at 2 and 5 h after the addition of the indicated concentrations of IPTG. $YdaM_N$-$KinA_C$ activity was measured as Spo0A activity based on the PspoIIA-lacZ reporter as described for Fig. II-2. All experiments were performed at least three times independently, and average values and standard deviations are plotted.

II.3.5 The chimeric kinase distributes uniformly throughout the cytoplasm, similar to wild-type KinA

Second, to investigate whether the subcellular localization of $YdaM_N$-$KinA_C$ is related to the shift in the peak activity, possibly by increasing the local concentration of the kinase in SM medium at earlier times than in LB medium, we examined the cellular distribution pattern of the GFP-fusion protein with fluorescence microscopy, under the same culture conditions as above. As shown in figure II-7, we found that $YdaM_N$-$KinA_C$-GFP localized uniformly in the cytosolic compartment at the effective IPTG concentration (200 μM) in both LB and SM media, and no significant differences in localization patterns were detected between these two conditions, as was observed in the KinA-GFP strain (Fujita and Losick 2005, Eswaramoorthy, Dinh et al. 2010). These results suggest that the shift in the peak activity was not due to the decreased local concentration of the chimeric kinase in LB medium.

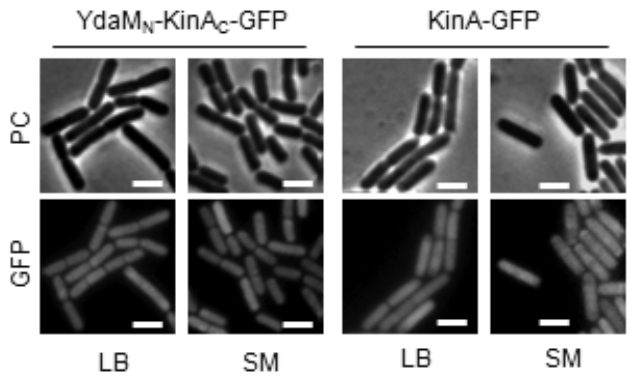


Figure II.8: Fluorescence microscopy of the cellular localization of YdaM$_N$-KinA$_C$-GFP in the IPTG-inducible system.

Cells of the strains harboring the IPTG-inducible YdaM$_N$-KinA$_C$-GFP (MF4201) and KinA-GFP (MF3352) were cultured in LB and SM media in the presence of IPTG (200 µM for YdaM$_N$-KinA$_C$-GFP and 10 µM for KinA-GFP) for 2 h to steady state level. Fluorescence microscopy was carried out as indicated in Materials and Methods. GFP and the corresponding phase contrast (PC) images shown are representative of more than 500 cells in the whole cell population inspected in three separate fields of view. Bar, 2 µm.

II.3.6: The chimeric kinase forms a homotetramer, similar to wild-type KinA.

Recently, using biochemical means, it has become evident that KinA forms a homotetramer as a stable and functional kinase (Eswaramoorthy, Guo et al. 2009). In the light of these results, we wonder whether the YdaM$_N$-KinA$_C$ chimeric kinase forms a tetramer as a functional unit and the tetramer formation is preferentially reduced in the cells cultured in LB medium, resulting in the delay of the peak activity. To address these issues, we performed chemical cross-linking experiments. For this, crude cell extracts were prepared from cells expressing YdaM$_N$-KinA$_C$-GFP in LB and SM media and incubated in the presence or absence of BMH, a thiol-specific cross-linker (Eswaramoorthy, Guo

et al. 2009). The N terminal of YdaM ($YdaM_N$) has three cysteines (C88, C172, and C234) that are potentially cross-linkable by BMH, if they are located near each other in the complex. we note that the C terminus of KinA ($KinA_C$) has no cysteine residues, so that it cannot be used for the cross-linking reaction with BMH and thus, the complex formation, if detected, is absolutely dependent on the N-terminus. The calculated molecular masses of each of the protein samples are listed in Fig. II-8A. The crude extracts containing $YdaM_N$-$KinA_C$-GFP ($YdaM_N$-$KinA_C$) were prepared from cells grown in LB medium. Then, the samples were treated with or without cross-linker. In the presence of cross-linker, larger bands at around 300 kDa were detected (Fig. II-8B, lane 6). In contrast, only a discrete band at around monomer size (estimated size, 80 kDa) was detected in the absence of the cross-linker (Fig. II-8B, lane 5). Furthermore, $YdaM_N$-GFP migrated at around 200 kDa in the presence of the cross-linker (Fig. II-8B, lane 8), while only the monomer (55 kDa) was detected in the absence of the cross-linker (Fig. II-8B, lane 7). These shifted bands at high molecular mass positions corresponded well with the tetramer sizes of each of the tested samples (Fig. II-8A). As positive controls, we used both KinA-GFP and $KinA_N$-GFP to verify that the results were reproducible as reported previously (Eswaramoorthy, Guo et al. 2009), showing that KinA predominantly forms a tetramer (Fig. II-8B, lanes 1 to 4). As a negative control, both in the presence and absence of the cross-linker, only a discrete single band corresponding to the monomer size of GFP was detected in the crude extracts from cells expressing GFP alone, suggesting that two cysteine residues in GFP are not involved in complex formation under our

tested conditions (Fig. II-8B, lanes 9 and 10) (Yang, Moss et al. 1996). Our previous results indicate that neither Spo0F nor Spo0A is detected in the immunoprecipitated fraction, suggesting that KinA does not form a stable complex with other phosphorelay components (Eswaramoorthy, Guo et al. 2009). Therefore, some minor bands detected in the cross-linked samples might not be the complex with the other phosphorelay component. Rather, they might be due to the formation of nonspecific protein complexes, such as inter- or intramolecular cross-linking formations in different ways, depending on the sulfhydryl involved.

Next, I examined whether the functional tetramer of $YdaM_N$-$KinA_C$-GFP is preferentially formed in SM medium (compared to LB medium), which may explain the shift in the peak activity shown in Fig. II-2A. Cells expressing $YdaM_N$-$KinA_C$-GFP were grown in LB and SM media for 2 h and 5 h in the presence of IPTG (200 μM). Then, crude cell extracts were prepared and incubated in the presence or absence of cross-linker. As shown in Fig. II-8C, in the presence of cross-linker, the tetramer form of $YdaM_N$-$KinA_C$-GFP was detected in each of the tested conditions and there were no significant differences of the complex formations in all tested samples prepared from cells grown in LB and SM media. These results suggest that the shift in the peak activity is not due to the destabilization of the complex formation in LB medium. In conclusion, although various attempts were made, the mechanism by which the timing of the chimeric kinase activity is regulated either positively or negatively, depending on the medium used, remains unknown.

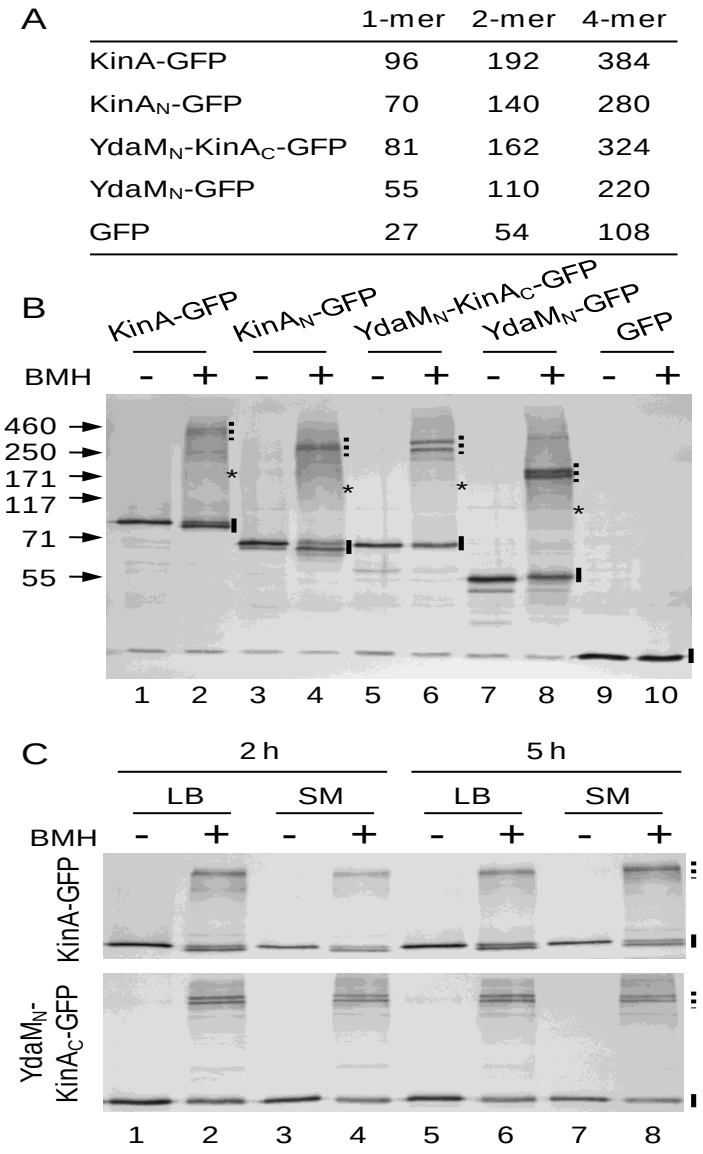


Figure II.8: Cross-link analysis of YdaM$_N$-KinA$_C$ complex formation in cells cultured in LB and SM media.

(A) Molecular masses and possible multimeric combinations of each sample. (B) Cells harboring the IPTG-inducible GFP-tagged protein construct were cultured in LB in the presence of IPTG (10 μM for KinA-GFP and GFP, 200 μM for

Fig II.8 cont.
$YdaM_N$-$KinA_C$-GFP). Cell extracts were prepared from each strain 2 h after IPTG addition and processed for the BMH cross-linking reaction as described in Materials and Methods (BMH+, even lanes). Samples processed in the absence of BMH were used as controls (BMH-, odd lanes). Strains expressing KinA-GFP (MF3352; lanes 1 and 2), $KinA_N$-GFP (MF3360; lanes 3 and 4), $YdaM_N$-$KinA_C$-GFP (MF4201; lanes 5 and 6), $YdaM_N$-GFP (MF4080; lanes 7 and 8), and GFP alone (MF2732; lanes 9 and 10) were examined for cross-linking experiments. Samples were separated by SDS-PAGE, followed by immunoblot analysis with anti-GFP antibodies. Solid bars, asterisks, and dashed bars depict the expected sizes of monomer, dimer, and tetramer forms, respectively. (C) Cells expressing KinA-GFP (top panel) or $YdaM_N$-$KinA_C$-GFP (bottom panel) were cultured in LB (lanes 1, 2, 5, and 6) and SM (lanes 3, 4, 7, and 8) and collected at 2 h (lanes 1 to 4) or 5 h (lanes 5 and 6) after IPTG addition (10 μM for KinA-GFP, 200 μM for $YdaM_N$-$KinA_C$-GFP). Cell extracts were processed as described for panel B. Even and odd lanes contain samples with and without BMH, respectively. Solid and dashed bars depict the expected sizes of monomer and tetramer forms, respectively. Molecular masses (in kDa) of the protein size marker (Invitrogen) are indicated to the left.

II.3.7 Autophosphorylation activity of the chimeric kinase is independent of enzyme concentration, similar to wild-type KinA

We determined whether the autophosphorylation of the chimeric kinase occurs in a protein concentration-dependent manner. Generally, an intramolecular reaction would exhibit saturation kinetics, so that the activity of the autophosphorylation reaction would not be enzyme concentration dependent (Kuret and Schulman 1985). In other words, if the activity of the tetrameric kinase is dependent on the protein concentration, the functional subunit assembly might be dependent on the concentration of the individual protomers. In order to test this, the autophosphorylation activity was monitored as a function of enzyme concentration over a 10-fold range of concentrations (Fig. II-9). A plot of the

activity at various concentrations showed a constant level, indicating that the rate of $YdaM_N$-$KinA_C$ autophosphorylation is independent of enzyme concentration (Fig. II-9AC). As a control, I examined the purified KinA, as described above, and obtained essentially reproducible results as reported in the literature (Fig. II-9B and D) (Grimshaw, Huang et al. 1998). In our previous study with a dominant negative assay, I showed that autophosphorylation of KinA is in a transphosphorylation manner (Eswaramoorthy, Guo et al. 2009). Taking together all the data presented above and considering the similar properties of $YdaM_N$-$KinA_C$ and KinA, we suggest that the autophosphorylation activity of each kinase is likely to result from an intramolecular transautophosphorylation in a stable tetramer as a functional unit.

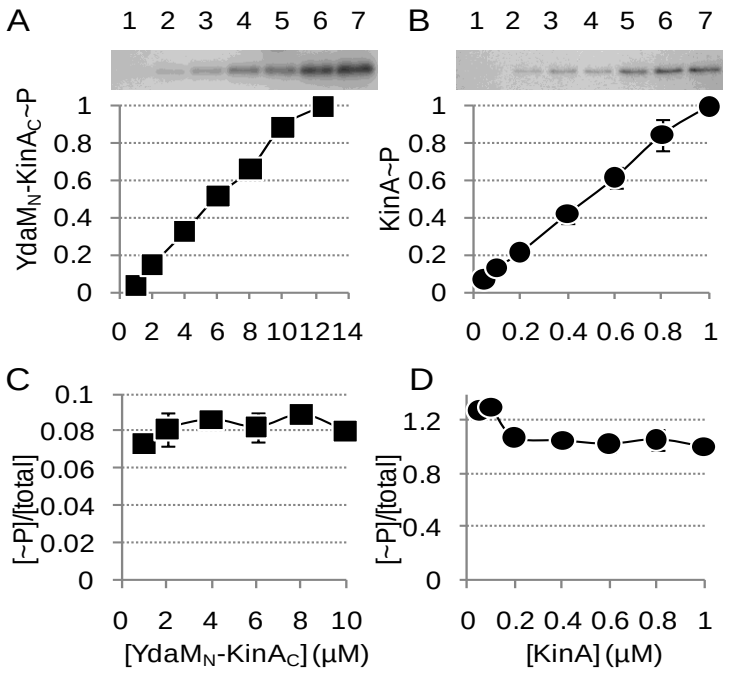


Figure II.9: YdaM$_N$-KinA$_C$ is autophosphorylated in a protein concentration-independent manner in vitro.

(A) The indicated concentrations (in μM) of purified YdaM$_N$-KinA$_C$ were incubated with [γ-32P]ATP as described in Materials and Methods. Reaction mixtures were analyzed by SDS-PAGE and autoradiography (top panel). Phosphorylation levels of YdaM$_N$-KinA$_C$ (YdaM$_N$-KinA$_C$~P) plotted on the y-axis were defined as the ratio of [32P]YdaM$_N$-KinA$_C$ present at the indicated concentration of YdaM$_N$-KinA$_C$ versus the level of [32P]YdaM$_N$-KinA$_C$ at the highest concentration of YdaM$_N$-KinA$_C$ (lane 7). (B) The indicated concentrations of purified KinA were examined as described for panel A. (C) Phosphorylation levels of YdaM$_N$-KinA$_C$ shown in panel A at the indicated concentrations of YdaM$_N$-KinA$_C$ were divided by the same range of concentrations. Thus, the ratio of phosphorylated YdaM$_N$-KinA$_C$ over total YdaM$_N$-KinA$_C$ is expressed as [~P]/[total] on the y-axis, and the value is plotted as a function of the protein concentration on the x-axis. (D) The data for KinA autophosphorylation were processed as described for panel C.

II.4 Concluding remarks and perspectives

Evidence is presented in this chapter that the replacement of the sensor domain of KinA with a foreign domain of YdaM from *E. coli* results in a massive induction of sporulation initiation by a concentration-dependent mechanism through phosphorelay. The resulting chimeric kinase forms a homotetramer, as was reported for KinA (Eswaramoorthy, Guo et al. 2009, Eswaramoorthy, Duan et al. 2010, Eswaramoorthy and Fujita 2010). Although the chimeric kinase shows delayed activation of Spo0A under nutrient-rich conditions compared with starvation conditions, with unknown mechanisms, the peak activity levels of Spo0A (as an indirect measure of the kinase activity) and the resulting sporulation efficiencies are essentially the same under these two conditions. These results and our published studies (Eswaramoorthy, Duan et al. 2010, Eswaramoorthy and Fujita 2010) suggest that, regardless of the origin of the protein, tetramer formation mediated by the N-terminal domain is sufficient for the kinase activity catalyzed by the C-terminal domain, irrespective of nutrient availability. The prior studies of the well-known sensor kinases, such as the EnvZ osmosensor and the nitrogen sensor NRII kinase/phosphatase, indicate that, even under conditions of overexpression from multicopy plasmids, the signal (ligand) is still required for the proper response of the kinases (Atkinson and Ninfa 1993, Waukau and Forst 1999). More recently, overexpression of KinC, an alternative histidine kinase involved in biofilm formation, does not induce massive pellicle formation (biofilm growth) (Lopez and Kolter 2010). These prior results suggest that the overexpression of the kinase is not capable of overriding the

sensor control in vivo, if a ligand is required for the activation process. Thus, our results suggest that massive entry into sporulation by inducing the synthesis of the chimeric kinase is not simply due to the overexpression of the chimeric kinase. Rather, our data support a model in which the threshold level of the kinase, which is constitutively active and signal independent, acts as a molecular switch regulating the entry into sporulation. The mechanism(s) that control the cellular level of the kinase remains to be determined.

CHAPTER III: TRIGGERING SPORULATION IN *BACILLUS SUBTILIS* WITH ARTIFICIAL TWO-COMPONENT SYSTEMS REVEALS THE IMPORTANCE OF PROPER SPO0A ACTIVATION DYNAMICS.

Triggering sporulation in Bacillus subtilis with artificial two-component systems reveals the importance of proper Spo0A activation dynamics.

Vishnoi M, Narula J, Devi SN, Dao HA, Igoshin OA, Fujita M.

Mol Microbiol. 2013 Oct;90(1):181-94. doi: 10.1111/mmi.12357. Epub 2013 Aug 23.

The gradual increase of the Spo0A activity is controlled by the integrated signal processing through phosphorelay after nutrient starvation. Here, we showed that simultaneous induction of KinC, a kinase that can directly phosphorylate Spo0A, and Spo0A itself from separately controlled inducible promoters can efficiently trigger sporulation even under nutrient rich conditions. Here, using a synthetic biology approach, I found that KinC, in a dose-dependent manner, triggers efficient entry into sporulation under nutrient rich conditions. We showed that the KinC level in the engineered cells undergoing sporulation is higher than that in the wild-type cells, suggesting that the expression level of KinC is important to cause sporulation. Furthermore, when expressed certain levels, KinC was able to directly transfer the phosphate moiety from the kinase to the sporulation master regulator Spo0A and trigger sporulation by bypassing a requirement for a normal sporulation four-component phosphorelay composed of the kinase, two phosphotransferases (Spo0F and Spo0B), and Spo0A. However, when artificially induced the synthesis of the two components (KinC and Spo0A) simultaneously, Spo0A is activated prematurely by KinC in a two-component manner, resulting in impaired sporulation efficiency. Based on these findings, we propose a model in which the evolutionally conserved phosphorelay acts as a sporulation.

III.1 Introduction

Upon nutrient starvation, a majority of *Bacillus subtilis* cells differentiate to produce spores. Extensive studies have elucidated a detailed genetic network of genes involved in an early stage of sporulation (Burbulys, Trach et al. 1991, Hoch 1993, Grossman 1995, Sonenshein 2000). At the heart of this network is a phosphorelay (Fig. III-1A), in which phosphate is transferred from multiple histidine protein kinases (KinA-E) to a master transcription regulator Spo0A through two intermediate phosphotransferases (Spo0F and Spo0B) (Burbulys, Trach et al. 1991, Hoch 1993, Fawcett, Eichenberger et al. 2000). Transcriptionally, the phosphorelay genes encoding KinA, Spo0F, and Spo0A are controlled by the phosphorylated form of Spo0A (Spo0A~P) directly and indirectly via multiple feedback loops (Fig. III-1B). This complex network appears to be the integration point for putative extracellular and intracellular signals that trigger entry into the sporulation program in response to starvation (Hoch 1993, Grossman 1995).

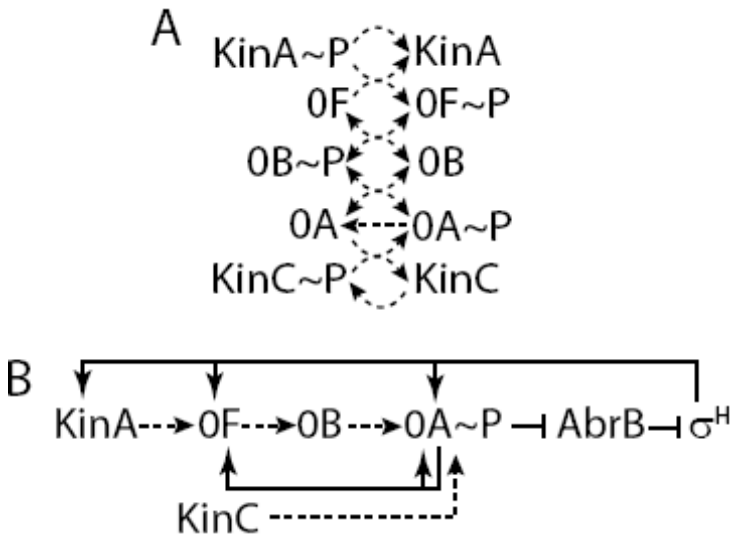


Figure III.1: The sporulation network phosphorelay.

Solid arrows and T-shaped bars indicate positive and negative gene regulatory interactions respectively. Dashed arrows indicate phosphotransfer interactions.

A. Post-translational interactions in the wild-type phosphorelay regulatory network. Sensor histidine kinases (KinA and KinC) autophosphorylate and provide phosphate to activate the master regulator Spo0A (0A). KinA transfers phosphate to 0A via Spo0F (0F) and Spo0B (0B), whereas KinC can transfer phosphate to 0A directly.

B. Transcriptional regulatory interactions. Phosphorylated Spo0A (0A~P) becomes a positive regulator for sporulation genes, including those for 0A itself and 0F. 0A~P also indirectly activates the expression of the gene for σH, which is essential for sporulation, by repressing AbrB, the repressor of σH.

The initial phase of starvation is marked by a gradual increase in the level of Spo0A~P, which triggers the expression of many genes essential for sporulation (Fujita and Losick 2003, Fujita, Gonzalez-Pastor et al. 2005, Fujita and Losick 2005). These observations are the motivation for the long-standing hypothesis that sporulation cell-fate can be predicted based on whether or not a cell has a threshold level of Spo0A~P (Hoch 1993, Grossman 1995). I have

recently demonstrated that such a Spo0A~P threshold is justified considering the ultrasensitive (switch-like) response of a cascade of feed-forward loops downstream of Spo0A~P (Narula, Devi et al. 2012). However this Spo0A~P threshold model of cell-fate determination does not explain the experimental observation that rapid accumulation of active Spo0A actually has an adverse effect on sporulation (Fujita, Gonzalez-Pastor et al. 2005, Fujita and Losick 2005). These studies raise two important questions about the mechanistic connection between Spo0A~P dynamics and sporulation cell-fate: (1) Why does accelerated Spo0A~P accumulation impair sporulation efficiency? and (2) How does the design of the sporulation phosphorelay ensure proper Spo0A~P accumulation dynamics? In this study I took a synthetic biology approach to answer these questions.

Interrogating naturally-occurring networks with methods of synthetic biology has become a widely used approach (Bashor, Horwitz et al. 2010, Elowitz and Lim 2010). The relationships between network architecture and its function can be uncovered more easily by rewiring naturally occurring networks and decoupling interactions or feedback loops than through gene disruption/deletion (Eldar, Chary et al. 2009, Eswaramoorthy, Duan et al. 2010, Kuchina, Espinar et al. 2011). I have previously used this approach by building an Artificial Sporulation Initiation (ASI) system in which the phosphate flux of the phosphorelay activating Spo0A is artificially regulated by expressing KinA from an IPTG-inducible promoter (Fujita and Losick 2005, Eswaramoorthy, Duan et al.

2010). This system allowed us to uncouple the signal-sensing mechanisms in the phosphorelay from the signal-processing mechanisms and revealed important design properties of the signal processing modules in the sporulation network (Eswaramoorthy, Guo et al. 2009, Eswaramoorthy, Duan et al. 2010, Narula, Devi et al. 2012).

Here we took this approach further and used novel synthetic networks to control the temporal dynamics of Spo0A~P accumulation. I employed a system in which the kinase KinC is expressed from an IPTG-inducible promoter and Spo0A is expressed from a xylose-inducible promoter. Since KinC can transfer phosphate to Spo0A directly (Kobayashi, Shoji et al. 1995, LeDeaux and Grossman 1995), both the level of Spo0A and its phosphorylation rate can be easily modulated externally in this system. Using this system, I first determined the relationship between sporulation efficiency and the level of expression of KinC and Spo0A. Surprisingly, I found that sporulation efficiency depends non-monotonically on the levels of these proteins despite the fact that Spo0A activity increases monotonically with increases in Spo0A and KinC. We were able to predict and confirm the specific biochemical mechanism underlying the adverse effect of accelerated Spo0A~P accumulation on sporulation in our artificial system. Finally, we modified our inducible KinC-Spo0A and demonstrated that the positive transcriptional feedback in the wild-type phosphorelay is sufficient to ensure proper temporal accumulation of Spo0A~P and efficient sporulation.

III.2 Materials and Method

III.2.1 Strains, plasmids and oligonucleotides

All strains for experiments were derived from the B. subtilis prototrophic strain PY79 (Youngman, Zuber et al. 1985). Details of strains used in this study are listed in Supplementary Table III-1 in the supplementary material. The strain harbouring the divIVAΩlinker-cfp was a gift from Prahathees Eswaramoorthy and Kumaran S. Ramamurthi. All plasmid constructions (Table III- 2) were performed in Escherichia coli DH5α using standard methods. The oligonucleotide primers used for plasmid construction are listed in Table III-3.

Table III-1. Strains

Sporulation assay		
Strain	Genotype or description	Reference
MF1913	*kinA*ΩP$_{hy\text{-}spank}$-*kinA cm*	(Fujita and Losick 2005)
MF1915	*kinC*ΩP$_{hy\text{-}spank}$-*kinC cm*	(Fujita and Losick 2005)
MF2406	*kinC*ΩP$_{hy\text{-}spank}$-*kinC cm, Δspo0F::tet*	This study
MF4419	*kinC*ΩP$_{hy\text{-}spank}$-*kinC cm, Δspo0B::tet*	This study

β-Galactosidase assay		
Strain	Genotype or description	Reference
MF750	*thrC*::P$_{spoIIG}$-*lacZ erm*	(Eswaramoorthy, Guo et al. 2009)
MF4317	*kinC*ΩP$_{hy\text{-}spank}$-*kinC cm*, *amyE*::P$_{xylA}$-*spo0A spc*, *Δspo0F::tet*, *Δspo0A::kan*, *thrC*::P$_{spoIIG}$-*lacZ erm*	This study
MF4531	*amyE*::P$_{hy\text{-}spank}$-*kinC spc*, *ΔkinA::tet*, *ΔkinB::kan*, *ΔkinC::cm*, *thrC*::P$_{spoIIG}$-*lacZ erm*	This study

Fluorescence and immunoblot assay		
Strain	Genotype or description	Reference
MF929	*kinA*Ω*kinA-gfp kan*	
MF2659	*kinC*Ω*kinC-gfp spc*	This study
MF1996	*kinA*ΩP$_{hy\text{-}spank}$-*kinA-gfp spc kan*	
MF2734	*kinC*ΩP$_{hy\text{-}spank}$-*kinC-gfp spc kan*	This study
MF4318	*kinC*Ω*Phy-spank-kinC cm, amyE::PxylA-spo0A spc, Δspo0F::tet, Δspo0A::kan, thrC::PspoIIG-gfp erm*	This study
MF4581	*amyE*::P$_{xylA}$-*cfp spc*, *thrC*::P$_{hy\text{-}spank}$-*yfp erm*	This study
MF4812	*amyE::PxylA-spo0A spc, thrC::Phy-spank-kinC erm, divIVA*Ω*linker-cfp cat, ∆spo0A::kan, ∆spo0F::tet*	This study

Table III-2. Plasmids used in this study

Plasmid	Description	Reference
pMF311	*amyE*::P*xylA-spo0A*	This study
pMF604	*amyE*::P*xylA-cfp spc*	This study
pMF607	*thrC*::$P_{hy\text{-}spank}$-*yfp erm*	This study

Table III-3. Oligonucleotide primers used in this study

Primer	Sequence	Construct
om24	5'-ccgctcgaggctgtctgattttaaaggcaa-3'	(*Xho*I) *kinC-gfp*
om29	5'-gccgaattcacatctgtcagcggtttcctccag-3'	(*Eco*RI) *kinC-gfp*
omf288	5'-ggcgtcgacacataaggaggaactactatggagaaaattaaagtttgtgttgc-3'	(*Sal*I) P_{xylA}-*spo0A*
omf318	5'-gccgcatgcttaagaagccttatgctctaacctcagc-3'	(*Sph*I) P_{xylA}-*spo0A*
om138	5'-gccgtcgacacataaggaggaactactatggtttcaaaaggcgaagaactg-3'	(*Sal*I) P_{xylA}-*cfp*
om139	5'-gccgcatgcttacttataaagttcgtccatgccaag-3'	(*Sph*I) P_{xylA}-*cfp*
om137	5'-gccgtcgacacataaggaggaactactatgagtaaaggagaagaactt-3'	(*Sal*I) $P_{hy\text{-}spank}$-*yfp*
omf317	5'-gccgcatgcttatttgtatagttcatccatgcc-3'	(*Sph*I) $P_{hy\text{-}spank}$-*yfp*

III.2.2 Media and culture conditions

To induce the synthesis of the protein of interest in B. subtilis cells, an isopropyl-β-d-thiogalactopyranoside (IPTG)-inducible hyper-spank promoter (Phy-spank) (Fujita and Losick 2005) and/or a xylose-inducible promoter (PxylA) (Gueiros-Filho and Losick 2002, Wagner, Marquis et al. 2009) were used (kind gifts from D. Rudner, Harvard Medical School). The inducer (IPTG and/or xylose) was added at the indicated concentration to the culture in LB medium during the exponential growth phase (optical density at 600 nm, 0.5). Sporulation of the wild-type strain was induced by the procedure of the resuspension method (Sterlini and Mandelstam 1969).

III.2.3 Sporulation efficiency and β-galactosidase assays

Sporulation efficiency was determined in overnight (16–18 h) culture, as cfu per ml (spores) after heat treatment by incubation at 80°C for 10 min, compared with cfu per ml (viable count) of the pre-heat treatment sample. Assays of β-galactosidase activity were performed as described previously (Eswaramoorthy, Guo et al. 2009).

III.2.4 Immunoblot analysis

For KinC-GFP, cells of the KinC strain (a gene for KinC is placed under the control of an IPTG-inducible Phy-spank promoter) harbouring kinC-gfp (MF2734) were cultured to mid-exponential phase (OD600 = 0.5) in LB, and IPTG was added at indicated concentrations. Cells were harvested at 2 h after induction. Cells of the wild-type strain harbouring kinC-gfp (MF2659) were cultured the same as above, except for the IPTG addition, as nutrient rich conditions. Cells of MF2659 were cultured in hydrolysed casein (CH) growth medium and resuspended in Sterlini & Mandelstam (SM) medium as sporulation conditions (Sterlini and Mandelstam 1969). For Spo0A, the KinC-Spo0A strain (MF4318, a gene for KinC is placed under the control of an IPTG-inducible Phy-spank promoter and a gene for Spo0A is placed under the control of a xylose-inducible PxylA promoter) was cultured as above, and xylose was added at indicated concentrations. MF2659 was used for the wild-type Spo0A protein. Cell extracts for immunoblot analysis were prepared from 2 h after IPTG addition in LB or 2 h culture in SM medium by sonication. Immunoblot analysis was

performed as described previously (Fujita and Losick 2002). Polyclonal anti-GFP (Rudner and Losick 2002), anti-Spo0A (Fujita 2000) and anti-σA (Fujita 2000) antibodies were used to detect corresponding proteins or GFP-tagged proteins. The intensities of each band after immunostaining were quantified with a FluorChem digital imaging system (Alpha Innotech). σA was served as an internal standard control for normalization (Fujita 2000). The protein levels were normalized to both the levels of σA and then the levels of each of the corresponding proteins in the wild-type strain (MF2659).

III.2.5 Fluorescence microscopy

Fluorescence microscopy was performed as described previously (Eswaramoorthy, Guo et al. 2009). The membrane and DNA were stained with FM4-64 and DAPI (4', 6-diamidino-2-phenylindole) respectively. Image acquisition and analysis were performed with Slidebook (Intelligent Imaging Innovations). Single cell fluorescence intensities (arbitrary pixel units) were quantified using MicrobeTracker Suite (Sliusarenko, Heinritz et al. 2011) and custom MATLAB code.

III.3 Results

III.3.1 KinC governs entry into sporulation in a concentration-dependent manner

We constructed a strain expressing KinC under an IPTG-inducible hyper-spank promoter (hereafter referred to as Phy-spank). To determine the effective

level of KinC for efficient entry into sporulation, the KinC-inducible strain (MF1915, referred as KinC strain) was cultured in LB in the presence of varying concentrations of IPTG. Sporulation efficiency was determined by measuring the fraction of heat-resistant colony forming units (CFU). We note that in these nutrient rich media, the wild-type strain fails to sporulate (Eswaramoorthy, Duan et al. 2010). We found that, at IPTG concentrations 10 of 10μM, sporulation was efficiently induced to the level comparable to that in the wild-type strain (i.e., without IPTG-inducible construct) under normal sporulation conditions. Second, to determine the effect of KinC induction on Spo0A activity I constructed a reporter system consisting of lacZ gene expression driven by the Spo0A-dependent spoIIG promoter (Fujita and Losick 2005, Eswaramoorthy, Guo et al. 2009). We then introduced this reporter system into the KinC strain (MF4531) to examine β-galactosidase activity under the same conditions as above. We confirmed that Spo0A was efficiently activated similarly to that seen in the sporulating wild-type strain (Table III.1). We note that, without IPTG, none of the kinases can activate Spo0A to a level that is sufficient to trigger sporulation in LB medium (LeDeaux and Grossman 1995, de Jong, Veening et al. 2010, Eswaramoorthy, Duan et al. 2010). These results show that artificially induced expression of KinC can be used to control Spo0A activation and entry into sporulation. Increase in KinC triggers sporulation in a phosphorelay-independent manner. We determined whether inducing the synthesis of KinC would bypass the requirement of the phosphorelay intermediate phosphotransferases for sporulation as suggested previously (Kobayashi, Shoji et al. 1995, LeDeaux and

Grossman 1995). To demonstrate this, a null mutation of spo0F (MF2406) or spo0B (MF4419) was introduced into the KinC strain, and the resulting strains were tested for sporulation efficiency in LB in the presence of varying concentrations of IPTG. We found that in the presence of 10μM or higher concentrations of IPTG, sporulation efficiency (10^{-1}) of the resulting strain was on the same order as that of the wild-type strain under normal sporulation conditions (Tables III.4). In contrast, inducing the synthesis of KinA triggered massive induction of sporulation in wild-type background (MF1913), but no sporulation initiation was observed in the spo0F or spo0B null genetic background as shown previous (Fujita, Gonzalez-Pastor et al. 2005, Eswaramoorthy, Erb et al. 2011). Taken together the results from our phosphorelay bypass systems (Δspo0F (MF2406) and Δspo0B (MF4419)) show that direct phosphotransfer from KinC to Spo0A can produce sufficient Spo0A~P for efficient sporulation.

Table III-4. Sporulation in the KinC strain (MF4531) in the presence of different IPTG concentrations.

Cells of MF4531 were collected at 3h after IPTG addition and assayed for β-Galactosidase activity from PspoIIG-lacZ as indicated in Materials and Methods. β-gal activities shown correspond to the average of three independent

experiments.

IPTG (μM)	CFU/ml		Efficiency	β-gal (Miller units)
	Viable cells	Spores		
0	5.5×10^{8}	1.7×10^{6}	3.1×10^{-3}	15
2	7.7×10^{8}	9.8×10^{5}	1.3×10^{-3}	24
4	7.5×10^{8}	5.1×10^{5}	6.8×10^{-4}	27
6	5.2×10^{8}	3.3×10^{5}	6.3×10^{-4}	75
8	6.1×10^{8}	5.4×10^{7}	8.9×10^{-2}	125
10	5.7×10^{8}	3.2×10^{8}	5.6×10^{-1}	163
wt	5.3×10^{8}	3.4×10^{8}	6.4×10^{-1}	159

III.3.2 KinC governs entry into sporulation in a concentration-dependent manner

We constructed a strain expressing KinC under an IPTG-inducible hyper-spank promoter (hereafter referred to as Phy-spank). To determine the effective level of KinC for efficient entry into sporulation, the KinC-inducible strain (MF1915, referred as KinC strain) was cultured in LB in the presence of varying concentrations of IPTG. Sporulation efficiency (Table III-4) was determined by measuring the fraction of heat-resistant colony forming units (CFU). We note that in these nutrient rich media, the wild-type strain fails to sporulate (Eswaramoorthy, Duan et al. 2010). I found that, at IPTG concentrations 10 of 10μM, sporulation was efficiently induced to the level comparable to that in the wild-type strain (i.e. without IPTG-inducible construct) under normal sporulation conditions (Table III-4). Second, to determine the effect of KinC induction on Spo0A activity I constructed a reporter system consisting of lacZ gene expression driven by the Spo0A-dependent spoIIG promoter (Fujita 2000, Fujita

and Losick 2005, Eswaramoorthy, Guo et al. 2009). We then introduced this reporter system into the KinC strain (MF4531) to examine β-galactosidase activity under the same conditions as above. We confirmed that Spo0A was efficiently activated similarly to that seen in the sporulating wild-type strain (Table III-4). We note that, without IPTG, none of the kinases can activate Spo0A to a level that is sufficient to trigger sporulation in LB medium (LeDeaux and Grossman 1995, de Jong, Veening et al. 2010, Eswaramoorthy, Duan et al. 2010). These results show that artificially induced expression of KinC can be used to control Spo0A activation and entry into sporulation. Increase in KinC triggers sporulation in a phosphorelay-independent manner. We determined whether inducing the synthesis of KinC would bypass the requirement of the phosphorelay intermediate phosphotransferases for sporulation as suggested previously (Kobayashi, Shoji et al. 1995, LeDeaux and Grossman 1995). To demonstrate this, a null mutation of spo0F (MF2406) or spo0B (MF4419) was introduced into the KinC strain, and the resulting strains were tested for sporulation efficiency in LB in the presence of varying concentrations of IPTG. We found that in the presence of 10μM or higher concentrations of IPTG, sporulation efficiency (10^{-1}) of the resulting strain was on the same order as that of the wild-type strain under normal sporulation conditions (Table III-4). In contrast, inducing the synthesis of KinA triggered massive induction of sporulation in wild-type background (MF1913), but no sporulation initiation was observed in the spo0F or spo0B null genetic background as shown previously (Fujita and Losick 2005, Eswaramoorthy, Erb et al. 2011). Taken together the

results from our phosphorelay bypass systems Δspo0F (MF2406) and Δspo0B (MF4419)) show that direct phosphotransfer from KinC to Spo0A can produce sufficient Spo0A~P for efficient sporulation.

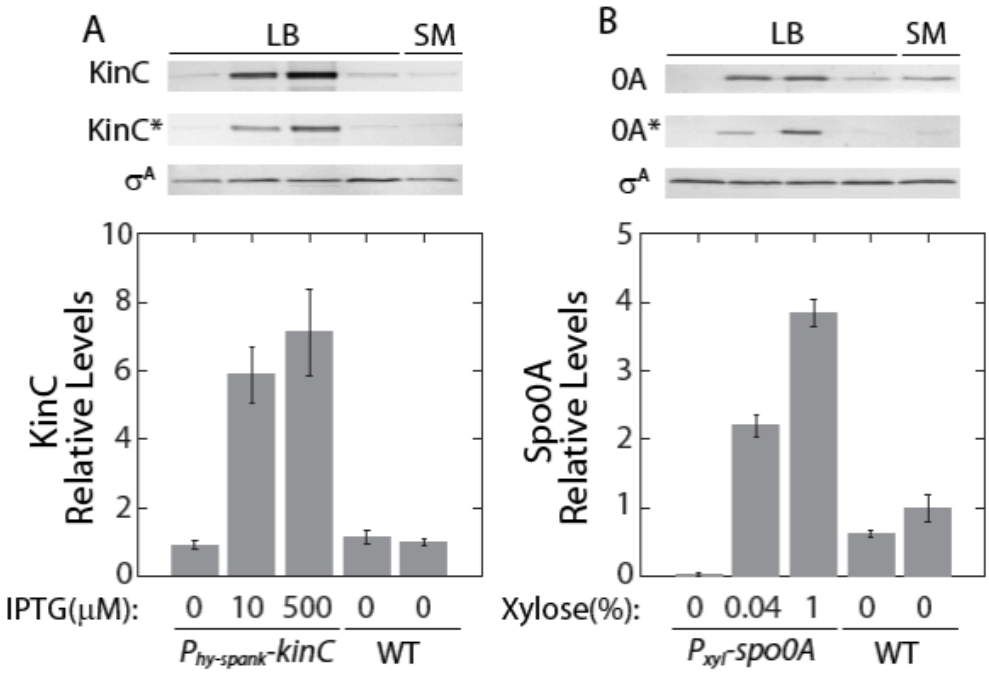


Figure III.1 Protein levels of KinC and Spo0A in the IPTG- and xylose-inducible strain.

(A) For the determination of KinC level, cells of the KinC (MF2734) and wild-type (MF2659) strains, both harboring kinC-gfp, was cultured and then processed for immunoblots as described in Materials and Methods. In order to make measurements in a non-saturation range, protein samples were diluted before processing for immunoblots (0.125μg total, KinC* panel). (B) For the determination of Spo0A level, cells of the KinC-Spo0A strain (MF4317) were cultured as above and xylose was added at indicated concentrations (0, 0.04, and 1%). Samples of MF2659 were used for the detection of wild-type Spo0A protein. Total protein samples (2μg total, top panel) were diluted and processed for immunoblot (0.125 μg total, 0A* panel). Protein levels were normalized to the constitutively expressed σ^A and then to the level at 500μM IPTG in LB conditions.

Fig III.1 cont

Relative protein levels are shown in the bottom graphs. All experiments were performed at least three times independently. Gray bars and errorbars represent the means and standard deviations under each condition.

III.3.3 KinC-Spo0A artificial two-component system triggers entry into sporulation

To investigate the importance of the dynamics of Spo0A~P accumulation, we perturbed these dynamics artificially. To control the dynamics of accumulation of Spo0A~P we needed to directly control both the level of Spo0A and its phosphorylation rate. To this end, we constructed a strain in which genes for the kinase KinC and Spo0A are independently placed under the control of IPTG-inducible (Phy-spank for KinC) or the xylose inducible (PxylA for Spo0A) promoters (KinC-Spo0A strain hereafter) (Fig. III-2A). We chose KinC (instead of other kinases, such as KinA, etc.) for two reasons. First, it has been previously shown that induced expression of KinC can be used to achieve high sporulation efficiencies irrespective of culture conditions (Fujita and Losick 2005); see also Table III-4). Second, since KinC can directly phosphorylate Spo0A, thus bypassing the need for Spo0F and Spo0B (Kobayashi, Shoji et al. 1995, LeDeaux and Grossman 1995), it offers an easy and direct method of modulating the rate of Spo0A phosphorylation. We also introduced a spo0F deletion into this KinC-Spo0A-inducible strain to ensure direct phosphotransfer from KinC to Spo0A. By restricting phosphate flow in this manner and conducting experiments

in rich media we were able to minimize the effects of starvation related signals and modulators acting on the phosphorelay.

Using this system we evaluated the effect of different concentrations of IPTG and Xylose (that control the synthesis of KinC and Spo0A, respectively) on sporulation efficiency in nutrient-rich conditions. To this end we defined and measured the fraction of total colony-forming units (total CFU=viable cells and spores) that were heat-resistant (heat-resistant CFU=spores; see Experimental procedures for more details). We found that in the double-induction strain sporulation efficiency depends non-monotonically on the levels of both IPTG and xylose (Fig. III-2B): sporulation efficiency was low in the absence of inducers and increased to reach a maximum of about 30% at the optimal combination of 10μM IPTG and 0.04% xylose (see Fig. III-2B). This sporulation efficiency is comparable to that observed in the wild-type cells in sporulation medium (50-70%, Table III-4). However, my results show that sporulation was impaired by further increasing either the concentration of IPTG or xylose (Fig. III-2B and Table III-4). Going from 10μM IPTG to 500μM while keeping xylose at the optimal level of 0.04% decreases sporulation efficiency to ~9%. Similarly, going from 0.04% xylose to 1% xylose while keeping KinC induction at the optimal level (at 10μM IPTG) decreases efficiency to ~5%. Moreover, I found that simultaneously overexpressing both KinC and Spo0A by adding excess amounts of two inducers resulted in even greater impairment of sporulation (sporulation efficiency ~0.1% at 500μM IPTG and 1% xylose Fig. III-2B and Table III-4). Thus, I established an

artificial two-component sporulation system (harboring independently inducible copies KinC and Spo0A) and found that only an optimal combination of Spo0A and KinC expression can be used to trigger an efficient entry into sporulation. To understand the non-monotonic dependence of sporulation efficiency on IPTG and xylose levels, I quantified the cellular levels of KinC and Spo0A in the KinC-Spo0A strain. I constructed strains expressing a functional KinC-GFP and the wild-type Spo0A under these two inducible promoters, respectively. Using this strain, I quantified the amount of KinC and Spo0A proteins at 2h after the addition of inducers with immunoblot analysis using anti-GFP and anti-Spo0A antibodies, respectively. For comparison, the exponentially growing and sporulating cells expressing KinC-GFP from native promoter were cultured in LB and SM media, respectively, and the cell extracts were prepared and quantified with immunoblotting. As shown in Fig. III-1, at 10μM IPTG concentration KinC-GFP levels were approximately 5-fold higher than those in the wild-type strain cultured either in rich (LB) or sporulation (SM) conditions. The Spo0A level at 0.04% xylose was approximately 2-fold higher than that in the sporulating wild-type strain (Fig. III-1B). At higher concentrations of the inducers (500μM IPTG and 1% xylose), the synthesis of KinC and Spo0A proteins was increased further (Fig. III-1A, B). Thus, the conditions that trigger efficient sporulation correspond to overexpression of both proteins and therefore may not be relevant for wild-type sporulation induction. Nevertheless, the double induction system can enable insights into how level and dynamics of Spo0A~P affect sporulation.Considering the above results, the low sporulation efficiency seen in our experiments at low

IPTG and xylose concentrations (<10μM IPTG and <0.04% xylose) was expected and may be explained by insufficient expression of KinC and/or Spo0A for sporulation. However, the decrease in sporulation efficiency at high inducer concentrations (>10μM IPTG and >0.04% xylose) was somewhat surprising. One possible explanation for this negative effect is that overexpression of KinC and Spo0A with excess IPTG and xylose (>10μM IPTG and >0.04% xylose) may somehow result in a decrease of Spo0A activity, thereby impairing sporulation. To test this, I used the PspoIIG-lacZ reporter and determined Spo0A activity in this strain at different inducer concentrations. As shown in Fig. III-2C, D, in the KinC-Spo0A system, β-galactosidase activity increases monotonically with IPTG and xylose concentrations. Furthermore, I found that the Spo0A activity is higher under conditions of simultaneous overexpression of both KinC and Spo0A (500μM and 1% xylose), as compared to the optimal conditions for sporulation (10μM IPTG and 0.04% xylose; Table III-4). Therefore, the impairment of sporulation by the induced overexpression of KinC and Spo0A is not due to a decrease in Spo0A activity.

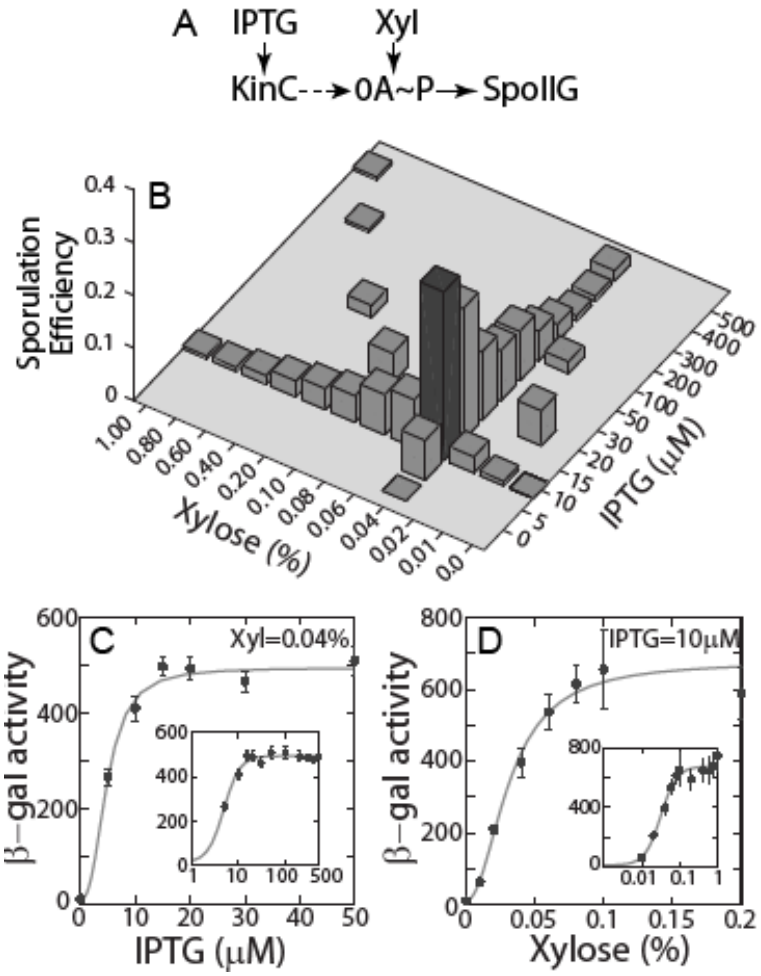


Figure III.2 Simultaneous overproduction of KinC and Spo0A reduces sporulation efficiency.

A. The KinC-Spo0A artificial two-component system (strain MF4317).

B. Bars indicate the results of sporulation efficiency measurements at various combinations of IPTG and xylose inducer concentrations (see Experimental procedures). The black bar indicates the optimal combination of IPTG and xylose for sporulation in this strain.

C and D. PspoIIG-lacZ reporter was used to measure the effects of KinC and 0A induction on 0A~P levels in the KinC-Spo0A strain. C. Induction of KinC expression from a Phy-spank promoter using IPTG leads to a monotonic increase in 0A~P levels (dots and error bars show the mean and standard deviation of three independent measurements of β-galactosidase activity in Miller

Fig III.2 cont.

units). D. Induction of Spo0A expression from a PxylA promoter using xylose leads to a similar monotonic increase in 0A~P levels (dots). Solid lines in (C) and (D) show Hill-equation fits to the measured responses. Insets in (C) and (D) show the same data on a log-scale for inducer concentrations.

III.3.4 Accelerated Spo0A~P accumulation leads to premature repression of DivIVA which impairs chromosome segregation

The thresholds for DivIVA can be explained by the essential requirement for this protein during sporulation. In the early phase of sporulation, accumulation of σ^H leads to asymmetric septation by promoting increased expression of FtsZ (Gholamhoseinian, Shen et al. 1992). DivIVA plays an important role around this time since it is essential for proper chromosome segregation; divIVA mutants have impaired chromosome segregation, which leads to formation of chromosome-free compartments and poor sporulation efficiency (Thomaides, Freeman et al. 2001, Soufo, Soufo et al. 2008). Based on these facts, we reasoned that early repression of DivIVA in conditions of KinC and Spo0A overexpression may mimic the phenotype of divIVA mutants, resulting in the low sporulation efficiency due to lack of the proper chromosome segregation during sporulation.

We predicted that accelerated Spo0A~P accumulation resulting from overproduction of KinC and Spo0A causes the earlier onset of reduction in the expression of high threshold Spo0A-directly repressed genes such as *divIVA.* To verify this experimentally I measured the protein level of endogenous DivIVA

using immunoblotting for a functional DivIVA–CFP fusion protein (Patrick and Kearns 2008, Eswaramoorthy, Erb et al. 2011) in the KinC-Spo0A strain (MF4812). I found that, in contrast to the optimum conditions of 10 μM IPTG and 0.04% xylose, DivIVA expression at T3 is significantly reduced under conditions of overproduction of KinC and Spo0A (500 μM IPTG and 1% xylose; see Fig. III-3).

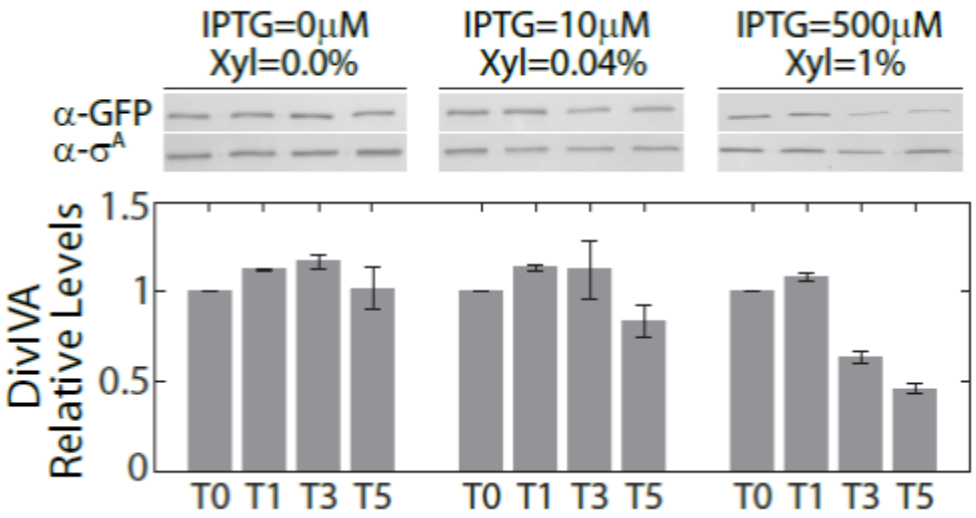


Figure III.3 Early onset of divIVA repression at high IPTG and xylose concentrations.

Level of DivIVA was assayed using cells expressing an endogenous DivIVA-CFP fusion protein in the KinC-Spo0A strain (MF4812) at 0μM IPTG, 0% xylose, 10μM IPTG, 0.04% xylose and 500μM IPTG, and 1% xylose at indicated time-points (0, 1, 3, and 5 hours after induction). The upper panel shows representative immunoblots for each condition (2 μg total proteins). The lower panel shows the quantification of these immunoblots. Gray bars show the mean values of three independent experiments with standard deviations. Values are normalized to the constitutively expressed σ^A and then those at time zero (T0) in each case.

To evaluate the effect of Spo0A and KinC overexpression on DivIVA levels and on chromosome segregation in single cells, I performed fluorescence microscopy experiments with a functional DivIVA–CFP fusion construct (Eswaramoorthy, Erb et al. 2011). I found that DivIVA–CFP levels in single cells were significantly lower at 500 μM IPTG and 1% xylose compared with 10 μM IPTG and 0.04% xylose (Fig. III-4). Moreover, DivIVA–CFP was irregularly localized in the cells under the high inducer conditions (Fig.III-5). Further, using DAPI (4-,6-diamidino-2-phenylindole dihydrochloride) to stain for DNA, I found that, in approximately 35% of the cells, DNA was improperly segregated at 500 μM IPTG and 1% xylose as compared with approximately 8% at 10 μM IPTG and 0.04% xylose (Fig. III-4B–G and Fig. III-5). Thus, our results suggest that low sporulation efficiency under conditions of overproduction of KinC and Spo0A in the artificial two-component system could be explained at least in part by the improper chromosome segregation, resulting from premature repression of DivIVA.

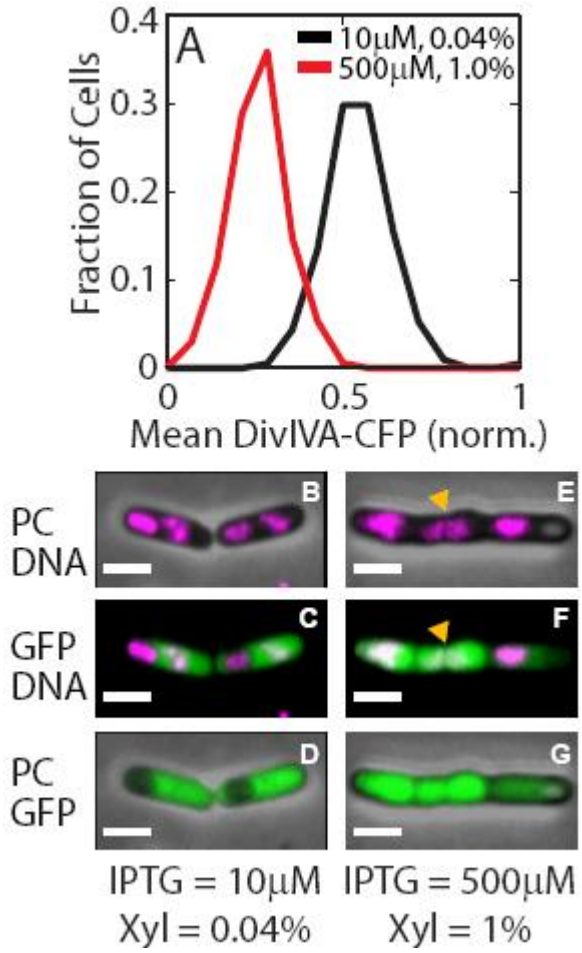


Figure III.4 Overproduction of KinC and Spo0A results in early repression of DivIVA and improper chromosome segregation.

A. Histograms for DivIVA levels at T2 after the addition of 0.04% xylose and 10 μM IPTG (black) and 1% xylose and 500 μM IPTG (red). DivIVA level in single cells was measured by expressing a functional DivIVA–CFP fusion in the KinC-Spo0A strain. DivIVA–CFP fluorescence levels were normalized to the maximum level seen in both conditions. B–G. The KinC-Spo0A strain harbouring a gfp reporter gene under the control of the spoIIG promoter and stained with DAPI for DNA was cultured in the presence of (B–D) 0.04% xylose and 10 μM IPTG; and (E–G) 1% xylose and 500 μM IPTG and examined with fluorescence microscopy. Typical cells under each condition are shown. GFP and DAPI (DNA) are pseudo-coloured with green and magenta respectively. Yellow carats indicate cells with improper chromosome segregation. PC, phase-contrast image. Scale bar: 2 μm.

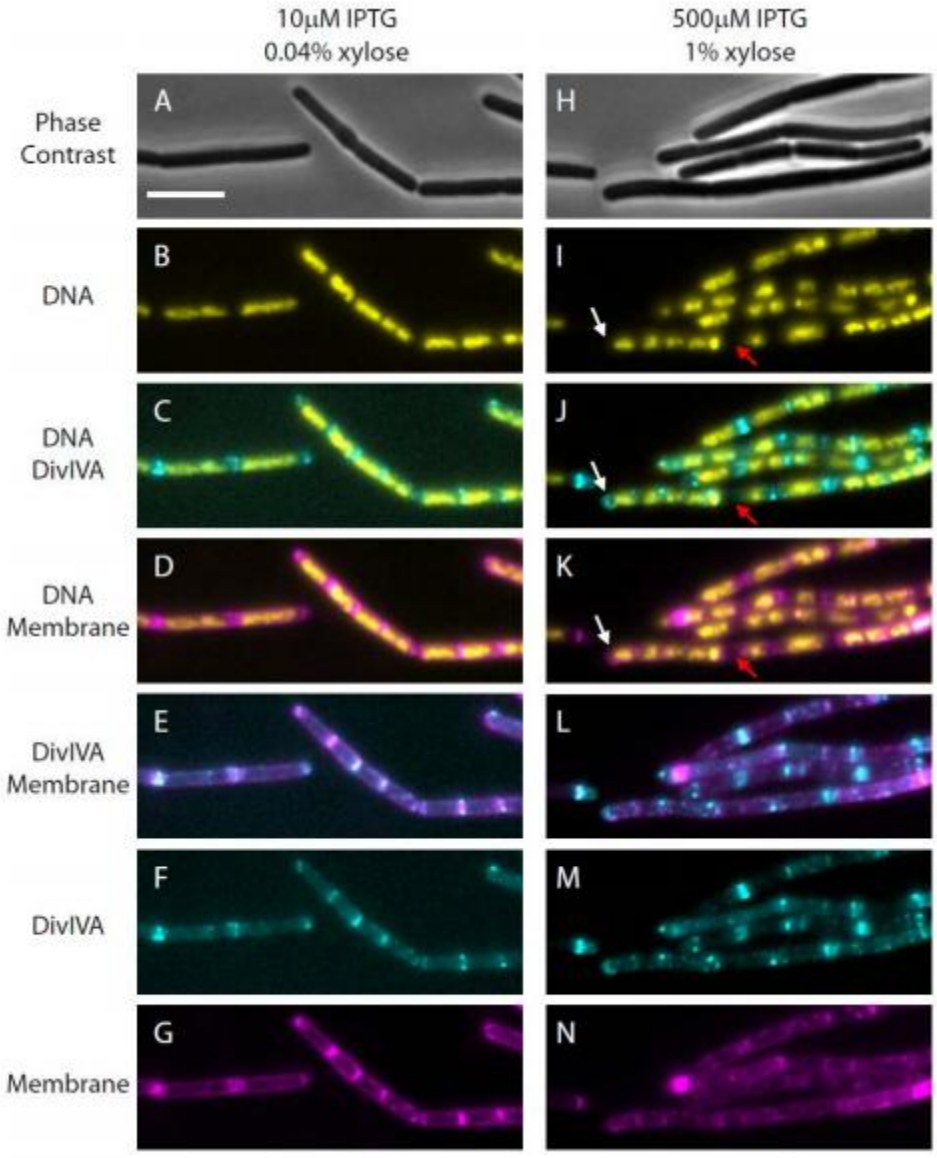


Figure III.5 Fluorescence microscopy of KinC-Spo0A strain.

Cells of MF4812 expressing an endogenous DivIVA fused to CFP (cyan) in the KinC-Spo0A strain were harvested at two hours after induction with inducers. (A-G) 10µM IPTG and 0.04% xylose. (H-N) 500µM IPTG and 1% xylose. Membrane and DNA were visualized with FM4-64 (magenta) and DAPI (yellow), respectively. White arrows (IJK) show an empty forespore while its mother cell contains a

Fig III.5 cont.

stubby nucleoid with its end is not attached to DivIVA or polar septum. Red arrows (IJK) show empty cell formed as a result of improper chromosome segregation. The numbers of abnormal cell types are shown in Supplementary Figure S3. Scale bar: 5μm

III-3-5 Autoregulation of spo0A ensures proper temporal co-ordination of high and low threshold gene expression

Our results show that the impairment of sporulation by rapid accumulation of Spo0A~P can be explained by the premature repression of its targets such as DivIVA before σ^H is fully activated. I also noted that in the wild-type phosphorelay the σ^H-mediated positive feedback to spo0A may limit the accumulation of Spo0A~P until σ^H is fully activated. Taking all this into account I hypothesized that putting spo0A under native promoter may be sufficient to ensure proper Spo0A~P dynamics and prevent premature activation/repression of the Spo0A regulon.

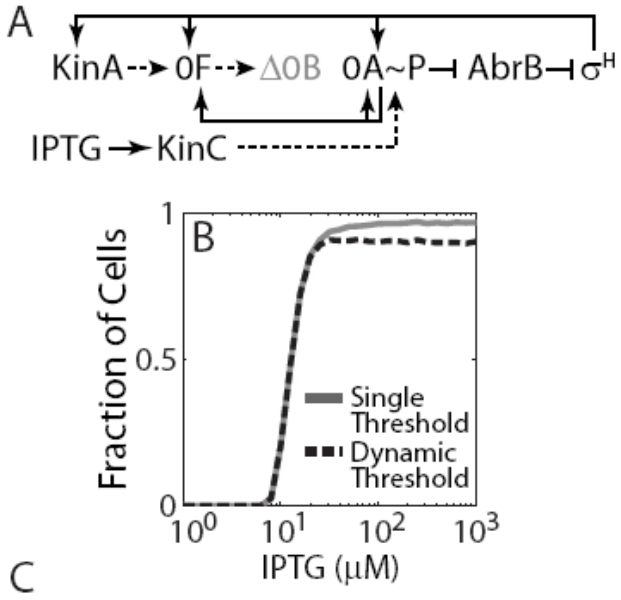
To test this hypothesis, I first constructed a strain where KinC is expressed from the IPTG inducible Phy-spank promoter, spo0A is regulated by its native promoters, and the phosphotransferase spo0B is deleted to ensure only direct phosphotransfer from KinC to Spo0A (Δspo0B, Fig. III-6A). The expression of spo0A is regulated by multiple Spo0A binding sites that regulate both σ^A-dependent and σ^H-dependent promoters (Chastanet and Losick 2011). Thus native spo0A regulation results in direct feedback from Spo0A to itself (both positive and negative loops) and indirect positive feedback via σ^H.

However, the autoregulation of spo0A expression ensures that this onset does not precede the activation of σ^H. Consequently, sufficient levels of high threshold repressed genes like DivIVA are always available at the appropriate time point in the sporulation programme. To examined how sporulation depends on the level of kinC. I tested for the sporulation efficiency in LB media in the presence of varying concentration of IPTG. As expected from the our previous result, I found that with spo0A under its native promoter, sporulation efficiency at 500µM IPTG was significantly higher (9×10^{-1}) than that in the KinC-spo0A strain (10^{-2}) at high 500µM IPTG and 1% xylose (Table III-5 and Figure III-6C).

Table III-5. Sporulation Efficiency and Spo0A activity in the KinC-Spo0A double induction (MF4317) strain.

Cells of MF4317 were collected at 3h after IPTG and Xylose addition and assayed for β-Galactosidase activity from PspoIIG-lacZ as indicated in Materials and Methods. β-gal activities shown correspond to the average of three independent experiments. Sporulation efficiency was determined as indicated in Materials and Methods.

IPTG (µM)	Xylose (%)	CFU/ml		Efficiency	β-gal (Miller units)
		Viable cells	Spores		
10	0.04	4.4×10^{8}	1.3×10^{8}	3.0×10^{-1}	408
10	1	4.4×10^{8}	2.2×10^{7}	5.0×10^{-2}	545
500	0.04	5.7×10^{8}	5.2×10^{7}	9.1×10^{-2}	488
500	1	6.3×10^{8}	6.2×10^{6}	9.8×10^{-3}	598



C

KinC Δ*spo0B*			
IPTG (μM)	CFU/ml		Sporulation Efficiency
	Viable cells	Spores	
0	6.6×10^{8}	1.9×10^{4}	2.8×10^{-5}
10	5.7×10^{8}	1.4×10^{8}	2.4×10^{-1}
500	9.5×10^{7}	8.2×10^{7}	8.6×10^{-1}

Figure III.6: σH-mediated feedback prevents decrease in sporulation efficiency at high KinC levels.

A.KinC induction strain with spo0B deletion and spo0A expressed from native promoter (MF4419). B. Predicted fraction of sporulating cells as a function of IPTG in this strain. Fraction of cells that sporulate in this strain was calculated using the results of stochastic simulations at different IPTG concentrations and the Single Threshold (grey curve) and Dynamic Thresholds (black dashed curve) models. C. Results of sporulation efficiency measurements at various concentrations of IPTG in the KinC induction strain harbouring spo0B deletion mutation (MF4419). Note that sporulation efficiency increases with IPTG.

III-4 Discussion

I have constructed a genetically engineered strain that decouples and rewires the sporulation phosphorelay network thereby establishing an artificial two-component system of sporulation comprising KinC and Spo0A. In contrast to the previously reported systems (Fujita, Gonzalez-Pastor et al. 2005, Eswaramoorthy, Duan et al. 2010, Chastanet and Losick 2011), our artificial two-component system offers unique control over the kinetics of Spo0A~P accumulation and thus is a powerful tool to investigate why Spo0A~P accumulation needs to be gradual for efficient sporulation.

We found that at a certain combination of Spo0A and KinC protein expression levels (10 μM IPTG and 0.04% xylose, Fig. III-2B), Spo0A~P gradually accumulated to high enough levels so that cells sporulate efficiently. However, increases in KinC or Spo0A expression beyond this condition can significantly speed-up the rate of Spo0A~P accumulation, shift the onset of high threshold gene expression to earlier time points, and as a result, decrease sporulation efficiency (500 μM IPTG and/or 1% xylose, Fig. III-2B).

Several genes repressed by Spo0A~P are involved in cell division, DNA replication, cell-shape determination and protein synthesis (Molle, Fujita et al. 2003, Fujita, Gonzalez-Pastor et al. 2005, Eswaramoorthy, Erb et al. 2011). Thus, overproduction of KinC and Spo0A in the artificial two-component system could cause abnormalities in cell division, DNA replication and protein synthesis, and as a result, adversely affect sporulation. Among the genes repressed by

Spo0A~P, divIVA may be the most obvious candidate to explain the importance of proper timing. Although it is repressed during sporulation, DivIVA plays an important role in chromosome segregation in the early phase of sporulation (Thomaides, Freeman et al. 2001, Wu and Errington 2003). In conditions of Spo0A and KinC overexpression, divIVA is repressed prematurely (Fig. III-3), resulting in improper chromosome segregation and increased incidence of chromosome-free compartments (Fig. III-4). Similar phenotypes have been observed in sporulation-defective divIVA mutant (Thomaides, Freeman et al. 2001). Thus, premature repression of DivIVA could at least partially explain our observation of decreased sporulation efficiency in conditions of high KinC and Spo0A expression. As divIVA is repressed at high levels of Spo0A~P (Fujita and Losick 2005), gradual accumulation of Spo0A~P in the wild-type strain under starvation conditions ensures that it is repressed only after it has served its function during the early phase of sporulation – a period which also includes the activation of low-threshold genes such as σH by Spo0A~P (Fujita, Gonzalez-Pastor et al. 2005). I also found that simultaneous overproduction of KinC and Spo0A results in swollen cell morphologies and changes the staining pattern of cell membranes with lipophilic fluorescent dye, FM4-64. The swollen morphologies may be explained by the early repression of a gene mreB. This gene is repressed (indirectly) by high levels of Spo0A~P during sporulation (Fujita and Losick 2005) and is responsible for cell shape determination as a prokaryotic homolog (Defeu Soufo and Graumann 2005, Formstone and

Errington 2005). The abnormal staining pattern of the membrane can be attributed to inappropriate fatty acids synthesis, since Spo0A~P has been shown to control fatty acids synthesis and maintain membrane lipid homeostasis during early stages of sporulation (Pedrido, de Ona et al. 2013). Thus accelerated and inappropriate activation of Spo0A appears to have pleiotropic effects on various cellular functions. However, the mechanism by which these resulting aberrant morphologies could interfere with sporulation remains unclear.

The artificial induction KinC-Spo0A two-component system demonstrates the importance of gradual accumulation of Spo0A~P. To explain how the structure of the phosphorelay network plays a role in ensuring gradual Spo0A~P accumulation, We put these results in a broader context. In principle, there could be two mechanisms to slow-down Spo0A~P accumulation during the onset of sporulation: (1) limit the phosphate flux that activates Spo0A and (2) limit the amount of Spo0A available for activation.

My experiments with the inducible strains reported here concur with the importance of mechanism in modulating Spo0A activation. Results for KinC-Spo0A induction strains indicate that kinase overexpression strains are limited by the amount of Spo0A available for activation. The simultaneous overproduction of both Spo0A and KinC can overcome this limitation and further speed up Spo0A activation and thereby reduce sporulation efficiency. However, the high sporulation efficiency of KinC induction strain with native spo0A promoter suggests that the Spo0A feedback loops ensure gradual Spo0A~P accumulation.

Notably, feedback architecture is a common feature of many phosphorelays (Williams, Boucher et al. 2005, Williams and Cotter 2007) and may thus represent a common strategy for proper dynamical control in variety of functional settings.

CHAPTER IV: IN VIVO FUNCTIONAL CHARACTERIZATION OF THE TRANSMEMBRANE HISTIDINE KINASE KINC IN *BACILLUS SUBTILIS*

Seram Nganbiton Devi, Monika Vishnoi, Brittany Kiehler, Lindsey Haggett, and Masaya Fujita*

In response to nutrient deprivation, *Bacillus subtilis* cells undergo differentiation to form metabolically inert spores. This process requires the involvement of a multiple phosphorelay component, wherein phosphate is transferred to a master transcription regulator Spo0A from five histidine kinases (KinA-KinE) via two intermediate phosphotransferases, Spo0F and Spo0B. In this study, I hypothesized that Spo0A activity necessary for entry into sporulation is regulated by the relative activities of KinA and KinC. Using conventional genetic approaches, I found that, KinC preferentially and positively controls the expression of the cannibalism (a mechanism to delay sporulation) genes during early sporulation stage in a manner dependent on phosphorelay, resulting in the delay of sporulation. Furthermore, using an artificial induction of KinC system, I found that sporulation is efficiently induced by overexpression of KinC even under nutrient rich conditions. These results suggest that (1) when KinC is fully activated during early phase of starvation, cells produce cannibalism toxin proteins and form biofilm by expressing only low level of Spo0A~P, mainly through KinC, resulting in avoidance of sporulation decision-making; (2) the level of fully active KinC is not sufficient to achieve the threshold of Spo0A~P needed for entry into sporulation during this period; and (3) when KinA activity increases gradually later on, cells ultimately end up forming spores with high levels of Spo0A~P.

IV.1 Introduction

Upon nutrient starvation, *Bacillus subtilis* cells undergo a series of distinct developmental phases, including competence, biofilm formation, cannibalism (a mechanism to delay sporulation) and sporulation, Extensive studies have elucidated a detailed genetic network of genes involved in these events after starvation (Burbulys, Trach et al. 1991, Grossman 1995, Sonenshein 2000, Piggot and Hilbert 2004). The heart of this network is a phosphorelay, in which phosphate is transferred from multiple histidine protein kinases to a master transcription regulator Spo0A through two intermediate phosphotransferases (Spo0F and Spo0B) (Burbulys, Trach et al. 1991). The biological significance of the multi-component phosphorelay is thought to provide multiple entry points for a wide variety of environmental signals (Hoch 1993, Grossman 1995). In this view, environmental signals are recognized and processed by the components of phosphorelay and dedicated phosphatases such as RapA for phosphorylated Spo0F (Spo0F~P) and Spo0E for phosphorylated Spo0A (Spo0A~P) (Jiang, Shao et al. 2000). As an initial event, an yet unidentified environmental or intracellular starvation signal(s) is supposed to be recognized by one or a combination of the N-terminal “sensor” domains of the five histidine kinases, resulting in autophosphorylation of the histidine residue within the C-terminal “catalytic” domain (8, 9). Therefore, the unknown environmental stimuli ultimately appear to be processed for Spo0A phosphorylation via the phosphorelay network, although no direct evidence to support this notion has been provided to date.

Upon starvation, initially, cells start producing SpoA~P at relatively low levels, resulting in switching on the low-threshold Spo0A~P regulated genes, including those involved in cannibalism and biofilm formation. During extended times of starvation, cellular levels of Spo0A~P increase gradually and reach to a threshold for expression of genes that are directly involved in sporulation such as SpoIIA and spoIIG (Fujita et al., 2005). Therefore, cannibalism and biofilm formation in *B. subtilis* are known as alternative developmental processes that are independent of sporulation but are controlled by the master regulator Spo0A~P upon starvation.

While KinA and KinB are known to be primarily responsible for sporulation (LeDeaux and Grossman 1995, LeDeaux, Yu et al. 1995, Jiang, Shao et al. 2000), a gene for a transmembrane kinase KinC has been identified as a suppressor of a sporulation defective null mutation of spo0K (gene for oligopeptide permease required for sporulation) (LeDeaux and Grossman 1995) or of a missense mutation of spo0A (E14V) (Kobayashi, Shoji et al. 1995). Under these genetic conditions, KinC becomes active in promoting sporulation by directly transferring a phosphate group from KinC~P to Spo0A or its mutant protein in a two-component manner (Kobayashi, Shoji et al. 1995, LeDeaux and Grossman 1995). These results suggest that KinC plays some role in entry into sporulation under certain conditions.

More recently, using an undomesticated *B. subtilis* strain, KinC has been demonstrated to contribute for triggering biofilm formation and cannibalism during

the transition phase from growth to sporulation. Under such conditions, surfactin, a cyclic lipopeptide antibiotic is secreted into the medium by the same population of cells, thereby inducing membrane leakage. By adding of KCl (150 mM) to the culture medium, biofilm formation is diminished. These observations suggest that KinC is activated by lowering the intracellular potassium concentration (Lopez, Fischbach et al. 2009, Lopez, Vlamakis et al. 2009, Lopez, Vlamakis et al. 2009). Furthermore, another paper reported that KinC, together with a homolog of Flotillin-1 (a eukaryotic protein found exclusively in lipid rafts) (Browman, Hoegg et al. 2007), localizes to lipid rafts of membrane and the KinC activity is dependent on lipid raft integrity (Lopez and Kolter 2010). Taken together, these results suggest that, in the undomesticated strain, the activity of KinC is regulated by multiple processes in a manner dependent on the environmental conditions, resulting in different cell-fate outcomes. By contrast, using the domesticated strain that lacks ability to synthesize surfactin (Nakano, Marahiel et al. 1988), Hobbs, 2006) demonstrated that cells of the kinC mutant strain was shown to sporulate faster than the wild-type strain and similar to mutants of cannibalism toxin operons (skf and sdp) (Gonzalez-Pastor, Hobbs et al. 2003). These results suggest that, in the domesticated strain, genes involved in cannibalism are positively regulated by KinC, but independent of surfactin. Thus, it is remain unknown how KinC becomes active to induce cannibalism in the domesticated strain. Furthermore, how KinC, in addition to the major kinases KinA and KinB, controls the level of Spo0A~P during the course of cell growth

and differentiation, including cannibalism, biofilm formation and sporulation, remains unknown.

As a first step towards solving these problems, it is essential to characterize the relationship between structure and function of KinC. Until now, the conventional biochemical approaches were not successful due to the difficulty of purification of the membrane-bound kinase. Furthermore, in vivo functional characterization was not possible due to lack of proper procedures to monitor KinC activity in a quantitative manner.

To overcome these limitations, here, I established an in vivo quantitative and functional assay system for KinC. Using an IPTG-inducible KinC expression system in combination with null mutations of kinA and kinB, I performed in vivo domain-based step-wise deletion analyses and determined the minimum functional domain of KinC. Furthermore, In vivo chemical cross-linking experiments revealed that KinC forms a functional tetramer mediated by PAS domain, which is known to be involved in protein–protein interactions in the sensor kinases of the bacterial two-component systems (Taylor and Zhulin 1999). Thus, our results provide further insights into the structure and function of KinC.

IV.2 Materials and Mathods

IV.2.1 Strain and plasmid construction.

B. subtilis strain PY79 was used as the parental strain for all experiments (Youngman, Perkins et al. 1984). Detailed genotypes of all strains are shown in

Table IV-1. Details of the constructions are available upon request. *B. subtilis* strains were constructed by transformation with either chromosomal DNA or plasmid DNA as described by Harwood and Cutting (1990). The standard recombinant DNA techniques including plasmid DNA construction and isolation using *E. coli* DH5α were performed as described by Sambrook and Russell (2001). The plasmids used in this study are listed in Table IV-2. The oligonucleotide primers used are listed in Table IV-3. Plasmid pDR111 (25) was a gift from David Rudner (Harvard Medical School). B. subtilis strain harboring ΔkinC::cm was a gift from Kazuo Kobayashi (Nara Institute of Science & Technology).

Table IV.1. List of Strains

Colony morphology		
Strain	Genotype or description	Reference
MF1237	*ΔkinA::tet*	(Eswaramoorthy, Guo et al. 2009)
MF1847	*ΔkinB::kan*	(Eswaramoorthy, Guo et al. 2009)
MF1845	*ΔkinC::cm*	Lab stock*
EG168	*Δ(skfABCDEF)::tet*	(Gonzalez-Pastor, Hobbs et al. 2003)
EG407	*Δ(sdpABC)::spc*	(Gonzalez-Pastor, Hobbs et al. 2003)

β-Galactosidase assay		
Strain	Genotype or description	Reference
MF1821	*amyE::Psdp-lacZ cm*	(Gonzalez-Pastor, Hobbs et al. 2003)
MF1826	*Δspo0F::tet, amyE::Psdp-lacZ cm*	This study
MF1827	*Δspo0B::tet, amyE::Psdp-lacZ cm*	This study
MF1831	*Δspo0A::spc, amyE::Psdp-lacZ cm*	This study
MF1923	*ΔkinA::tet, amyE::Psdp-lacZ cm*	This study
MF1924	*ΔkinB::kan, amyE::Psdp-lacZ cm*	This study
MF1925	*ΔkinC::erm, amyE::Psdp-lacZ cm*	This study
MF290	*amyE::PspoIIG-lacZ spc*	(Fujita and Losick 2003)
MF1354	*ΔkinA::tet, amyE::PspoIIG-lacZ spc*	This study
MF1870	*ΔkinB::kan, amyE::PspoIIG-lacZ spc*	This study
MF1872	*ΔkinC::erm, amyE::PspoIIG-lacZ spc*	This study
MF4531	*amyE::Phy-spank-KinC spc, ΔkinA::tet, ΔkinB::kan, ΔkinC::cm, thrC::PspoIIG-lacZ erm*	This study
MF4495	*amyE::Phy-spank-KinC$^{\Delta TM1}$ spc, ΔkinA::tet, ΔkinB::kan, ΔkinC::cm, thrC::PspoIIG-lacZ erm*	This study
MF4496	*amyE::Phy-spank –KinC$^{\Delta TM1+2}$ spc, ΔkinA::tet, ΔkinB::kan, ΔkinC::cm, thrC::PspoIIG-lacZ erm*	This study
MF4539	*amyE::Phy-spank-KinC$^{\Delta PAS}$ spc, ΔkinA::tet, ΔkinB::kan, ΔkinC::cm, thrC::PspoIIG-lacZ erm*	This study
MF4468	*amyE::Phy-spank-KinC$_C$ spc, ΔkinA::tet, ΔkinB::kan, ΔkinC::cm, thrC::PspoIIG-lacZ erm*	This study

GFP fusion protein

Strain	Genotype or description	Reference
MF929	*kinAΩkinA-gfp kan*	(Fujita and Losick 2005)
MF2659	*kinCΩkinC-gfp spc*	This study
MF3352	*ΔkinA::tet, ΔkinB::cm, amyE::*$P_{hyper\text{-}spank}$*-kinA-gfp spcR kanR*	This study
MF4547	*ΔKinA::tet, ΔkinB:cm, ΔkinC::erm, amyE::*$P_{hy\text{-}spank}$*-kinC-gfp spc kan*	This study
MF4548	*ΔKinA::tet, ΔkinB:cm, ΔkinC::erm, amyE::*$P_{hy\text{-}spank}$*-kinC*$^{\Delta TM1}$*-gfp spc kan*	This study
MF4549	*ΔKinA::tet, ΔkinB:cm, ΔkinC::erm, amyE::*$P_{hy\text{-}spank}$*-kinC*$^{\Delta TM1+2}$*-gfp spc kan*	This study
MF4550	*ΔKinA::tet, ΔkinB:cm, ΔkinC::erm, amyE::*$P_{hy\text{-}spank}$*-kinC*$^{\Delta PAS}$*-gfp spc kan*	This study
MF4609	*ΔKinA::tet, ΔkinB:cm, ΔkinC::erm, amyE::*$P_{hy\text{-}spank}$*-kinC*$_C$*-gfp spc kan*	This study

***A gift from Kazuo Kobayashi (Nara Institute of Science & Technology)**

Table IV.2. Plasmids used in this study.

Plasmid	Description	Source
pMF378	*'kinC-gfp kan*	This study
pMF600	*amyE::Phy-spank-kinC spc* in pDR111	This study
pMF585	*amyE::Phy-spank-kinC*$^{\Delta TM1}$ *spc* in pDR111	This study
pMF586	*amyE::Phy-spank-kinC*$^{\Delta TM1+2}$ *spc* in pDR111	This study
pMF595	*amyE::Phy-spank-kinC*$_C$ *spc* in pDR111	This study
pMF601	*amyE::Phy-spank-kinC*$^{\Delta PAS}$ *spc* in pDR111	This study

Table IV.3. Oligonucleotide primers used in this study.

Primer	Sequence
om24	5'-ccgctcgaggctgtctgattttaaaggcaa-3'
om29	5'-gccgaattcacatctgtcagcggtttcctccag-3'
om118	5'-gccaagcttacataaggaggaactactatgaaaaacccgattaattggcctgttgat-3'
om119	5'-gccaagcttacataaggaggaactactatgaaggataacgaatggaagtat-3'
om122	5'-gccgctagctcagctgtctgattttaaaggca-3'
om123	5'-gccctcgagatgagtaaaggagaagaactt-3'
om124	5'-cgggcatgcttatttgtatagttcatccat-3'
om134	5'-ctgggattccctcgccagttcagaaagctgtttatacttc-3'
om135	5'-ctggcgagggaatcccag-3'
om136	5'-gccaagcttacataaggaggaactactatgagaaaatatcaagctcgt-3'
op11	5'-gccaagcttacataaggaggaactactatgagaaaatatcaagctcgtatc-3'

IV.2.2 Media and culture conditions.

To induce the synthesis of KinA, KinC, or green fluorescent protein (GFP) under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible hyper-spank promoter (Phy-spank) (Britton, Eichenberger et al. 2002) in the engineered B. subtilis cells, IPTG was added to Luria-Bertani (LB) cultures during the exponential growth phase [optical density at 600 nm (OD600) of 0.5] as the rich medium conditions. Sporulation of the wild-type B. subtilis strain was induced by resuspension in Sterlini and Mandelstam (SM) medium (Sterlini and Mandelstam 1969). Threonine supplement (40 μg/ml) was added to the culture for strains harboring reporter genes at the thrC locus in SM medium. Sporulation of colonies was examined on DS (Difco sporulation) agar medium as described previously (Harwood and Cutting, 1990).

IV.2.3 Sporulation efficiency and β-galactosidase assays.

Sporulation efficiency was determined in 16-h cultures as colony-forming units (CFUs) per milliliter after incubation at 80°C for 10 min compared with CFUs of the pre-heat treatment sample. Assays of β-galactosidase activity were performed as described previously (Harwood and Cutting, 1990).

IV.2.4 Immunoblot analysis.

Whole-cell lysates for immunoblot analysis were prepared by sonication. Protein concentration was measured by Bradford method (Pierce). Total proteins were subjected to SDS-PAGE (16% acrylamide) and transferred to a

nitrocellulose filter. Immunoblot analysis was done with polyclonal anti-GFP (a gift from David Rudner) (Rudner and Losick 2002), and anti-σ^A (Fujita 2000) antibodies. Alkaline phosphatase coupled secondary antibodies were used for recognizing the primary antibodies and detected using substrate nitro blue tetrazolium/5-bromo-4-choloro-3-indolyl phosphate (NBT/BCIP) (Promega). The intensities of each band were quantified with an image analyzer (FluorChem; Alpha Innotech). σ^A, a constitutively expressed protein, was used as a loading control. The protein levels were normalized to both the endogenously expressed KinC-GFP and σ^A in the wild-type strain.

IV.2.5 Protein purification.

All His-tagged proteins were expressed in E. coli BL21(DE3) and purified as described previously (Fujita and Losick 2003, Eswaramoorthy, Dravis et al. 2011).

IV.2.6 In vitro phosphorylation.

Phosphorylation reactions were performed as described previously (Fujita and Losick 2003, Eswaramoorthy, Dravis et al. 2011).

IV.2.7 Protein cross-linking.

Protein cross-linking with bis-maleimidohexane (BMH; Pierce) was performed as described previously (Eswaramoorthy, Guo et al. 2009).

IV.2.8 Fluorescence microscopy.

Fluorescence microscopy was performed as described previously (Eswaramoorthy, Guo et al. 2009). To randomize the sampling, all cells within a field were analyzed by use of Slidebook software (Intelligent Imaging Innovations, Inc.).

IV.2.9 Plasmid construction

All plasmids were constructed in *E. coli* DH5α using basic molecular cloning methods (Burbulys, Trach et al. 1991). pMF600 was constructed by PCR amplifying the coding region of kinC with primers om136 and om122 (1-1284 bp; corresponding to amino acid residues 1-428) using chromosomal DNA of B. subtilis PY79 as a template. The PCR-amplified DNA was digested with HindIII and NheI, and cloned into the HindIII and NheI sites of pDR111 (Hoch 1993). The resulting plasmid containing Phy-spank-kinC with flanking amyE sequences was integrated at the amyE locus in *B. subtilis* by transformation.

pMF585 was constructed by PCR amplifying the coding region of kinCΔTM1 with primers om118 and om122 (87-1284 bp; corresponding to amino acid residues 29-428) using chromosomal DNA of B. subtilis PY79 as a template. The PCR-amplified DNA was digested with HindIII and NheI, and cloned into the HindIII and NheI sites of pDR111 (Hoch 1993). The resulting plasmid containing Phy-spank-kinC$^{\Delta TM1}$ with flanking amyE sequences was integrated at the amyE locus in *B. subtilis* by transformation.

pMF586 was constructed by PCR amplifying the coding region of kinC$^{\Delta TM1+2}$ with primers om119 and om122 (198-1284 bp; corresponding to amino acid residues 66-428) using chromosomal DNA of *B. subtilis* PY79 as a template. The PCR-amplified DNA was digested with HindIII and NheI, and cloned into the HindIII and NheI sites of pDR111 (Hoch 1993). The resulting plasmid containing Phy-spank-kinC$^{\Delta TM1+2}$ with flanking amyE sequences was integrated at the amyE locus in *B. subtilis* by transformation.

pMF595 was constructed by PCR amplifying the coding region of kinC$_C$ with primers op11 and om122 (603-1284 bp; corresponding to amino acid residues 201-428) using chromosomal DNA of *B. subtilis* PY79 as a template. The PCR-amplified DNA was digested with HindIII and NheI, and cloned into the HindIII and NheI sites of pDR111 (Hoch 1993). The resulting plasmid containing Phy-spank-kinC$_C$ with flanking amyE sequences was integrated at the amyE locus in *B. subtilis* by transformation.

pMF601 was constructed by amplifying the coding region of kinC$^{\Delta PAS}$ with primers om136, om134 (1-234 bp; corresponding to amino acid residues 1-78), and om135 and om122 (612-1284 bp; corresponding to amino acid residues 204-428) using chromosomal DNA of *B. subtilis* PY79 as a template in a by joining PCR reaction (Piggot and Hilbert 2004). The PCR-amplified DNA was digested with HindIII and NheI, and cloned into the HindIII and NheI sites of pDR111 (Hoch 1993). The resulting plasmid containing Phy-spank-kinCΔPAS with flanking amyE sequences was integrated at the amyE locus in B. subtilis by transformation.

pMF378 was constructed by amplifying the C-terminal coding region of kinC (denoted by ‘kinC) with primers om24 and om29 using chromosomal DNA of *B. subtilis* PY79 as a template. The PCR-amplified DNA was digested with EcoRI and XhoI and cloned into the EcoRI and XhoI sites of pKL168 (Sonenshein 2000). The resulting plasmid containing the C-terminal coding region of kinC fused to gfp (kinC-gfp) was integrated by single crossover at the 3′end of kinC or its variant genes in *B. subtilis*.

IV.3 RESULTS

IV.3.1 Construction of a quantitative assay system for KinC activity in vivo

In the undomesticated strain of *B. subtilis*, the cytoplasmic PAS domain of KinC was demonstrated to be important for sensing the effect of potassium leakage in response to surfactin and activatation of the autokinase activity, leading to biofilm formation (Browman, Hoegg et al. 2007). In contrast, in the domesticated strain, the production of surfactin is defective due to mutations in genes required for its synthesis (Nakano, Marahiel et al. 1988). Thus, without surfactin production, the potassium concentration appears to be relatively constant in the domesticated strain. Even under such conditions, KinC is active and involved in cannibalism, a mechanism to delay sporulation (Hobbs, 2006). Therefore, it is of interest to know how KinC is activated independent of decreased intracellular potassium concentration in the domesticated strain in the absence of surfactin.

To date, it has been technically difficult to monitor KinC activity biochemically because the autokinase activity of histidine is not directly measurable due to the instability of phosphohistidine. Therefore, generally, to monitor the histidine kinase activity, indirect assay using a lacZ reporter gene under the control of Spo0A~P is performed. However, Spo0A-directed genes are controlled not only by KinC but also by KinA and KinB. Thus, in the wild-type background, it is not possible to characterize the true nature of the signaling pathways involved in KinC. In conclusion, so far, the only measurable effect of KinC in the domesticated strain is an accelerated sporulation phenotype in the kinC mutant strain (Hobbs, 2006). To overcome these technical issues and to provide an improved method for the in vivo quantitative assay of the KinC activity, I took advantage of the fact that sporulation is efficiently induced by the artificially increased synthesis of KinC harboring an IPTG-inducible kinase construct (Fujita and Losick 2005, Eswaramoorthy, Guo et al. 2009). First, to exclude the possible involvement of the sporulation kinases KinA and KinB, I constructed a strain harboring the IPTG-inducible KinC in the kinA and kinB double knockout strain. Second, to monitor the Spo0A activity as an indirect measure of the KinC activity, the PspoIIG-lacZ reporter was introduced into the engineered strain.

Under rich medium conditions (LB), Spo0A activity and sporulation efficiency increased dependent on the IPTG concentrations. These results were similar to those in our previous reports.(Fujita and Losick 2003, Eswaramoorthy, Duan et al. 2010). Based on these results, the optimum concentration of IPTG to

induce efficient sporulation (~10^8/ml, the similar levels to the wild-type sporulating cells) with the synthesis of KinC was determined to be 10 μM. I note that this artificial KinC system is fully functional to trigger sporulation under rich medium conditions when the KinC synthesis is achieved by adding IPTG. Thus, I can exclude the potentially confounding effect(s) of unidentified starvation signal(s),.

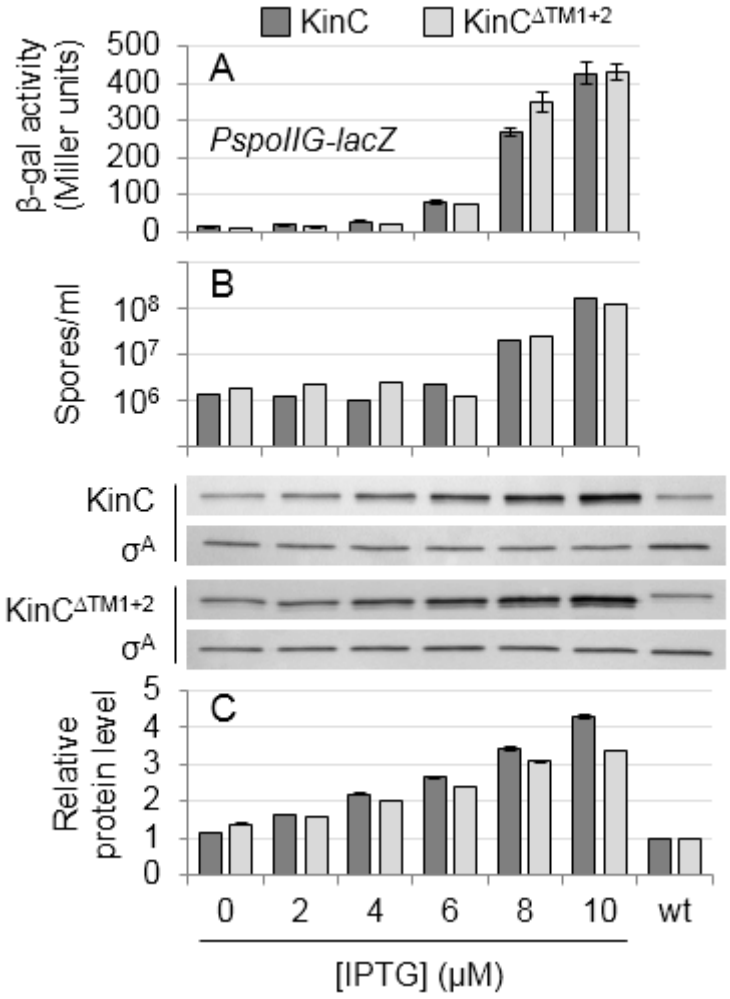


Figure IV.1 Determination of the threshold level of KinC and the soluble KinC (KinC$^{\Delta TM1+2}$) for sporulation.

IPTG concentration was increased over a narrow range (0-10 μM) in order to determine the sufficient protein levels of KinC and KinC$^{\Delta TM1+2}$ for sporulation under rich conditions (LB medium). Strains and culture conditions are indicated in the legend to Fig. IV-2. β-Galactosidase activities (Miller units) from *PspoIIG-lacZ* were measured as indicated in the legend to Fig. IV-2. MF2659 (wild-type lacking the IPTG-inducible construct) was used as a wild-type control to normalize the protein levels. Note that the wild-type strain does not sporulate under rich medium conditions. Thus, β-galactosidase activity and spore count are not detectable in the wild-type strain.

Lopez et al. reported that addition of 150 mM KCl (a level that is close to the intracellular concentration) to the undomesticated B. subtilis cells cultured diminishes biofilm formation (Lopez, Fischbach et al. 2009). Accordingly, They proposed that KinC is activated in response to the decreased concentration of the cation when the membrane leakage is induced by surfactin. Although the surfactin production is defective in the domesticated strain, I tested the effect of potassium on KinC activity and found that the Spo0A activity measured by the Spo0A-controlled PspoIIG-lacZ reporter and sporulation efficiency did not change in the presence of high concentration of KCl (150 mM) under our tested conditions (data not shown). These results indicate that KinC activity is independent of potassium concentration and sporulation is efficiently triggered solely by the increased level of KinC in our artificial strain of the domesticated background.

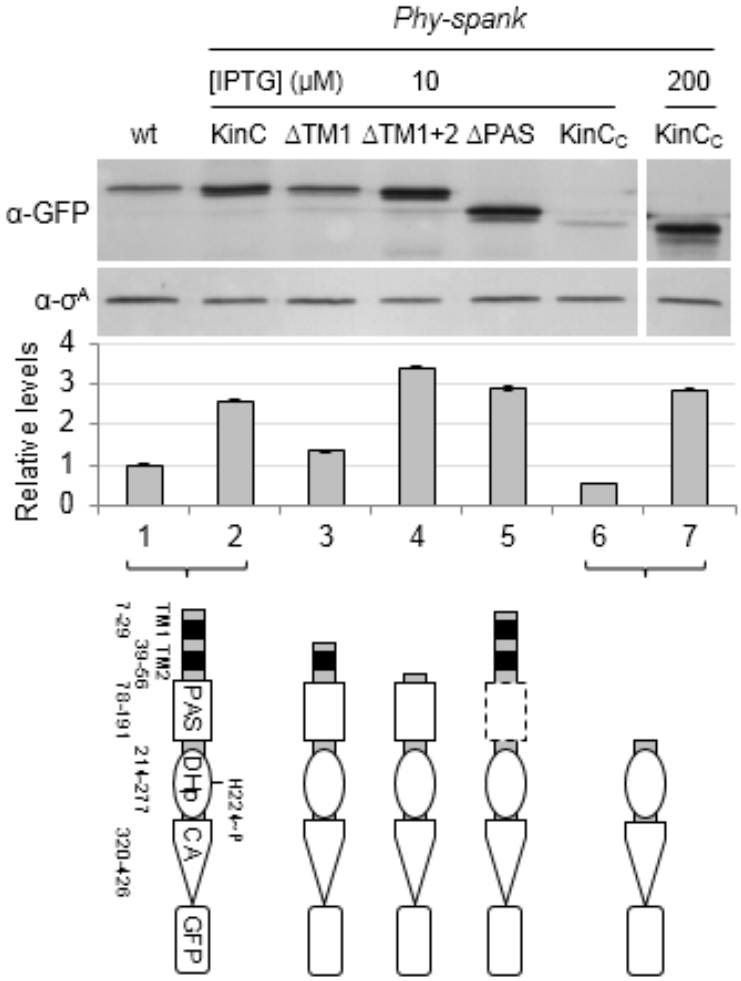


Figure IV.2 Determination of the relative protein levels of KinC and its mutants.

Cell extracts of strains expressing GFP fusion proteins were prepared at T2 after IPTG addition in LB culture and were processed for immunoblotting with anti-GFP (α-GFP) antibodies. Cells of the wild-type strain harboring *kinC-gfp* (MF2659) were cultured for 2h after the mid-exponential phase (OD600=0.5) in LB medium. Protein concentration was measured by Bradford method and total proteins were loaded on 16% SDS PAGE gel (2 μg of total proteins per lane). Strains are indicated in the legends to Figs. IV-4 and 5. A constitutively expressed σ^A protein was used as a loading control. The protein levels were

Fig IV.2 cont.
quantified and normalized to both the levels of σ^A and the levels of the wild-type KinC-GFP (lacking the IPTG-inducible construct).

Finally, to confirm the expression levels of KinC at varying concentrations of IPTG, I performed immunoblot analyses using a C-terminal GFP-tagged KinC under the control of Phy-spank (Fig. IV-2A). In the presence of varying concentrations of IPTG from 0 to 10 μM, the level of KinC-GFP increased proportionally (Fig. IV-1C).

I also constructed a strain harboring KinC-GFP under the control of its own promoter at the endogenous locus in the wild-type background and used as a control (Figs. IV-3). At 10 μM IPTG, the level of KinC-GFP in the engineered strain showed approximately four fold higher than that in the wild-type strain (Fig. IV-1C). I confirmed that, when 10 μM IPTG was added for induction, the KinC-GFP construct showed similar levels of sporulation efficiency to those of the strain expressing the untagged protein (data not shown). These results indicate that KinC-GFP is functional. I note that, when grown in rich medium conditions, Spo0A activity measured by the Spo0A-controlled PspoIIG-lacZ reporter in the wild-type strain (that is lacking the IPTG-inducible construct) is not detectable, resulting in no sporulation is triggered (data not shown) (Eswaramoorthy, Duan et al. 2010). Thus, using this new system, I established a functional relationship of KinC level, Spo0A activity, and sporulation efficiency. One of the important advantages of this system is that I can exclude the involvement of the possible

starvation signal(s) by using rich medium conditions, under which no starvation can occur.

Table IV.4. Viable cells and spores in the IPTG-inducible strain

IPTG (μM)	KinC			KinC$^{\Delta TM1+2}$			KinC$^{\Delta TM1}$			KinC$^{\Delta PAS}$		
	CFU/ml		Efficiency	CFU/ml		Efficiency	CFU/ml		Efficiency	CFU/ml		Efficiency
	Viable	Spore		Viable	Spore		Viable	Spore		Viable	Spore	
0	6.5×10^8	1.4×10^6	2.2×10^{-3}	5.9×10^8	1.8×10^6	3.1×10^{-3}	3.5×10^8	<10	ND	3.5×10^8	<10	ND
2	6.9×10^8	1.2×10^6	1.7×10^{-3}	5.4×10^8	2.3×10^6	4.3×10^{-3}	-	-	-	-	-	-
4	8.1×10^8	1.0×10^6	1.2×10^{-3}	6.4×10^8	2.5×10^6	3.9×10^{-3}	-	-	-	-	-	-
6	6.5×10^8	2.2×10^6	3.4×10^{-3}	6.8×10^8	1.2×10^6	1.8×10^{-3}	-	-	-	-	-	-
8	4.9×10^8	2.0×10^7	4.1×10^{-2}	6.3×10^8	2.5×10^7	3.9×10^{-2}	-	-	-	-	-	-
10	6.9×10^8	1.6×10^8	2.3×10^{-1}	6.2×10^8	1.3×10^8	2.1×10^{-1}	4.5×10^8	1.3×10^2	2.9×10^{-7}	3.3×10^8	<10	ND
20	7.3×10^8	6.7×10^7	9.2×10^{-2}	8.6×10^8	2.9×10^7	3.4×10^{-2}	-	-	-	-	-	-
50	7.6×10^8	1.2×10^7	1.6×10^{-2}	7.0×10^8	5.4×10^7	7.7×10^{-2}	5.0×10^8	8.2×10^3	1.6×10^{-5}	4.2×10^8	4.6×10^3	1.1×10^{-5}
100	6.5×10^8	3.7×10^6	5.7×10^{-3}	7.8×10^8	4.4×10^7	5.6×10^{-2}	4.3×10^8	7.1×10^3	1.7×10^{-5}	2.8×10^8	2.6×10^3	9.2×10^{-6}
200	7.0×10^8	1.4×10^6	2.0×10^{-3}	6.7×10^8	5.4×10^7	8.1×10^{-2}	5.7×10^8	5.2×10^3	9.1×10^{-6}	2.7×10^8	7.4×10^3	2.7×10^{-5}
500	7.6×10^8	1.0×10^6	1.3×10^{-3}	7.5×10^8	6.2×10^7	8.3×10^{-2}	5.3×10^8	5.5×10^3	1.0×10^{-5}	1.7×10^8	1.3×10^4	7.6×10^{-5}
1000	-	-	-	-	-	-	5.2×10^8	4.7×10^3	9.0×10^{-6}	7.2×10^7	1.5×10^4	2.1×10^{-4}

IV.3.2 The PAS domain, but not the transmembrane domain, is required for KinC activity

Using the above described system, I attempted to determine the minimum functional domain of KinC required for the kinase activity. For this, I constructed a series of domain deletion mutants of KinC and tested Spo0A activities and sporulation efficiencies in each of the constructs. Previously, it was not possible to quantitatively analyze the kinase activity because the expression levels of the mutant proteins are often less stable than the wild-type protein (14). Thus, one of the most important advantages of our system is that I was able to test whether the same level of KinC and its mutant activities are achieved when a similar level of each of the tested proteins is synthesized in a quantitative manner.

As shown in Fig. IV-1 and Fig. IV-3, when 10 μM IPTG was added to the culture medium, sporulation was efficiently triggered by inducing the synthesis of KinC and KinC$^{\Delta TM1+2}$, whereas not by other three constructs, KinC$^{\Delta TM1}$, KinC$^{\Delta PAS}$, and KinC$_C$. These results suggested that, among the tested mutants, only KinC$^{\Delta TM1+2}$ is enzymatically active in vivo. Next, expression levels of KinC and its mutant proteins were examined by immunoblot analysis. For this, I constructed a series of C-terminal GFP-tagged KinC mutant proteins under the control of an IPTG-inducible promoter (Phy-spank) (Fig. IV-3). Results of immunoblot analysis indicated that the expression levels of the enzymatically active KinC$^{\Delta TM1+2}$, as well as the wild-type KinC, were approximately proportional to the concentrations of the added inducer (Fig.IV- 1). The Spo0A activities and the number of sporulating cells increased in a manner dependent on the IPTG concentrations. However, at 10 μM IPTG, expression levels of other non-active mutant proteins (KinC$^{\Delta TM1}$, KinC$^{\Delta PAS}$, and KinC$_C$) were significantly lower than those of the active kinase proteins. Then, I wondered whether sporulation is triggered by the increased amount of the mutant proteins, KinC$^{\Delta TM1}$, KinC$^{\Delta PAS}$, and KinC$_C$. The effect of the dosage of each mutant protein on Spo0A activity and sporulation was examined with increased inducer concentrations. As shown in Fig. IV-1CD, in the presence of the high dose of IPTG (200 - 1000 μM), the levels of KinC$^{\Delta TM1}$ and KinC$^{\Delta PAS}$ proteins increased to 7-15 times greater than that in the wild-type. Under such conditions, the Spo0A activity and sporulation efficiency increased to the detectable levels (103 - 10^4/ml), but not to the same extent as the wild-type protein. I note, at 200 μM IPTG concentration, that the level of KinC$_C$ protein

increased three fold greater than that in the wild-type strain. However, under these conditions, cells showed neither detectable Spo0A activity nor sporulation efficiency (Fig. IV-3E).

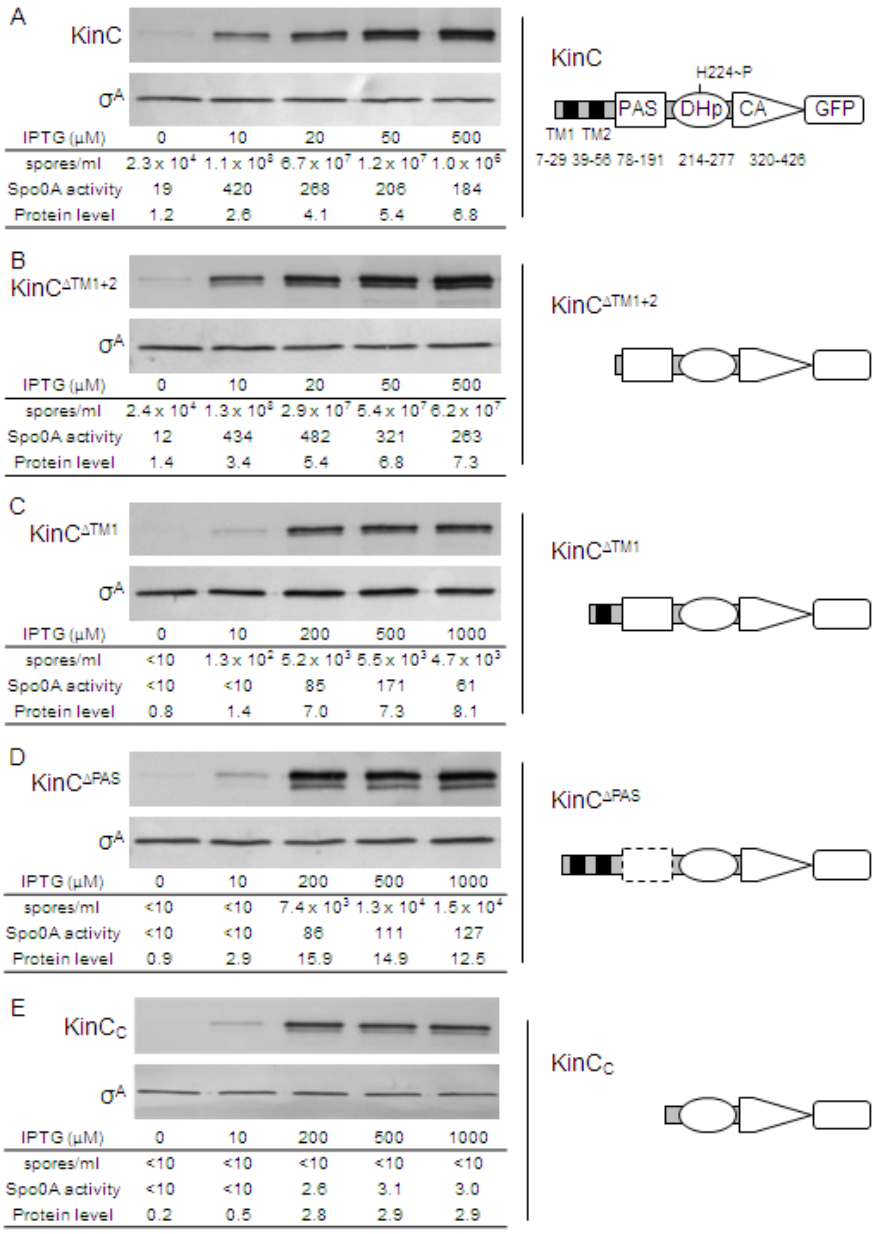


Figure IV.2 Protein dosage effect of KinC and its derivatives on sporulation.

Cells of the IPTG inducible strain for the GFP fused full length KinC and its mutants were cultured to mid-exponential phase (OD600=0.5) in LB medium and

Fig.IV.2 cont.

IPTG was added as indicated concentrations at the bottom of each panel. IPTG concentration was increased over a wide range (0-1000 μM) in order to increase the protein level of KinC or its mutants. Schematic diagram of each of the proteins is depicted at the right hand side of each panel (A-D). β-Galactosidase activities (Miller units) from PspoIIG-lacZ were measured at T3 after IPTG addition in LB culture as a measure of Spo0A activity. The same culture was used for the determination of number of spores per ml culture at 16 h after IPTG addition. Strains used for β-galactosidase activities are as follows: (A) MF4531 (full-length KinC), (B) MF4496 ($KinC^{\Delta TM1+2}$), (C) MF4495 ($KinC^{\Delta TM1}$), and (D) MF4539 ($KinC^{\Delta PAS}$). Expression of full-length KinC or its mutant proteins was examined by immunoblot analysis. Cell extracts of strains expressing GFP fusion proteins were prepared at T2 after IPTG addition in LB culture and were processed for immunoblotting with anti-GFP (α-GFP) antibodies. Protein concentration was measured by Bradford method and total proteins were loaded on 16% SDS PAGE gel (0.04 μg and 2 μg of total proteins per lane for anti-GFP and anti-σ^A antibodies, respectively). A constitutively expressed σ^A protein was used as a loading control. I note that each image in the set has a different exposure time to avoid saturation with high concentration of proteins in the presence of excess amounts of IPTG. The relative protein levels were quantified by running samples on the same gel and normalized to both the levels of σ^A and the levels of the wild-type KinC-GFP (MF2659, lacking the IPTG-inducible construct) shown in Fig. IV-1. Strains used for immunoblotting are as follows: MF4547 (full-length KinC-GFP), MF4549 (KinCΔTM1+2-GFP), MF4548 (KinCΔTM1-GFP), and MF4550 (KinCΔPAS-GFP). All strains were constructed in the kinA, kinB, and kinC triple deletion mutant background (See details in the strain table). All experiments were performed at least three times independently. The representative data are shown.

Finally, I demonstrated whether the soluble $^{KinC\Delta TM1+2}$ has an autokinase activity and the phosphoryl group can be transferred to Spo0A through phosphorelay in vitro. All the protein components were purified and used for in vitro phosphorylation assay. First, as reported previously (Jiang, Shao et al.

2000), I verified the autophosphorylation activity of purified KinC$^{\Delta TM1+2}$ in the presence of radiolabeled ATP (Fig. IV-4 lane 1). Second, in the presence of each of the phosphorelay components with KinC$^{\Delta TM1+2}$, I found that the phosphate group was transferred stepwise from KinC$^{\Delta TM1+2}$ to Spo0A via Spo0F and Spo0B (Fig. IV-4, lanes 2-4). As reported by LeDeaux and Grossman (LeDeaux and Grossman 1995), KinC, when overexpressed, is able to act directly on Spo0A. Similarly, I observed direct phosphorylation of Spo0A by KinC$^{\Delta TM1+2}$, but lesser extent than through the complete phosphorelay (Fig.IV-4, campare lanes 4 and 6). I verified that Spo0B phosphorylation by KinC$^{\Delta TM1+2}$ was not detectable (Fig.IV- 4, lane 5).

Taken together, these results indicate, under our experimental conditions, that the transmembrane domain of KinC is dispensable for the autokinase activity. Furthermore, the results suggest that the lesser activities of KinC$^{\Delta TM1}$, KinC$^{\Delta PAS}$, and KinC$_C$ as compared with KinC and KinC$^{\Delta TM1+2}$ are not due to the lower protein levels in the cells, but rather these proteins are not fully functional. Results of sporulation efficiencies with a set of strains at each condition tested are summarized in Table IV-4.

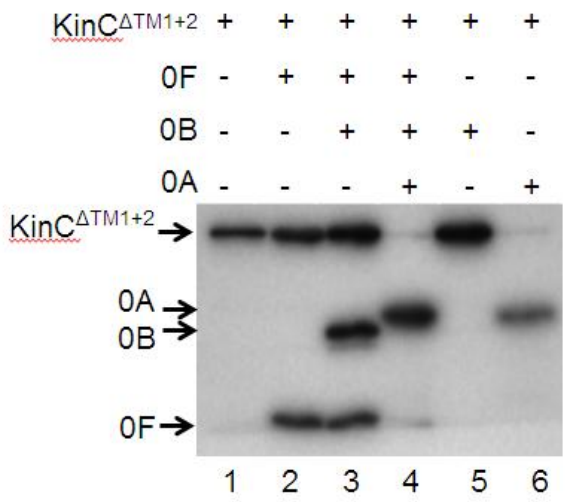


Figure IV.4: Autophosphorylation and phosphotransfer activities of KinC∆TM1+2.

The autophosphorylation and phosphotransfer activities of KinC∆TM1+2 were demonstrated in an in vitro reaction using [γ-32P]ATP. Reactions contained 6µM KinC$^{\Delta TM1+2}$ and 5µM each of the spo0F and spo0B and 10 µM of spo0A. Reaction conditions were as described in materials and method.

IV.3.3: KinC forms a homotetramer mediated by PAS domain

Many studies in the past have suggested that the PAS domain is involved in protein-protein interactions (Taylor and Zhulin 1999). Recently, I reported that the N-terminal domain of KinA having three PAS repeats (PAS-A, PAS-B, and PAS-C) forms a homotetramer and the ability to form a complex appears to be important for the autokinase activity (Eswaramoorthy, Guo et al. 2009). Similarly, in order to obtain insight into the KinC activity and its complex formation, I performed in vivo chemical cross-linking experiments. In general, characterization of the oligomeric state of a membrane protein is technically

difficult both in vivo and in vitro. Thus, I took advantage of the fact that the soluble KinC$^{\Delta TM1+2}$ is active as an autokinase. KinC$^{\Delta TM1+2}$ contains only a single cysteine residue (C118) in the PAS domain, while two cysteine residues (C316 and C395) are located near the C-terminus. Thus, I used bis-maleimidohexane (BMH), a thiol-specific cross-linker as a first attempt. Crude cell extracts were prepared from B. subtilis cells expressing KinC$^{\Delta TM1+2}$-GFP under the control of the IPTG-inducible promoter in the presence of IPTG (10 μM, at which the enzyme is active) in LB. Then, the cell extracts were incubated in the presence or absence of BMH. The calculated molecular mass of each of the protein species is listed in Fig. IV-5. In the presence of cross-linker, a discrete band corresponding to the tetramer size of KinC$^{\Delta TM1+2}$-GFP was detected, along with the smear layer (Fig. IV-5A, lane +). In contrast, only a discrete band at around monomer size was detected in the absence of the cross-linker (Fig. IV-5A, lane -). In the case of the C-terminal domain of KinC (KinC$_C$-GFP), no obvious tetramer complex was detected, while the monomer and smeared bands with some unclear ladders were observed in the presence of the cross-linker (Fig. IV-5B). As a control, I used KinA-GFP and confirmed that it predominantly forms a tetramer (Fig. IV-5C). This control experiment verified that the experimental conditions are well controlled as reported previously (Eswaramoorthy, Guo et al. 2009, Eswaramoorthy, Dravis et al. 2011). Therefore, these results indicate that the soluble and functional KinC$^{\Delta TM1+2}$ predominantly forms a tetramer, perhaps mediated by the cross-linked cysteine residue located at the position 118. I note that two cysteine residues in GFP are not involved in complex formation under

our tested conditions as demonstrated that only a monomer size of GFP is detected in the crude extracts from cells expressing GFP alone (data not shown) (Eswaramoorthy, Dravis et al. 2011). Based on these results, I propose that the full-length transmembrane KinC is a tetramer, mediated by the PAS domain, as a functional kinase.

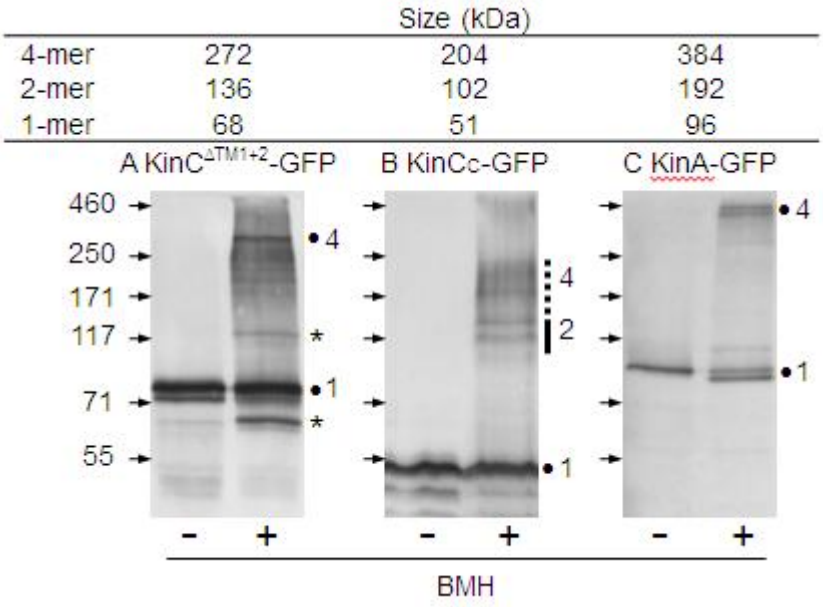


Figure IV.5: Cross-link analysis of the soluble KinC (KinC$^{\Delta TM1+2}$).

Strains expressing KinC$^{\Delta TM1+2}$-GFP (MF4549) (A), KinC$_C$-GFP (MF4609) (B), and KinA-GFP (MF3352) (C) under the IPTG-inducible promoter were examined for cross-linking experiments. Molecular masses and possible multimeric combinations of each sample are listed on the top. Cells harboring each of the IPTG-inducible GFP-tagged protein construct were cultured in LB medium in the presence of IPTG (10 µM). Cell extracts were prepared from each strain after 2h of IPTG addition in LB culture and processed for the BMH cross-linking reaction (+) as described in Materials and Methods. Samples processed in the absence of BMH were used as controls (-). Samples (total proteins corresponding to 0.33 ml of LB culture after IPTG addition per lane) were separated by 16% SDS-PAGE gel and followed by immunoblot analysis with anti-GFP antibodies. Solid bars depict the expected sizes of monomer, dimer, and tetramer forms, respectively. Asterisks indicate unknown forms of protein complexes (A and B). Dashed bar indicate the expected size of tetramer (B). The protein size marker (Invitrogen) was used to estimate the protein sizes on SDS-PAGE.

IV.3.4: KinC localizes as puncta along the cell length

In the previous studies using the undomesticated strain in combination with an IPTG-inducible fluorescent tagged-KinC, the activation of KinC required

localization of the kinase to membrane micro-domains, possibly analogous to lipid rafts in eukaryotic cells, with a heterogeneous localization (Lopez and Kolter GD2010) (Meile, Wu et al. 2006, Lopez and Kolter 2010). In the other report using the domesticated strain with a xylose-inducible fluorescent tagged-KinC, the protein was found to be associated with the membrane in a heterogeneous manner (Meile et al Proteomics 2006). To reproduce these results in our experimental system using the domesticated strain, I examined the membrane localization of KinC-GFP expressed from either the endogenous promoter or an IPTG-inducible promoter. In the wild-type strain expressing KinC-GFP from the endogenous promoter, the fluorescence signals were detected at the cell membrane as clusters of puncta or patches throughout the course of growth in LB medium (Fig. IV-6A and B and Fig. IV-7 A-B), as well as during early sporulation phase in SM medium (data not shown), distinct from the previous observations. To clarify the three-dimensional nature of the membrane localization of KinC-GFP, I carried out deconvolution microscopy for the wild-type cells expressing the GFP fusion protein in LB medium. The resulting image showed cells with occasional clustering of the fluorescent puncta (Fig. IV-6G). Similar localization patterns were reported with the membrane-associated GFP–MinD construct in B. subtilis (Barak, Muchova et al. 2008). Thus, these localization patterns appear to be the result of a spiral-like structure with 3-3.5 helical turns along the long axis of rod-shaped cell. In the control experiments, cells expressing the functional KinA-GFP from the endogenous locus were analyzed under sporulation conditions in SM medium (Fig. IV-7 CD and Fig. IV-6

D-F). Based on these results, I confirmed that KinA-GFP was largely detected in the cytosolic compartment as reported previously (Fujita and Losick 2005).

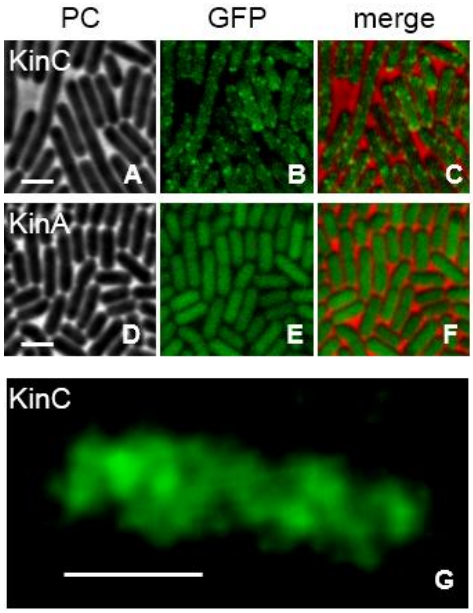


Figure IV.6. Images of fluorescent and deconvolution microscopy of KinC-GFP.

Cells of MF2659 (full-length KinC-GFP in wild-type lacking the IPTG-inducible construct) (A-C and G) and MF929 (KinA-GFP in wild-type lacking the IPTG-inducible construct) (D-F) were cultured as described in the legend to Fig. IV-7 and analyzed by fluorescence microscopy (A-F) or deconvolution system (G) (Intelligent Imaging Innovations). Scale bar, 2 μm.

Next, I examined the localization patterns in the IPTG-inducible KinC strain in LB medium in the presence of varying concentrations of IPTG. As shown in Fig. IV-6E and F, I found that in the presence of 10 μM IPTG, at which the inducer concentration is effective to trigger efficient sporulation (Fig. IV-1, and Fig.IV-2, and Table 4), the GFP-fusion protein was detected on the membrane more uniformly and continuously as compared with that expressed from the

endogenous locus in the wild-type strain (compare panels B and F in Fig. IV-7). In contrast to the membrane localization of full-length KinC, KinC$^{\Delta TM1+2}$ displayed a diffused distribution throughout the cytosol (Fig. IV-7MN), similar to KinA (Fig. IV-7CD). Furthermore, when overexpressed in the presence of 200 μM IPTG, KinC-GFP was detected more intensely (Fig. IV-7GH). Under this condition, discrete foci were observed frequently, near the membrane, most likely due to the aggregation of the overproduced protein (Fig. IV-7GH). I note that this particular localization pattern seems to be similar to that found in the report by Lopez and Kolter (Lopez and Kolter 2010).

Among the non-active mutant proteins, KinC$^{\Delta PAS}$ was detected as a membrane-anchored protein (Fig. IV-7Q-T). The other two non-active proteins (KinC$^{\Delta TM1}$ and KinC$_C$) were detected well only at 200 μM IPTG as discrete foci near the membrane and diffused throughout the cytosol, respectively (Fig.IV-7I-L and U-X).

Judging from these observations, I conclude that, in the domesticated wild-type strain, KinC localizes in a helical or spiral pattern along the long axis of rod-shaped cell, while it was previously reported that KinC localizes as a discrete focus in a heterogeneous manner on the specific membrane domain in the undomesticated strain (Meile, Wu et al. 2006, Lopez and Kolter 2010). The possible sources of the discrepancies of the protein localization between previous results and ours may be the differences of the strain systems and experimental conditions. I note that, in contrast to our endogenous expression system, the previous expression system for the fluorescent-tagged KinC protein

is under the control of a xylose-inducible promoter (Meile, Wu et al. 2006) or an IPTG-inducible promoter (Lopez and Kolter 2010). Thus, overexpression of the fluorescent-tagged protein in the presence of the inducer might cause the aggregation of the proteins, resulting in the discrete foci in their experimental conditions as I observed in this study (Fig. IV-7H).

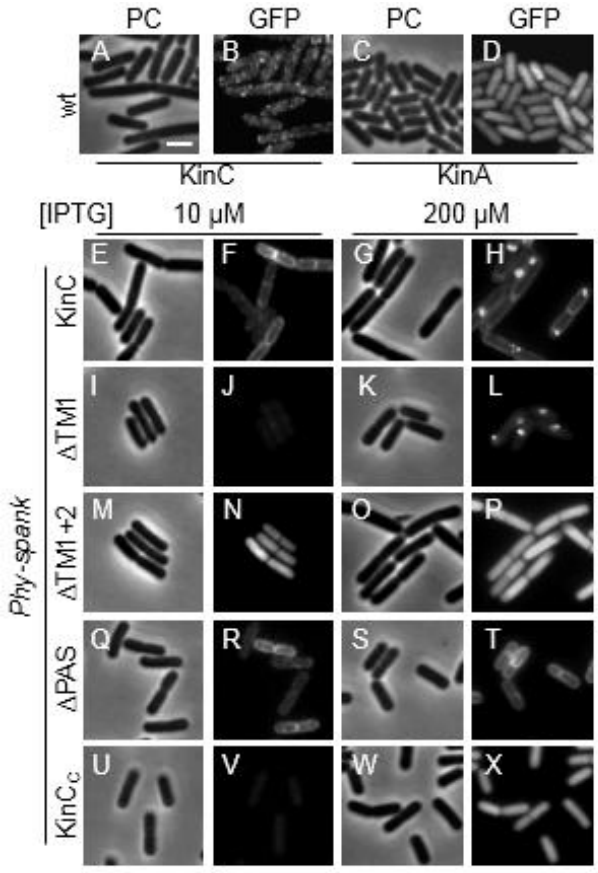


Figure IV-7: Fluorescence microscope image to show full length KinC-GFP and its mutant

Cells of the wild-type strain harboring kinC-gfp (MF2659) were cultured for 2h after the mid-exponential phase (OD600=0.5) in LB medium and analyzed by fluorescence microscopy (AB). Cells of the wild-type strain harboring kinA-gfp (MF929) were induced for sporulation in SM medium for 2h and analyzed by fluorescent microscopy (CD). Cells of the IPTG inducible strain for the GFP fused

Fig IV.7 cont.
full length KinC and its mutants were cultured to mid-exponential phase (OD600=0.5) in LB medium and IPTG was added as indicated concentrations (10 and 200 μM). Strains used are as follows: MF2659 (full-length KinC-GFP in wild-type lacking the IPTG-inducible construct), MF4547 (full-length KinC-GFP), MF4549 ($KinC^{\Delta TM1+2}$-GFP), MF4548 ($KinC^{\Delta TM1}$-GFP), MF4550 ($KinC^{\Delta PAS}$-GFP), and MF4609 ($KinC_C$-GFP). All strains were constructed in the kinA, kinB, and kinC triple deletion mutant background (See details in the strain table). Cells were harvested at 2h after IPTG addition and then analyzed by fluorescence microscopy (E-X). Images are shown in a series of two panels: phase contrast (left) and GFP (right). Scale bar: 2 μm.

IV.3.5 KinC preferentially and positively controls the expression of cannibalism genes during early sporulation phase.

I attempted to investigate the role and significance of KinC during growth and sporulation in the domesticated strains. For this, I began by confirming the results of Hobbs, in which kinC mutant was shown to sporulate faster than the wild-type strain and similar to mutants of cannibalism toxin operons (skf and sdp) (Gonzalez-Pastor, Hobbs et al. 2003). Cells of the wild-type strain and each of three kinase mutants (kinA, kinB, or kinC) were grown on solid Difco Sporulation (DS) medium. I found that the colony of the kinC mutant were whiter and more opaque than other kinase mutants (ΔkinA and ΔkinB), but similar to the mutants of skf and sdp (Δskf and Δsdp) (Fig. IV-8A). These results are essentially consistent with those of Hobbs (2006), suggesting that kinC mutant sporulates faster than the wild-type strain.

To test whether the expression of cannibalism genes is dependent on KinC, levels of phosphorylated Spo0A (Spo0A~P) were measured using a lacZ

transcriptional fusion to the sdp promoter in the wild-type and each of the three kinase mutant strains (ΔkinA, ΔkinB, or ΔkinC). The transcription of sdp is indirectly activated by Spo0A~P through AbrB, a transition state regulator (Fujita and Losick 2005). Under rich medium conditions (LB), a markedly reduced level of the β-galactosidase reporter activity from the sdp promoter was observed in the kinC null mutant (Fig. IV-8B). In contrast, in the kinA and kinB deletion mutants, β-galactosidase activities were similar or even higher than those in the wild-type control, respectively (Fig. IV-8B). These results suggest that expression of sdp is, at least in part, dependent on KinC under rich medium conditions. Next, I investigated the expression of sdp under sporulation conditions in SM medium and found that the β-galactosidase expression from the sdp reporter was significantly lower in the kinC mutant, as compared with the wild-type strain, indicating that it is largely dependent on KinC during sporulation (Fig. IV-8C). I note, as expected, that the level of β-galactosidase activity from sdp in the wild-type strain is approximately two times higher under sporulation conditions than that under rich medium conditions since Spo0A is more active under sporulation conditions (Fig. IV-8BC). The reporter activities in the kinA and the kinB mutant strains rose to higher levels than that in the wild-type sporulating cells, suggesting that the expression of sdp is derepressed with the relatively low activity of Spo0A in these mutant strains (Fujita, Gonzalez-Pastor et al. 2005). These results are consistent with the notion that: (1) sdp is repressed by AbrB during growth, (2) during early sporulation phase, AbrB is repressed at relatively lower levels of Spo0A~P; (3) as a result of the derepression of sdp gene

expression by decreasing the quantity of AbrB, Sdp level increases, resulting in delay of sporulation; and (4) in turn during later stages of sporulation, expression of sdp is directly repressed by higher dose of Spo0A~P to reinforce the progress of sporulation (Fujita, Gonzalez-Pastor et al. 2005). Therefore, these results indicate that KinC preferentially and positively controls the expression of cannibalism genes, thereby delaying sporulation.

KinC activates expression of sdp in a manner dependent on phosphorelay

Previous reports suggest that KinC can bypass the requirement of the intermediate phosphotransferases (Spo0F and Spo0B) under certain circumstances (Kobayashi, Shoji et al. 1995, LeDeaux and Grossman 1995, Lopez, Fischbach et al. 2009, Lopez and Kolter 2010), while the in vitro experiments indicate that Spo0F rather than Spo0A is predominantly phosphorylated by KinC (Jiang, Shao et al. 2000). To test whether expression of sdp is activated by KinC in a manner dependent on phosphorelay under the in vivo physiological conditions, the expression level of the lacZ transcriptional fusion to the sdp promoter was measured in a mutant for spo0F, spo0B, or spo0A as well as in a wild-type under growth and sporulation conditions. As shown in Fig. IV-8 BC, both under nutrient and sporulation conditions, the reporter activities from the sdp promoter in the spo0F, spo0B, or spo0A mutant were detected only at lower levels (less than 20 Miller units). These results indicate that, under physiological conditions, the threshold level of Spo0A for sdp expression is achieved by KinC through Spo0F and Spo0B in a manner

dependent on phosphorelay. Our in vitro phosphorylation results shown in Fig. 5 also support these in vivo data.

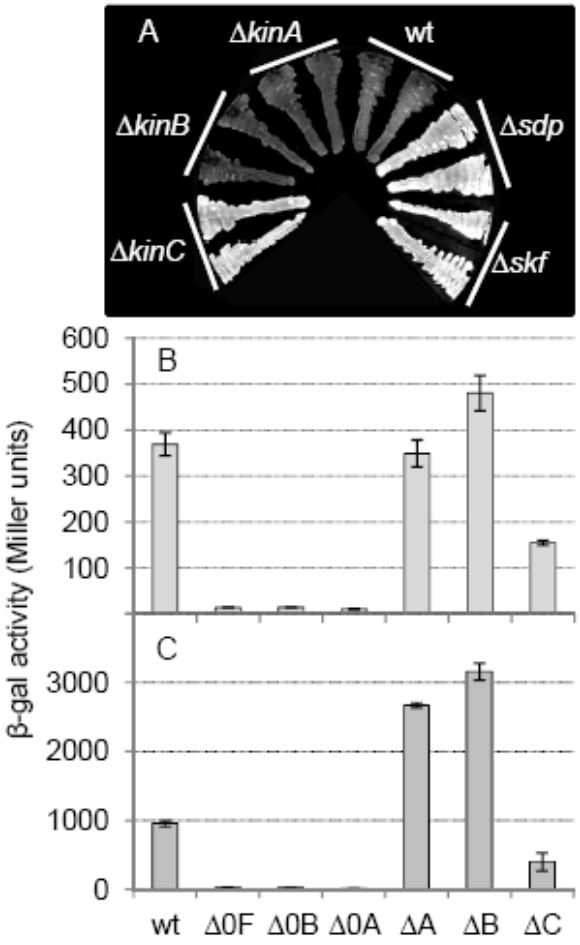


Figure IV.8: Expression of sdp operon is dependent on KinC in a manner dependent on phosphorelay, causing delay in sporulation.

Cells of kinC mutant sporulate more rapidly than the wild-type strain. Colonies of kinA (ΔkinA::tet, strain MF1237), kinB (ΔkinB::kan, MF1847), kinC (ΔkinC::cm, MF1845), skf [Δ(skfABCDEF)::tet; EG168] and sdp [Δ(sdpABC)::spc; EG407]

Fig IV.8 cont.
mutants, as well as the wild-type strain (PY79) were grown on solid sporulation medium for 16 hours at 37 °C. Mutant colonies of kinC, skf, and sdp were brighter than that of the wild-type strain, indicating that those mutants sporulate faster than the wild-type strain as shown previously (Gonzalez-Pastor, Hobbs et al. 2003). Time course of accumulation of β-galactosidase from Psdp-lacZ were measured in the wild-type strain (open circle, MF1821), in the spo0F mutant strain (Δ0F, MF1826), in the spo0B mutant strain (Δ0B, MF1827), in the spo0A mutant strain (Δ0A, MF831), in the kinA mutant strain (closed triangle, MF1923), in the kinB mutant strain (closed square, MF1924), and in the kinC mutant strain (closed diamond, MF1925) at two hours after the mid-exponential growth phase (OD600 of 0.5) under nutrient rich (LB) (B) and at two hour after resuspension of growing cells in sporulation medium sporulation (SM) (C).

IV.4 Discussion

The major findings in this study using the domesticated strain can be summarized as follows: (1) KinC preferentially contribute to the delay in sporulation by controlling the expression of cannibalism genes through Spo0A in a manner dependent on phosphorelay, (2) the PAS domain, but not the transmembrane domain, is required for KinC activity, (3) the soluble KinC (KinCΔTM1+2) forms a functional tetramer, (4) KinC is arranged in a spiral-like structure along the long axis of rod-shaped cell. Therefore, our results, in conjunction with the fact that the domesticated strain is deficient for the production of surfactin, suggest that (1) KinC becomes active irrespective of both the potassium status and the specific localization in a membrane microdomain composed of lipid raft, and (2) the PAS domain is involved in tetramer formation that is required for the kinase activity.

Using the domesticated laboratory strain, KinC was first suggested to be involved in sporulation in a Spo0A mutant (Kobayashi, Shoji et al. 1995, LeDeaux and Grossman 1995). Recently, in the undomesticated strain, the kinase was shown to induce exopolysaccharide matrix production, resulting in biofilm formation, in a quorum-sensing manner (Lopez, Fischbach et al. 2009, Lopez, Vlamakis et al. 2009, Lopez, Gontang et al. 2010, Lopez and Kolter 2010). Lopez and Kolter proposed that, in response to increased cell density, ComX pheromone triggers the ComP and ComA two-component system, resulting in the production of surfactin (Lopez, Fischbach et al. 2009, Lopez, Vlamakis et al. 2009, Lopez and Kolter 2010). Thereby, the intracellular concentration of potassium drops through the action of surfactin on membrane enhanced activity of KinC (Lopez, Fischbach et al. 2009), resulting in the enhanced activity of KinC (Lopez, Fischbach et al. 2009, Lopez, Vlamakis et al. 2009, Lopez, Gontang et al. 2010). Furthermore, lipid raft microdomain is required for the enhanced activity of KinC (Lopez and Kolter 2010). One of the interesting findings in this study is that KinC-GFP, when expressed from the endogenous locus, is localized as puncta along the long axis of rod-shaped cell, with patterns rather different from foci as reported before (Lopez and Kolter GD2010, (Lopez and Kolter 2010)). Our results suggest that the membrane localization of KinC may not be mediated by lipid raft microdomain, in contrast to the previous study using the undomesticated strain in combination with an IPTG-inducible KinC-CFP (Lopez and Kolter 2010). Further, Lopez and Kolter (2010) demonstrated that overexpression of KinC is not sufficient to induce biofilm formation in the absence

of lipid raft integrity (Lopez and Kolter 2010). Recently, I showed that gradual accumulation of Spo0A~P is essential for the proper temporal order of the Spo0A regulon expression (Vishnoi, Narula et al. 2013). Therefore, one of the possible reasons of their unsuccessful results is that cells overproducing KinC causes abnormally rapid increase in Spo0A activation by bypassing the requirement of phosphorelay. As a result, gradual activation/repression of many genes in the Spo0A regulon is not achieved, leading to improper cell growth and/or differentiation. In support of these notions, sporulation efficiency was decreased by adding higher concentrations of inducer (Table IV-4).

Lopez and colleagues (Lopez, Fischbach et al. 2009) also demonstrated that extracellular surfactin causes leakage of intracellular potassium and indirectly stimulates KinC activity by lowering the intracellular concentration of potassium, resulting in biofilm formation. In this context, they suggested that KinC, using the PAS domain, detects a drop of the intracellular potassium concentration and triggers the autokinase activity in the C-terminal catalytic domain. In their study, KinC and its derivatives were expressed from the native promoter. As a result, varied activities of each of these tested proteins were observed. Thus, they concluded that it is difficult to evaluate whether the KinC activity is intrinsic to the mutant protein or its expression level (Lopez, Fischbach et al. 2009). On the other hand, in this study using a quantitative in vivo assay for the kinase activity in combination with an IPTG-inducible system, I demonstrated that the PAS domain is essential for the autokinase activity by forming a homotetramer, regardless of the potassium concentration in the domesticated

strain. Thus, the reduction of the kinase activity by the deletion of the PAS domain demonstrated by Lopez et al. (Lopez, Fischbach et al. 2009) may be a result of the decrease in the active tetramer formation, but not due to insensitiveness of potassium with the removal of the sensor domain. However, these two mechanisms, sensing potassium and tetramer formation, are not distinguished by their experimental approach (Lopez, Fischbach et al. 2009, Lopez and Kolter 2010). Cannibalistic cells of the domesticated strain produce the Skf and Sdp peptide toxins to kill a fraction of their siblings during the early stages of sporulation (Gonzalez-Pastor, Hobbs et al. 2003). Synthesis of the toxin peptides is activated at early stages of sporulation by low threshold level of Spo0A~P, while genes directly involved in sporulation are regulated by high threshold level of Spo0A~P (Fujita, Gonzalez-Pastor et al. 2005). The deletion mutant of KinC phenocopies that of Skf or Sdp (Supplemental Fig. IV-8) (Hobbs, 2006). In the domesticated strain, surfactin production is deficient (Nakano, Marahiel et al. 1988). Taking all these facts together, in the domesticated strain, KinC appears to be active and sufficient to induce the synthesis of the killing peptides independent of the detergent and thus irrespective of the intracellular potassium concentrations.

During early stages of sporulation, low levels of Spo0A~P indirectly activate the expression of the sdp operon via repression of the repressor AbrB. In this process, genes controlled by AbrB might be temporally expressed under the control of the gradual increase in Spo0A~P. Although Spo0F is a more efficient substrate for KinC than Spo0A, it has been suggested that Spo0A can be directly

phosphorylated by KinC to some extent, which may be sufficient to repress AbrB expression to some degree (Jiang, Shao et al. 2000). However, our results indicate that transcription of the sdp operon is activated in a manner dependent on phosphorelay (Fig. IV-8). A wild-type strain harboring mutations in both kinA and kinB is not able to sporulate, indicating that KinC activity is not sufficient for triggering sporulation, However, when increased the protein level, it is sufficient to induce sporulation (Table IV-4) (Vishnoi, Narula et al. 2013). Based on these facts and observations, I speculate that two different levels (low and high) of Spo0A activation are achieved through two distinct pathways regulated by KinC and KinA, respectively as follows: (1) when KinC is fully activated during early phase of starvation, cells produce cannibalism toxin proteins and form biofilm by expressing only low level of Spo0A~P, resulting in avoidance of sporulation decision-making, (2) however, the level of fully active KinC is not sufficient to achieve the threshold of Spo0A~P needed for entry into sporulation during this period, and (3) when KinA activity increases gradually later on, cells ultimately end up forming spores with high levels of Spo0A~P (14). The above points will be the subject of further work.

CHAPTER V: SUMMARY

I used the artificial sporulation induction (ASI) system, in which the *kinA* gene is placed under the control of an inducible promoter. It was previously demonstrated that inducing the synthesis of KinA to mimic the wild-type level of KinA during sporulation conditions is sufficient to induce sporulation efficiently (Fujita and Losick 2005). Our group also found that the N-terminal PAS-A domain, previously believed to be essential, is dispensable for KinA activity (Eswaramoorthy, Guo et al. 2009, Eswaramoorthy and Fujita 2010), any two of the three PAS domains are sufficient to maintain KinA activity under the physiological conditions (Eswaramoorthy and Fujita 2010), and the N-terminal domain behaves in a dominant-negative fashion, impairing KinA activity, when overexpressed in the wild-type strain (Eswaramoorthy, Guo et al. 2009) and the C-terminal domain ($KinA_C$), containing DHp and CA domain, preferentially transfers the phosphate moiety from Spo0F~P to itself in a reverse reaction, resulting in sporulation deficiency, when overexpressed in the wild-type and Δ*kinA* strains (Eswaramoorthy, Guo et al. 2009). Based on these results, I hypothesized that the primary role of the N-terminal domain of kinase is to form a functional tetramer, but not for sensing an unknown starvation signal. . My results suggest that the replacement of the sensor domain of KinA with a foreign domain of YdaM from *E. coli* results in a massive induction of sporulation initiation by a concentration-dependent mechanism through phosphorelay. The resulting chimeric kinase forms a homotetramer, as was reported for KinA (Eswaramoorthy, Guo et al. 2009, Eswaramoorthy, Duan et al. 2010, Eswaramoorthy and Fujita 2010). Although the chimeric kinase shows delayed

activation of Spo0A under nutrient-rich conditions compared with starvation conditions, with unknown mechanisms, the peak activity levels of Spo0A (as an indirect measure of the kinase activity) and the resulting sporulation efficiencies are essentially the same under these two conditions. These results and our published studies (Eswaramoorthy, Duan et al. 2010, Eswaramoorthy and Fujita 2010) suggest that, regardless of the origin of the protein, tetramer formation mediated by the N-terminal domain is sufficient for the kinase activity catalyzed by the C-terminal domain, irrespective of nutrient availability.

I have also constructed a genetically engineered strain that decouples and rewires the sporulation phosphorelay network thereby establishing an artificial two-component system of sporulation comprising KinC and Spo0A. In contrast to the previously reported systems (Fujita, Gonzalez-Pastor et al. 2005, Eswaramoorthy, Duan et al. 2010, Chastanet and Losick 2011), our artificial two-component system offers unique control over the kinetics of Spo0A∼P accumulation and thus is a powerful tool to investigate why Spo0A∼P accumulation needs to be gradual for efficient sporulation

Results for KinC-Spo0A induction strains indicate that kinase overexpression strains are limited by the amount of Spo0A available for activation. The simultaneous overproduction of both Spo0A and KinC can overcome this limitation and further speed up Spo0A activation and thereby reduce sporulation efficiency. However, the high sporulation efficiency of KinC

induction strain with native spo0A promoter suggests that the Spo0A feedback loops ensure gradual Spo0A~P accumulation.

I also studied the role of KinC during the course of cell growth and sporulation. Using conventional genetic approaches, I found that, KinC preferentially and positively controls the expression of the cannibalism (a mechanism to delay sporulation) genes during early sporulation stage in a manner dependent on phosphorelay, resulting in the delay of sporulation. Furthermore, using an artificial induction of KinC system, I found that sporulation is efficiently induced by overexpression of KinC even under nutrient rich conditions. These results suggest that (1) when KinC is fully activated during early phase of starvation, cells produce cannibalism toxin proteins and form biofilm by expressing only low level of Spo0A~P, mainly through KinC, resulting in avoidance of sporulation decision-making; (2) the level of fully active KinC is not sufficient to achieve the threshold of Spo0A~P needed for entry into sporulation during this period; and (3) when KinA activity increases gradually later on, cells ultimately end up forming spores with high levels of Spo0A~P.

However, the level of fully active KinC is not sufficient to achieve the threshold of Spo0A~P needed for entry into sporulation during this period, and when KinA activity increases gradually later on, cells ultimately end up forming spores with high levels of Spo0A~P. The above points will be the subject of further work.

However, a question yet to be answered is how the Kinase level is increased during starvation conditions. Another interesting question to ask is how the level of Kinase in individual cells of the population contributes to the sporulation heterogeneity, whereby even in wild-type only a subpopulation of the cells undergoes sporulation.

V-I Proposed model for Spo0A~p activation

During early stages of sporulation, low levels of Spo0A~P indirectly activate the expression of the *sdp* operon via repression of the transition state regulator AbrB that represses the transcription of sdp operon during growth. In this process during the transition from growth to sporulation, genes controlled by AbrB might be temporally expressed under the control of the gradual increase in Spo0A~P over time of starvation. Although Spo0F is a more efficient substrate for KinC than Spo0A, it has been suggested that Spo0A can be directly phosphorylated by KinC to some extent, which may be sufficient to repress AbrB expression to some degree (Jiang, Shao et al. 2000). However, our results indicate that transcription of the *sdp* operon and biofilm formation are activated in a manner absolutely dependent on phosphorelay. A wild-type strain harboring mutations in both *kinA* and *kinB* is not able to sporulate, indicating that KinC activity is not sufficient for triggering sporulation. However, when the protein level is increased, it is sufficient to induce sporulation. (Vishnoi, Narula et al. 2013). Based on these facts and observations, we speculate that two different levels (low and high) of Spo0A activation are achieved through two distinct pathways

regulated by KinC and KinA, respectively, as follows: (1) when KinC is fully activated during early phase of starvation, cells produce cannibalism toxin proteins and form biofilm by expressing only low level of Spo0A~P, mainly through KinC, resulting in avoidance of sporulation decision-making; (2) the level of fully active KinC is not sufficient to achieve the threshold of Spo0A~P needed for entry into sporulation during this period; and (3) when KinA activity increases gradually later on, cells ultimately end up forming spores with high levels of Spo0A~P (Lopez, Fischbach et al. 2009). The above points will be the subject of further work.

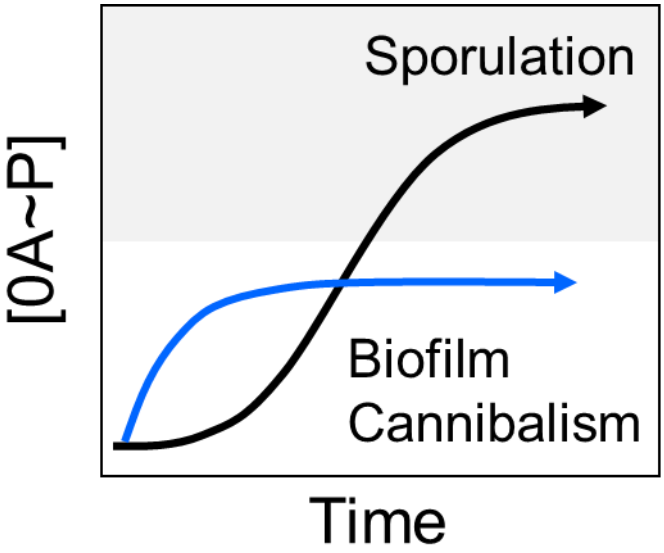


Figure V.1: Spo0A~P activation
At early times following nutrient deprivation,KinC is fully activated cells produce cannibalism toxin proteins and form biofilm by expressing only low level of Spo0A~P and then KinA activity increases gradually later on, cells ultimately end up forming spores with high levels of Spo0A~P

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