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Dissecting regulatory mechanisms of quorum sensing pathways in Bacillus subtilis

Patrick Hill

DISSECTING REGULATORY MECHANISMS OF QUORUM SENSING PATHWAYS IN *BACILLUS SUBTILIS*

A Dissertation Presented

by

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DEDICATION

To my family, friends new and old, and my ever-patient wife, Kelli.

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I would first like to thank my advisor, Kevin Griffith. I truly appreciate the opportunity I had to work with you, and for sticking with me all of these years. I would also like to thank all past members of the Griffith Lab group for everything from building strains and working out protocols, to just lending an ear. Thanks to Tom Steckbeck and Fabian Hoeller for all of their help and accepting me as I learned to be a better mentor. A very special thanks to Emily Roy for the constant support and keeping perfect notebooks and protocols that saved me more times than I'd like to admit.

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Finally, I need to thank my parents and Kelli. Without all of their support, guidance, and hope for me, I would never have persevered.

ABSTRACT

DISSECTING REGULATORY MECHANISMS OF QUORUM SENSING PATHWAYS IN *BACILLUS SUBTILIS*

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Living organisms generally share a small number of characteristics, among which include maintaining homeostasis, growth, and responding to changing environments. Wherever we find life, we typically observe this life performing these tasks. Likely no environment is truly barren, so organisms must be able to continue living in crowded conditions. Humans use their senses to determine the quality of their local environment. Individuals use languages, written, spoken and digital to communicate these findings to their neighbors. Bacteria have evolved complex systems to sense these conditions, and to trigger appropriate developmental programs to help them survive, grow, and respond in

changing environments. Bacteria both produce and sense signals about these density-dependent conditions in a process called quorum sensing.

Chapter 1 provides an introduction to the mechanisms utilized by bacteria referred to as quorum sensing. An overview is given of the history of the study of these mechanisms, as well as a review of molecules and strategies from both Gram-negative and Gram-positive organisms. Also discussed here are mechanisms of quorum quenching used by organisms in quorum sensing pathways. Next, we discuss in some detail the molecular mechanisms used by *Bacillus subtilis* to regulate pathways under control of the quorum response.

Chapter 2 describes work looking to further explain the mechanism of ComA activation. In this chapter, we use a genetic screen to identify constitutive mutants of ComA. We then characterize these mutants for their regulation by ComP and RapC and for their ability to bind DNA. These results were used in an attempt to generate a computational model of ComA activation. We take preliminary steps in validating this model by logically creating and testing combination mutants of ComA. The role of acetyl-phosphate in ComA activation is also briefly explored.

Chapter 3 explores the role of Rap proteins in regulating the biological processes of genetic competence, sporulation, motility and biofilm formation. We were able to characterize several Rap proteins as novel regulators of these pathways. We also were able to show that Rap60 uses a separate surface for interaction with ComA as compared to canonical Rap protein anti-activators of genetic competence.

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CHAPTER 1

INTRODUCTION TO DENSITY-DEPENDENT REGULATION OF BIOLOGICAL PROCESSES AND THE QUORUM RESPONSE

1.1 Benefits of being social

Sometimes, it seems as though we are under a constant barrage of decisions that need to be made. What clothes do I wear today? Do I drive to work, or is it nice enough to bike? If we are lucky, on Friday night, most of us are faced with a new decision: What do we choose to do this weekend to maximize fun and happiness? Do we go to the gym? What about a movie? Maybe we'll go check out that new restaurant in town. We have all been in a busy restaurant on a Saturday night. While guests get to relax, there are individuals responsible for noticing the characteristics of the crowd: How many guests are present? What size groups are they in? This information is used by the staff to work as a group to provide a great customer experience. An individual could never hope to seat guests, make drinks, prepare and serve meals, and clean up in order to turn the table over again an hour later. A host usually keeps track of how many tables are occupied, and brings guests to their place. A waitress takes orders and conveys meal choices to the kitchen. Line cooks and bartenders perform the specialized work of preparing food and drinks to our liking. The wait staff serves the food when ready, and relays information about customer satisfaction back to the kitchen. And when patrons leave, the bussers come along to clean and set the tables. All of these actions are part of running a restaurant and are important.

Communication is key to getting these actions to happen in the right time and place. Without such exquisite coordination, guests won't stay happy and consuming. Imagine if

the host stopped keeping track of the status of the tables. We would find ourselves fighting with a random family for our chance to sit. Similarly, we enjoy if we get our correct meal after the delicious spinach artichoke dip appetizer. Thus the timing and communication between the wait staff and the cooks needs to be working in perfect harmony. We do not even want to think about what would happen should the busser remove our plates before we are done eating. Even at our own table, we need to communicate and make decisions. For example, we decided on that tasty dip, instead of the mozzarella sticks. At the end of our meal, we need to decide how to split the bill.

Just like communication helps us to enjoy our night out, microorganisms are also helped by communication. Bacteria live in ever-changing environments. Populations of bacteria must handle varying availability of nutrients, space to grow, and competition from other microorganisms. When dealing with these changes, bacteria benefit from precise, coordinated communication. Pathogens need to know when it is best to produce proteins involved in colonization instead of virulence factors. If food and other resources are scarce, it will help the population to partake in survival strategies, including sporulation and horizontal gene transfer.

1.2 Early observations of group behavior among bacteria

1.2.1 Bioluminescence in *Vibrio fischeri*

In the late 1960s, *Vibrio fischeri* was observed to produce light in a manner coordinated with population density. This production of light was able to be triggered early by

growing the bacteria in conditioned media. At first, it was assumed that this effect was due to the growing cells removing a molecule that resulted in repression of the genes involved (Kempner and Hanson, 1968), but Nealson, Platt, and Hastings later showed transcription of genes involved in luciferase production to be the result of an autoinducer signaling molecule (Nealson, Platt and Hastings 1970; Eberhard *et al.*, 1981). This system was later revealed to be the LuxI-LuxR system (Engebrecht, Nealson and Silverman, 1983).

LuxI is responsible for synthesizing an acyl-homoserine lactone molecule (AHL) that functions as the autoinducer (Figure 1.1). Transcription of *luxI* is low at low cell density. Thus, the amount of autoinducer that has accumulated in the extracellular environment is low. At high cell density, a greater amount of autoinducer is present. AHL diffuses across the bacterial membranes and binds to the receptor and transcription factor, LuxR. LuxR binds a *lux* box DNA sequence, activating transcription of the *luxICDABE* operon (Figure 1.1). This operon encodes a number of genes involved in the production and function of the luciferase enzyme. Luciferase catalyzes the production of light by oxidizing an aldehyde and reducing FMN to FMNH2. FMNH2 in complex with LuxE produces light (Nealson and Hastings, 1979). The bacteria benefit by getting to live in the relatively nutrient rich light organ of the Hawaiian squid, while the squid gain protection from predators via counterillumination (Nealson and Hastings, 1979).

1.2.2 Fruiting body formation and sporulation in *Myxococcus xanthus*

Myxococcus xanthus, another Gram-negative bacteria, uses altogether different signaling pathways to control its fruiting body formation and sporulation. In response to starvation or exhaustion of prey, *M. xanthus* undergoes the stringent response (Kroos, 2017). Specifically, isoleucine, leucine, valine, and phenylalanine have been shown to be essential for vegetative growth on solid media (Bretscher and Kaiser, 1978). This stress pathway results in the accumulation of guanosine penta-/tetra-phosphate ((p)ppGpp) (Figure 1.2A). In response to the accumulation of (p)ppGpp, the cell produces A-Signal via a pathway involving the Asg (A-Signal Generating) gene products, AsgABCDE. A-Signal consists of short peptides and their constituent amino acids. This signal is sensed by the SasS-SasR two component system in a density-dependent manner.

The A-Signal pathway combines with that of a second density-dependent signaling system, the C-Signal pathway to trigger fruiting body formation and sporulation (Figure 1.2B). C-Signal is surface-bound and produced by the *csgA* gene product (Kim and Kaiser, 1990). This signal is sensed by surface receptors on cells that come in direct contact with the signal-presenting cell. At lower densities, A-Signal and C-Signal produce cell rippling and aggregation. At threshold densities, C-Signal leads cells in the fruiting bodies to differentiate into myxospores (Kim and Kaiser 1991). Myxospores differ from endospores in other spore-forming organisms, including *Bacillus subtilis*, in that the whole cell becomes the spore.

1.2.3 Competence development in *Streptococcus pneumoniae*

As early as the 1930s, Gram-positive organisms were also observed to exhibit group behavior (Dawson and Sia, 1931; Tomasz and Hotchkiss, 1964). *Streptococcus pneumoniae* coordinates the development of genetic competence with population density (Tomasz, 1966). Gram-positive organisms like *S. pneumoniae* tend to use peptides as signaling molecules. The signaling molecule utilized in the competence pathway is Competence Signaling Peptide (CSP) (Figure 1.3). CSP is processed from the longer ComC peptide (Havarstein, *et al.*, 1995). This processing is carried out by the ComAB ABC transporter, which then transports CSP into the extracellular environment (Claverys and Havarstein, 2002). At threshold densities, CSP binds its receptor kinase, ComD, causing the receptor to dimerize (Figure 1.3). The ComD dimer autophosphorylates. The response regulator, ComE, is activated when phosphorylated by ComD. Active ComE initiates transcription of both *comAB*, and *comCDE*, resulting in a positive feedback loop for competence activation (Pestova, *et al.*, 1996). Additionally, ComE~P activates transcription of *comX*, the alternative sigma factor for transcription of late competence genes (Lee and Morrison, 1999, Figure 1.3).

1.3 Cell-cell signaling in bacteria

1.3.1 Strategies for cell-cell signaling

In both restaurants and the microscopic world, communication needs several components to be successful. First, communication partners must have a common language. One may easily imagine potential frustrations that arise from ordering food while traveling abroad. Second, the partners must be able to communicate using the same medium. A toddler can

speak the same language as his or her parents, but cannot yet understand words in that same language that are written down, rather than spoken. Finally, for communication to be effective, it needs to happen at the right time. We all know how jarring it can be to have a friend chime in with a comment that the conversation passed by even a few minutes ago.

Bacteria have many of the same requirements for communication. While there exists some interspecies communication in the form of the “universal” autoinducer, AI-2, bacterial species tend to stick to their own language (Bassler and Losick, 2006). Gram-negative organisms typically communicate using acyl-homoserine lactone (AHL) molecules (Fuqua and Greenberg, 1998). One example of this type of communication was given above with *V. fischeri* and its LuxI-LuxR system. These AHLs provide species specificity in communication via unique R-groups. Gram-positive organisms do not use AHLs for interspecies communication. Instead, these bacteria, like *S. pneumoniae*, are most often found to communicate using short peptide pheromones. Bacteria, like humans, use different methods of communication. In the above example of *M. xanthus*, we see one potential method of using a direct hand-off mechanism, requiring cells to be in direct contact to pass information between cells (Blango and Mulvey, 2009). A more common mode of passing information is to use diffusible signals, such as 3-oxo-C6-homoserine lactone and CSP. Another example of a diffusible signal is the Phr peptide, PhrC, which is used to regulate genetic competence in *B. subtilis* (Lazazzera, 2000).

Often, these signaling molecules are utilized by bacteria to coordinate biological responses with population density. AHLs, peptides, and other diffusible signaling molecules are produced as cells grow and divide. As population densities increase, so does the concentration of signaling molecules (Figure 1.4). At threshold concentrations of signaling molecules, a response is triggered. This response is often a shift in which "program" the cell is running. This process of determining cell fate by population density is referred to as "diffusion monitoring" or "quorum sensing." Quorum sensing may also coordinate a response inversely with population density (Figure 1.4). An example of such a quorum sensing circuit is the EsaI-EsaR system of *Pantoea stewartii*, a Gram-negative plant pathogen. *P. stewartii* uses this system to produce and sense an AHL molecule. At low cell densities, extracellular polysaccharide (EPS) production is repressed. At higher cell densities, and thus higher concentrations of AHL this repression is relieved. It is believed that this system prevents early matrix production, which may interfere with the action of other *P. stewartii* virulence factors (von Bodman, Majerczak and Coplin, 1998).

1.3.2 Universal signaling molecules

Bacteria use diffusible signals not only for intraspecies communication, but also to communicate among various species. The molecule responsible for this interspecies signaling capability is called autoinducer 2 (AI-2) (Figure 1.5D). AI-2 was discovered as an additional signaling molecule capable of inducing bioluminescence in *Vibrio harveyi* (Bassler, *et al.*, 1994). AI-2 is produced by the enzyme LuxS (Surette, *et al.*, 1999). LuxS plays an important role in regular cellular metabolism as part of the activated methyl

cycle (AMC), producing homocysteine from S-ribosylcysteine (SRH). SRH is produced from S-adenosylmethionine (SAM) elsewhere in AMC metabolism. SAM is the major methyl donor in bacterial cells, and is also a precursor for other quorum sensing signaling molecules, AHL and CAI-1 (Miller and Duerre, 1968).

AI-2 has been shown to regulate a variety of pathways across both Gram-negative and Gram-positive organisms. These organisms may be grouped by the receptor into the LuxP family and the LsrB family (Pereira, *et al.*, 2013). LuxP-type AI-2 receptors are found primarily in *Vibrio* species. At high cell density, AI-2 binds the LuxP on the cell surface, which promotes phosphatase activity on LuxU~P (Chen, *et al.*, 2002). When LuxU is dephosphorylated, the non-coding RNA, Qrr1-5, is not transcribed, relieving inhibition of LuxR and promoting the expression of genes in quorum sensing pathways (Pereira, *et al.*, 2013). Organisms possessing the LsrB-type system respond in a different manner – by internalizing AI-2. Bacteria with the LsrB system are more diverse than those containing the LuxP system, and include Gram-negative enteric organisms such as *E. coli* and *Salmonella* spp, but also *Rhizobiaceae* and Gram-positive species among the *Bacillaceae* and *Streptococcus spp.* At high densities, AI-2 binds the periplasmic binding protein, LsrB (Miller, *et al.*, 2004). Next, AI-2 is imported via the LsrC/D/A channel, where it is phosphorylated by LsrK. Phospho-AI-2 blocks the activity of the repressor LsrR, creating a positive feedback loop for a quorum response. This system is implicated in the regulation of several biological pathways. In *E. coli* K12, AI-2 signaling regulates biofilm formation and motility. Pathogenesis in enteropathogenic *E. coli* may be regulated with

this system. AI-2 signaling has also been observed in regulating biofilm formation in Gram-positive organisms, such as *Bacillus cereus* and *S. pneumoniae* (Auger, *et al.*, 2006). Additionally, this universal signal is involved in the regulation of antibiotic resistance and haemolytic activity in *Streptococcus intermedius*.

1.3.3 Signaling molecules of Gram-negative organisms

Gram-negative organisms regulate a diverse set of pathways by quorum sensing mechanisms. One example is the above-mentioned bioluminescence in *Vibrio* species. Other examples include virulence factor production in *P. aeruginosa*, conjugation in the plant pathogen *A. tumefaciens*, and antibiotic production in *Erwinia curatovora* (Fuqua and Greenberg, 1998). Gram-negative organisms sense population density by means of diffusible signaling molecules. These molecules are acyl-homoserine lactone derivatives (AHLs) (Figure 1.5A). AHLs are produced by I-proteins which catalyze the lactonization of S-adenosyl-L-methionine (SAM) and the transfer of a specific acyl R-group from an acylated acyl carrier protein (acyl-ACP) (Watson, *et al.*, 2002). The products of this reaction are the species-specific AHL, 5'-methyl-thioadenosine and holo-ACP. Many of these I-proteins are homologs of *Vibrio* LuxI. At threshold densities, AHLs are sensed by binding to R-proteins, many of which are in the same family as *Vibrio* LuxR (Waters and Bassler, 2005). LuxR-type proteins are transcription factors which consist of an AHL-binding domain and a DNA-binding domain (Fuqua and Graeenberg, 1998). Upon activation by binding to AHL, LuxR-type proteins will initiate a new gene transcription profile, triggering a physiological response in the population. Commonly, these

transcription profiles will include a positive feedback loop. One example of such a loop is in the LuxI-LuxR system, where the *luxI* gene is under the control of LuxR.

1.3.4 Signaling molecules of Gram-positive organisms

Gram-positive organisms rely on short peptide pheromones to receive information about population density (Kleerebezem, *et al.*, 1997). These pheromones may be found as unmodified linear peptides, modified linear peptides, or even as ring structures (Waters and Bassler, 2005, Figure 1.5B). Linear peptide signaling molecules are typically produced in a long pro-peptide form, and can be processed either intra- or extra-cellularly into their shorter, active form. Examples of this processing include *S. pneumoniae* CSP, mentioned above, and Phr peptides, which are inhibitors of Rap anti-activator proteins in *B. subtilis*.

Pheromones may also be modified in their active form, including ComX, the signaling molecule of the ComP-ComA pathway from *B. subtilis* (Lopez and Kolter, 2010). ComX is prentylated on a tryptophan residue, which may help to increase its concentration at the cell surface (Lazazzera and Grossman, 1998). Peptide rings are used by *Staphylococcus aureus* as quorum sensing molecules (Figure 1.5B). *S. aureus* utilizes AIP to sense population density as part of the Agr system, which allows the bacteria to swich from a cellular program for attachment and colonization at low cell density to one for toxin and protease production at high cell density. As discussed in more detail below, *S. aureus* additionally uses AIP to differentiate among 4 different groups, based on AIP-type (Lyon,

et al., 2002). Additionally, *Streptomyces* soil bacteria use gamma-butyrolactones as signaling molecules, instead of peptides as with other Gram-positive species, to coordinate morphology and secondary metabolite production (Waters and Bassler, 2005).

1.4 Quorum quenching

As we can see, quorum sensing is quite important to the exquisite control of bacterial population physiology. This makes quorum sensing pathways likely targets for disrupting the growth and survival of these populations. Evolution has hit upon this strategy, as well. This disruption is referred to as quorum quenching. Quorum quenching has evolved to assist prokaryotes in their struggle for resources, and in eukaryotes to prevent colonization and disease. Among Gram-negative organisms, *V. paradoxus* has been found to open the lactone ring of other species' AHLs (Leadbetter and Greenberg, 2000). The bacteria can then use these AHLs as sources of carbon and nitrogen. Mechanisms of inactivating AHLs include opening the lactone ring by lactonases, cleaving the fatty acid chain from the lactone ring by acylases, and modifying the fatty acid chain by oxidoreductases (Figure 1.6A) *E. coli* and other enterics are able to disrupt the function of AI-2. These bacteria internalize the universal signaling molecule, which returns the extracellular environment to a low density of signal.

Gram-positive organisms have also been found to participate in quorum quenching. The opportunistic pathogen, *S.aureus* exists in four different groups. Each of these groups produces its own unique autoinducing peptides, which competitively inhibit the pathways

in other groups (Lyon, *et al.*, 2001, Figure 1.6B). Additionally, *B. subtilis* produces a protein called AiiA, which inactivates AHL signals from Gram-negative species by cleaving the signals' lactone rings (Dong, *et al*, 2001).

1.5 Two-component systems

Bacteria sense a wide range of signals and stimuli in their environment, including secondary metabolites like cyclic-AMP and ions, such as Fe^{2+} (Bijlsma and Groisman, 2003). Two-component systems (TCS) allow bacteria to sense further conditions, such as nutrient availability, redox state, osmolarity, antibiotics, and quorum sensing molecules (Laub and Goulian, 2007). TCS are nearly ubiquitous in bacterial genomes. Some bacteria even encode for roughly 200 such systems. A generic TCS contains a histidine kinase (HK) and response regulator (RR) (Figure 1.7). When a HK recognizes a particular signal, the catalytic domain utilizes ATP to autophosphorylate a critical histidine residue on the dimerization and histidine phosphotransferase domain (Dhp). The phosphoryl group is later transferred to a conserved aspartate residue in the receiver domain of a RR. This action typically results in a conformational change in the output domain. Usually, this change causes altered DNA binding affinity of the RR (Stock, *et al.*, 2000). While highly conserved residues in HKs and RRs bring catalytic residues of both proteins into close proximity, specificity is maintained through hypervariable regions surrounding the critical aspartate of the RR (Tzeng and Hoch, 1997). Despite the preference for specificity, there is evidence for both cross-talk between TCS pathways, and for phosphorylation by small molecule phosphodonors. One example of cross-talk

exists in the PhoB/PhoR system, which senses phosphate conditions in *E. coli*. In strains mutant for the sensor PhoR, PhoB-activated genes may be induced by both glucose and pyruvate, which trigger CreC. Additionally, PhoB has been shown to be activated by acetyl-phosphate (Wanner, 1993).

1.6 Quorum sensing systems in *Bacillus subtilis*

The Gram-positive soil bacterium, *B. subtilis*, utilizes several different quorum sensing systems to regulate important biological pathways. Genetic competence, degradative enzyme production, sporulation, and motility have all been shown to be regulated by population density (Figure 1.8). The review here will focus mostly on the activation and anti-activation of sporulation and genetic competence.

1.6.1 Sporulation phosphorelay

Sporulation is a key response to stresses, including starvation. The decision to undergo sporulation in *B. subtilis* begins with the integration of unknown signals by five different membrane-bound (KinA, KinB) and cytoplasmic (KinC, KinD, KinE) kinases (Figure 1.9). These five kinases all phospohorylate Spo0F. Spo0F~P initiates a phosphorelay system that results in the transfer of the phosphoryl group to Spo0A. Active Spo0A~P is the master transcritpional regulator for early sporulation. Spo0A~P binds a “Spo0A-box” in the promoter region of 121 target genes (Piggot and Hilbert, 2004). High levels of Spo0A~P result in the activation of early sporulation genes. Low levels of Spo0A~P result the stimulation of cannibalism, by the activation of *skf* and *sdp*, and biofilm

formation by activating the transcription of *sinI* and by repressing *abrB*. Rap proteins, including RapA, inhibit sporulation (Figure 1.9). These Rap proteins tend to be Spo0F~P phosphatases. Rap 60 inhibits KinA autophosphorylation, in addition to dephosphorylating Spo0F~P. Inhibition of sporulation by Rap proteins can be inhibited by quorum sensing of cognate Phr peptides.

1.6.2 Genetic competence

Genetic competence is initiated in *B. subtilis* by the ComP-ComA two-component system. The histidine kinase, ComP, senses the peptide signaling molecule ComX (Figure 1.10). ComX is an 11-amino acid pheromone that is modified by an isoprenyl group. Upon sensing ComX, ComP undergoes autophosphorylation. The phosphoryl group is passed to a critical aspartic acid residue on ComA, the master transcriptional regulator for the early genetic competence pathway. ComA~P binds a tripartite binding site upstream of the promoter regions of ComA-regulated genes, including those involved in genetic competence, and the production of degradative enzymes and antibiotics (Griffith and Grossman, 2008). Early genetic competence is inhibited by Rap proteins, such as RapC and RapF. As with sporulation, Rap activity is inhibited via quorum sensing of cognate Phr peptides (Figure 1.10).

While some biological programs controlled by quorum sensing mechanisms yield uniform results throughout a population, as with *V. fischeri* bioluminescence, other programs only get switched on in a fraction of cells. One example of this is the

development of genetic competence, or the ability to take up foreign DNA molecules for horizontal gene transfer events. This may or may not be a successful strategy for survival in any given situation, so only a small subpopulation of cells differentiate to acquire this capability. While some of these bacteria that have achieved a state of competence may integrate non-beneficial, or even deleterious, genes, those that take up genes that, say, allow for increased survival on a different type of nutrient, may proliferate and fill a new niche.

Late genetic competence is under the control of a second master transcriptional regulator, ComK. ComK is responsible for the transcription of genes involved in the translocation of DNA across the bacterial membrane. ComK is in turn regulated by at least five other transcription factors (CodY, AbrB, Rok, DegU, and Spo0A) and proteolytic control by ClpCP (Figure 1.11, Hamoen, *et al*., 2003). ComK may also bind its own promoter, creating a positive feedback loop on activation. This combined regulation results in a bimodal expression of competence, with only a small subset of the population achieving genetic competence (Lopez, *et al*., 2009).

1.6.3 Rap Proteins

Rap proteins are anti-activators that regulate various quorum sensing pathways in *B. subtilis* (Figure 1.12). These proteins were initially discovered inhibiting sporulation as Spo0A phosphatases disrupting the flux in the sporulation phosphorelay. Later, Rap proteins were found to regulate a number of other processes, including genetic

competence, degradative enzyme production, and mobilization of integrative conjugative elements (ICEs). Rap proteins themselves are subject to regulation by quorum sensing. Peptide pheromone signals referred to as Phrs bind Rap proteins. This event inactivates the Rap protein, and relieves Rap inhibition of biological processes. The *B. subtilis* genome contains 11 of these *rap-phr* quorum sensing pairs. Some endogenous Raps regulate genetic competence (Raps C, F, H, and K). Other Raps (Rap A, B, E, H, and J) regulate sporulation. RapG has been shown to regulate degradative enzyme production, and RapI regulates the Integrative Conjugative Element (ICE*Bs1*). In addition to endogenous *rap-phr* pairs, genes for other Rap proteins have been found on mobile genetic elements, such as plasmids and phages.

1.7 Thesis overview

In Chapter 1, I provided a general overview of the the quorum sensing mode of bacterial regulation. First, I relayed major early observations important to the discovery of quorum sensing. Next, I reviewed cell-cell signaling in bacteria, including different mechanisms and molecules used for this purpose, and two-component systems. Finally, I discussed quorum sensing in *Bacillus subtilis*, from sporulation to genetic competence to regulation by Rap anti-activator proteins.

In Chapter 2, I take genetic and biochemical approaches to attempt to elucidate a mechanism of ComA activation. A genetic screen isolated various constitutive mutants of ComA, which I characterized both *in vivo* and *in vitro*. I was able to classify these

mutants based on regulation by the kinase ComP and the anti-activator RapC. Biochemically, I found some of these mutants to be responsive to phosphorylation by the small molecule phosphodonor acetyl-phosphate.

In Chapter 3, I work to identify novel functions of Rap proteins and to characterize the plasmid-borne Rap60 by making point mutations which where tested *in vivo* and *in vitro*. I was able to uncover new regulation by several Rap proteins. I was also able to show that Rap60 likely utilizes a different binding surface for ComA than canonical competence-regulating Rap-proteins. This coincides with electromobility shift assay data that suggests a unique mechanism for Rap regulation of transcriptional activators.

1.8 Figures

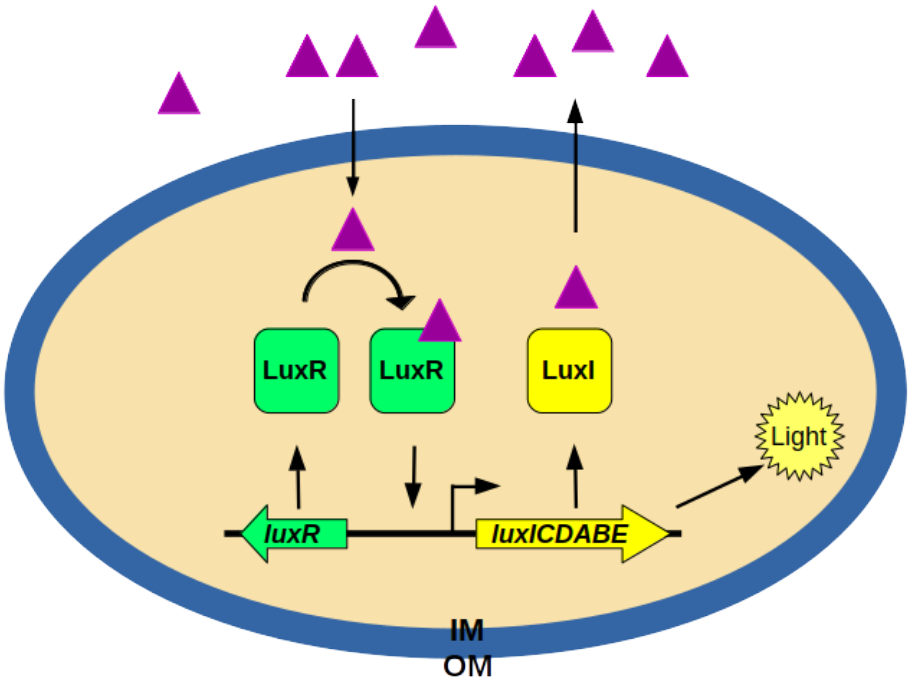

Figure 1.1. *Vibrio fischeri* LuxI-LuxR Quorum Sensing System. The AHL autoinducer (purple triangles) is produced by the LuxI autoinducer synthase. Autoinducer diffuses through the bacterial membrane and binds to the cytoplasmic autoinducer receptor and transcription factor, LuxR. The LuxR-AHL complex drives transcription of the *luxICDABE* operon, which results in the production of luciferase. IM – inner membrane, OM – outer membrane. (Adapted from Waters and Bassler, 2005)

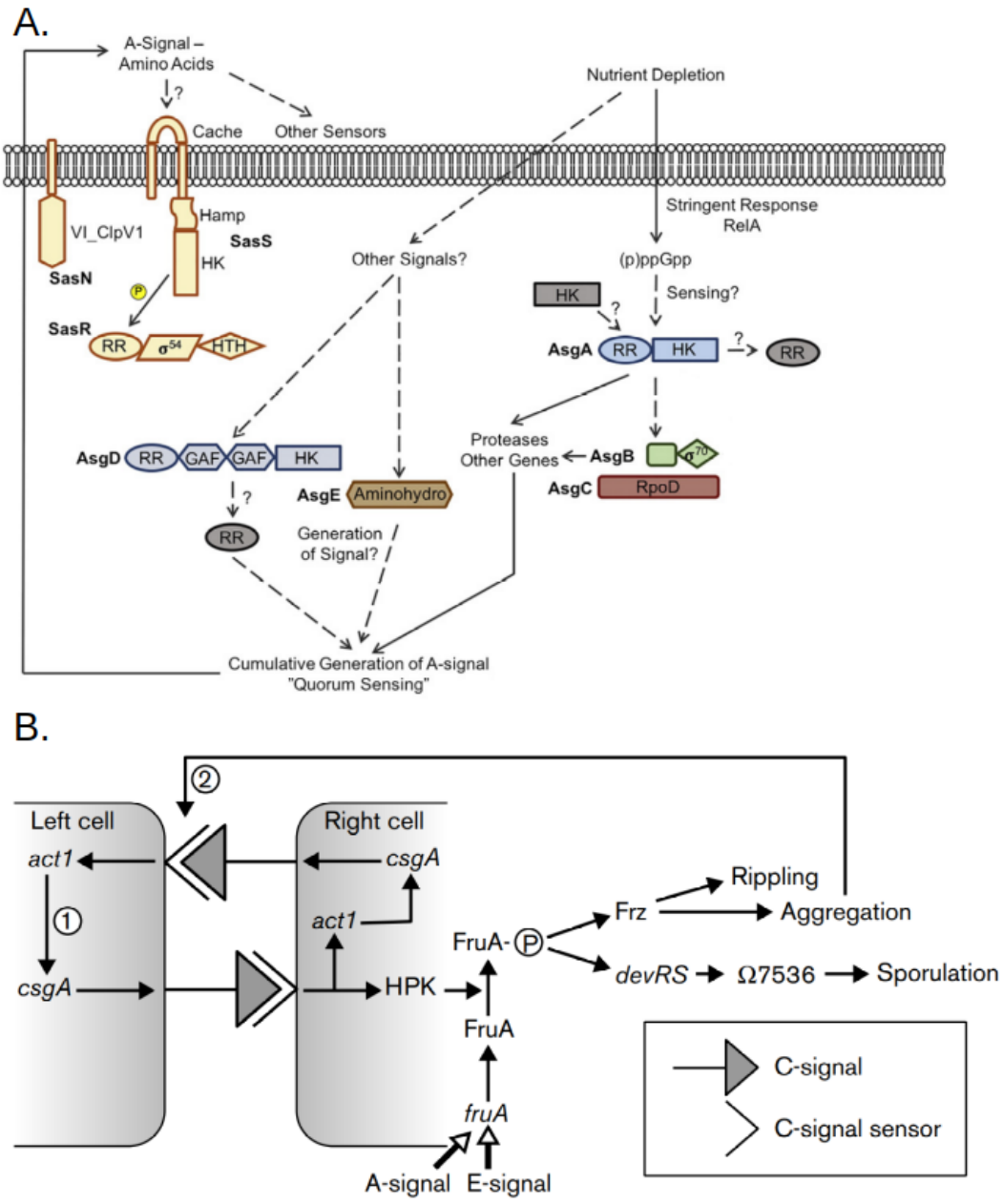

Figure 1.2. Fruiting body and sporulation signaling in *Myxococcus xanthus*. The A-Signal pathway (A) is triggered by the accumulation of the alamrone (p)ppGpp due to the stringent response to nutrient deprivation.. A-Signal is produced via the AsgABCDE pathway. The signal is sensed by SasS-SasR. The C-Signal pathway (Adapted from Bretl and Kirby, 2016). (B) leads to aggregation, and later sporulation, by a direct hand-off mechanism mediated by the C-Signal surface ligand and receptor. (Adapted from Jelsbak and Sogaard-Andersen, 2000).

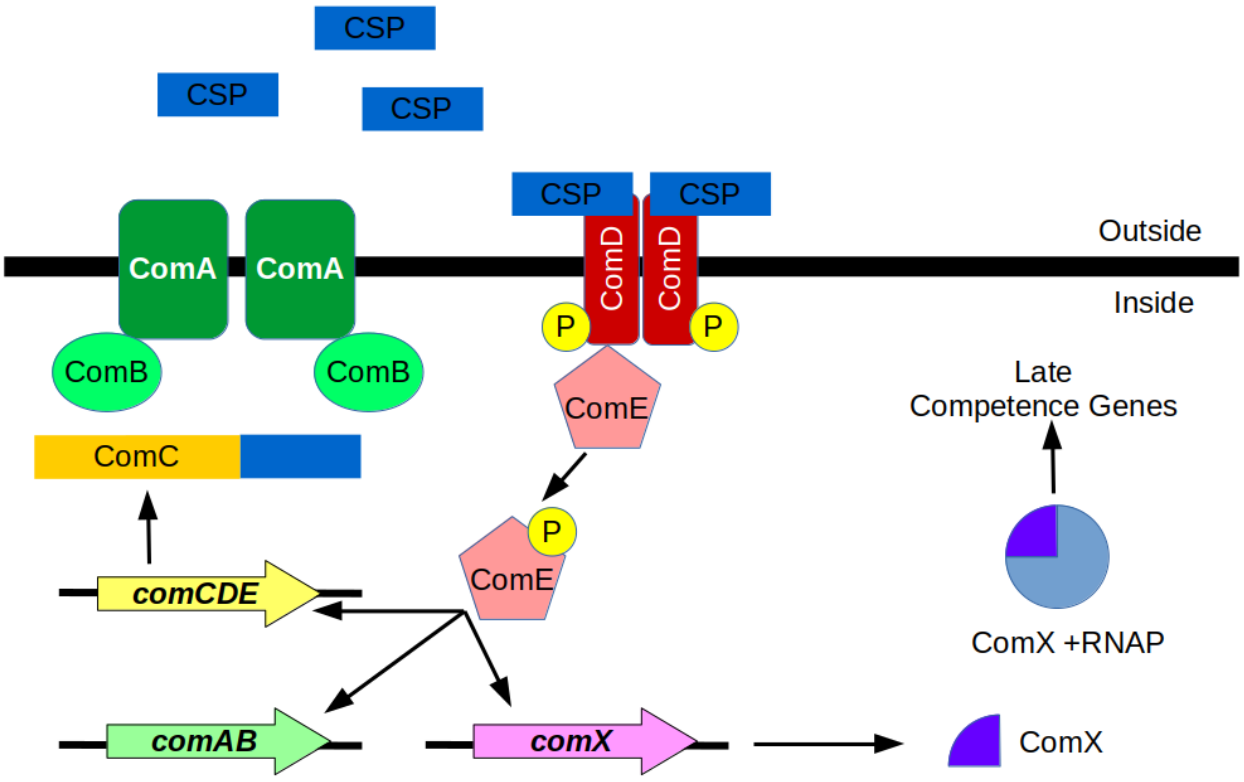

Figure 1.3. Competence development in *Streptococcus pneumoniae*. Competence Signaling Peptide (CSP) is cleaved from the longer ComC pro-peptide by the ComAB ABC transporter, which secretes CSP in to the extracellular environment. CSP is sensed by ComD, which activates the response regulator ComE. ComE~P drives the transcription of early competence genes and the competence-specific sigma factor, ComX. This process creates a positive feedback loop for competence development. (Adapted from Shanker and Federle, 2017).

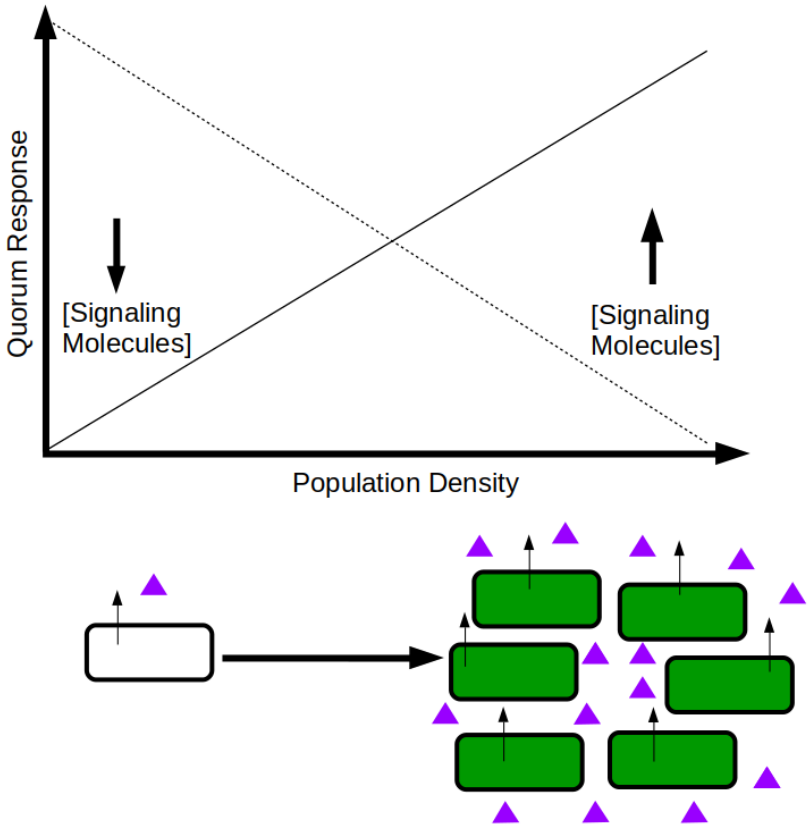

Figure 1.4. Coordination of quorum response with population density. Concentration of signaling molecules increases with population density. A biological response is triggered at a threshold concentration of signal (solid line). Responses may also be inversely coordinated with density (dashed line).

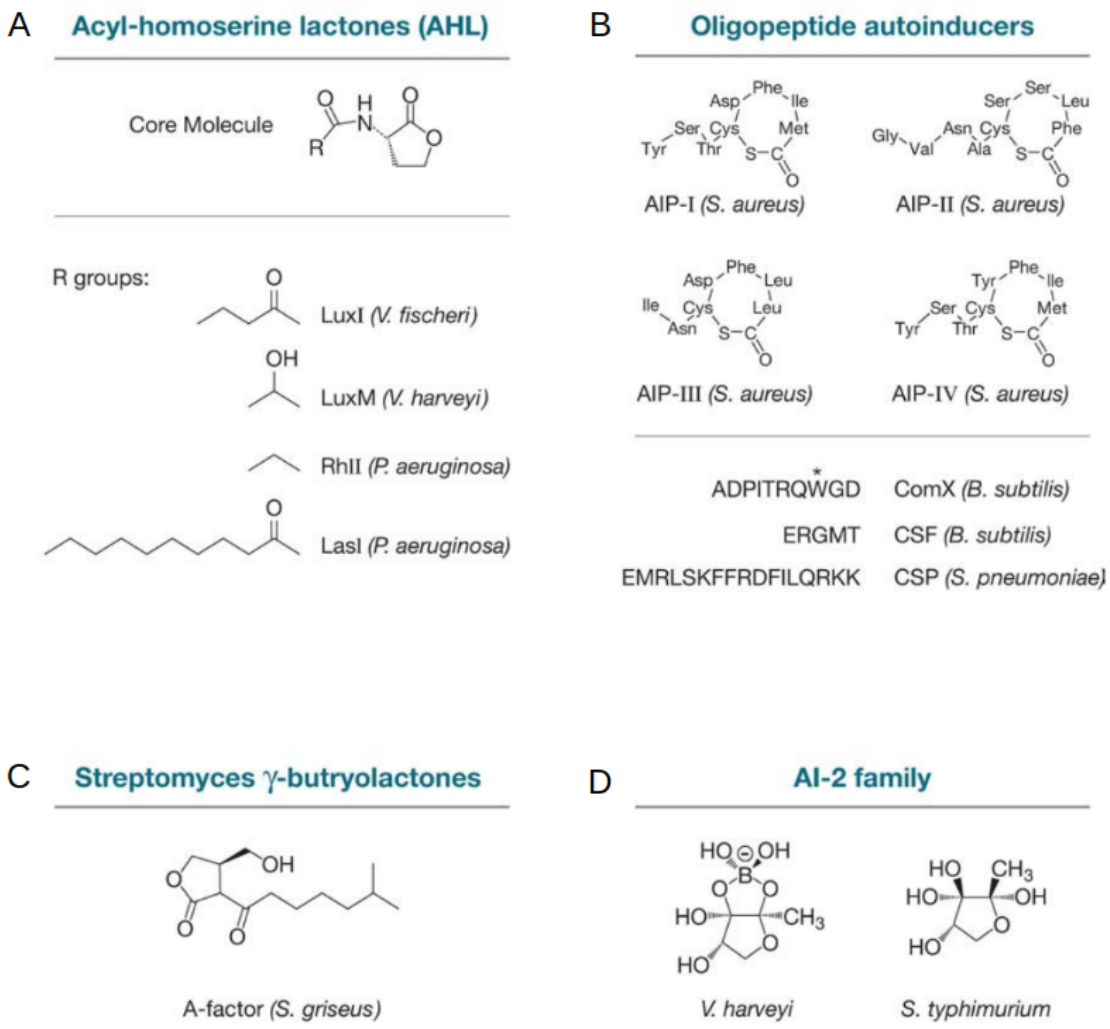

Figure 1.5. Bacterial signaling molecules. A. Core acyl-homoserine lactone molecule and R-groups from selected Gram-negative species. B. Ring, linear, and modified peptide signals from Gram-positive species. C. *Streptomyces* gamma-butryolactone signaling molcules. D. AI-2 universal signaling molecules (Bassler and Waters, 2005).

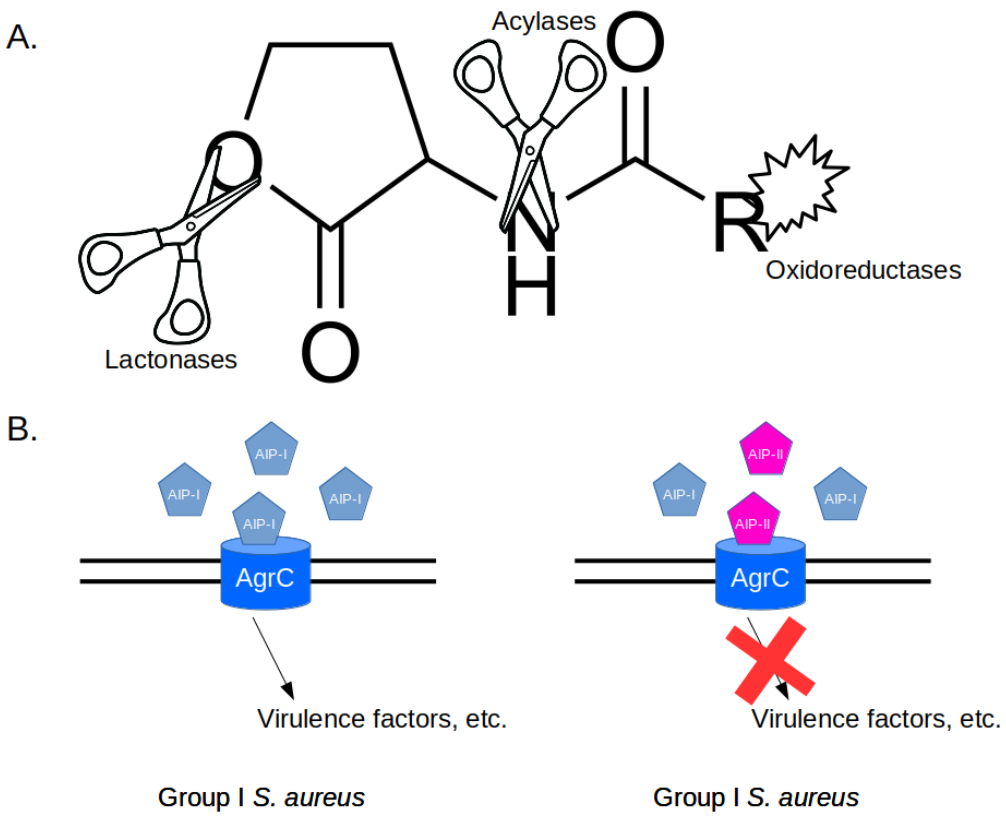

Figure 1.6. Examples of quorum quenching mechanisms. A. AHL molecules are inactivated by at least three mechanisms (left to right): Lactonases open AHL lactone rings. Acylases cleave the acyl chain from the lactone ring by hydrolyzing the amide bond. Oxidoreductases modify the acyl chain. B. *S. aureus* auto-inducing peptides (AIPs) of different groups competitively inhibit quorum sensing in other groups.

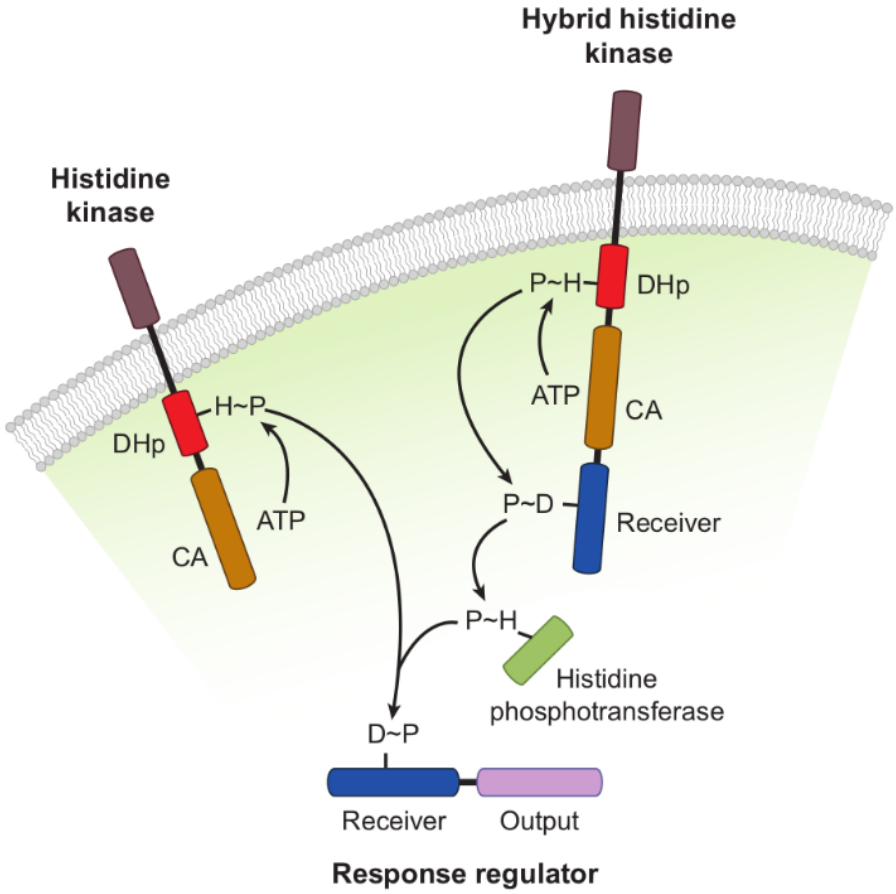

Figure 1.7. Schematic of two-component system function. Histidine kinases sense conditions on the cell surface. When a signal is perceived, the histidine kinase becomes autophosphorylated on a critical histidine residue of the dimerization and phosphotransferase domain (Dhp). This phosphate group is transferred to a conserved aspartic acid of a response regulator's receiver domain. Hybrid histidine kinases contain their own receiver domains, which participate in a phosphotransfer relay system before ultimately phosphorylating a response regulator. (Laub and Goulian, 2007).

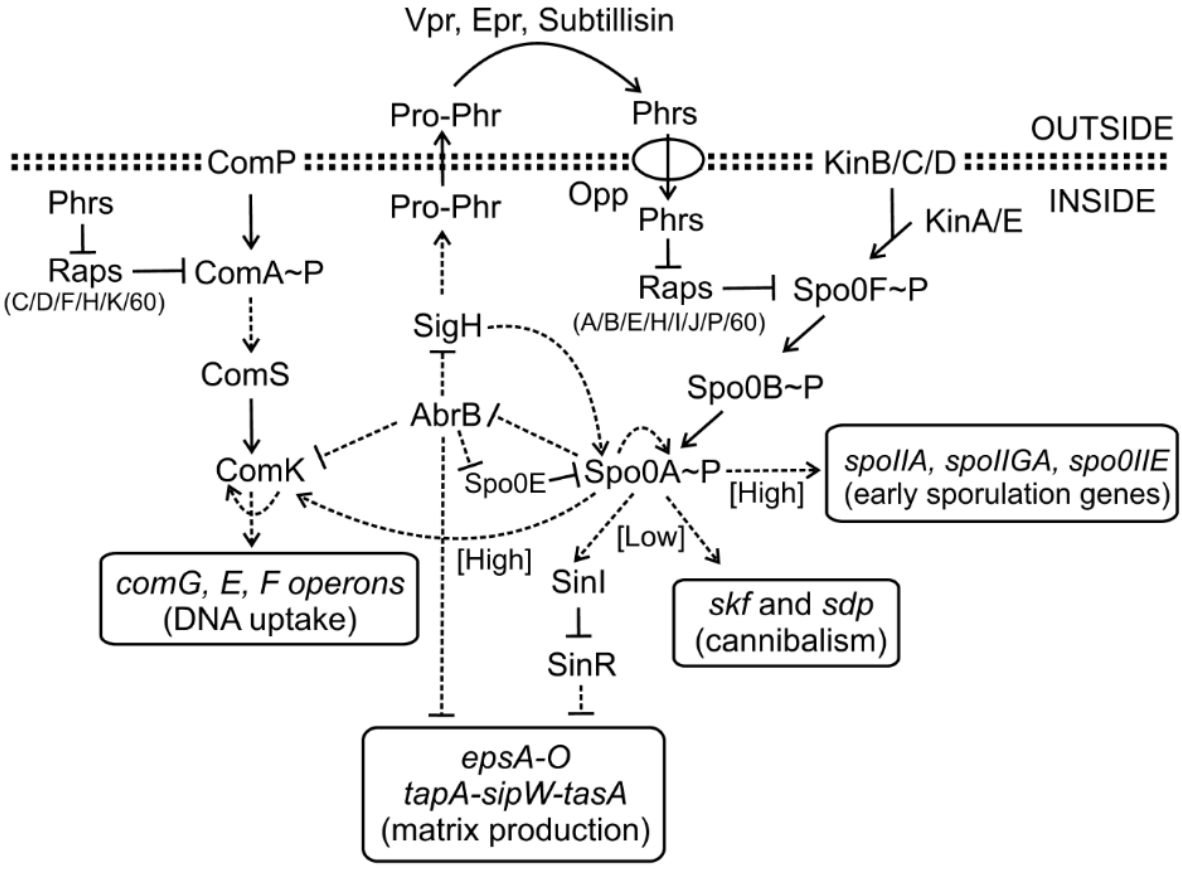

Figure 1.8. Overview of quorum sensing pathways in *B. subtilis*. Environmental and peptide signals are sensed by kinases, including ComP and KinA/B/C/D/E. These signals are transferred via phosphorylation to response regulators ComA and Spo0A. Genetic competence development is downstream of ComA, while sporulation, cannibalism, and matrix production are linked to varying levels of Spo0A~P. These processes are sensitive to anti-activation by Rap proteins. Rap proteins are regulated in a density dependent manner by Phr peptides. Phr peptides are secreted in a pro-peptide form, processed outside the cell by Vpr, Epr, and Subtilisin, and are transported into the cell by Opp. Arrows represent positive regulation; perpendicular lines represent negative regulation. Solid lines show protein-protein interaction; dashed lines show transcriptional control. (Boguslawski, Hill and Griffith 2015).

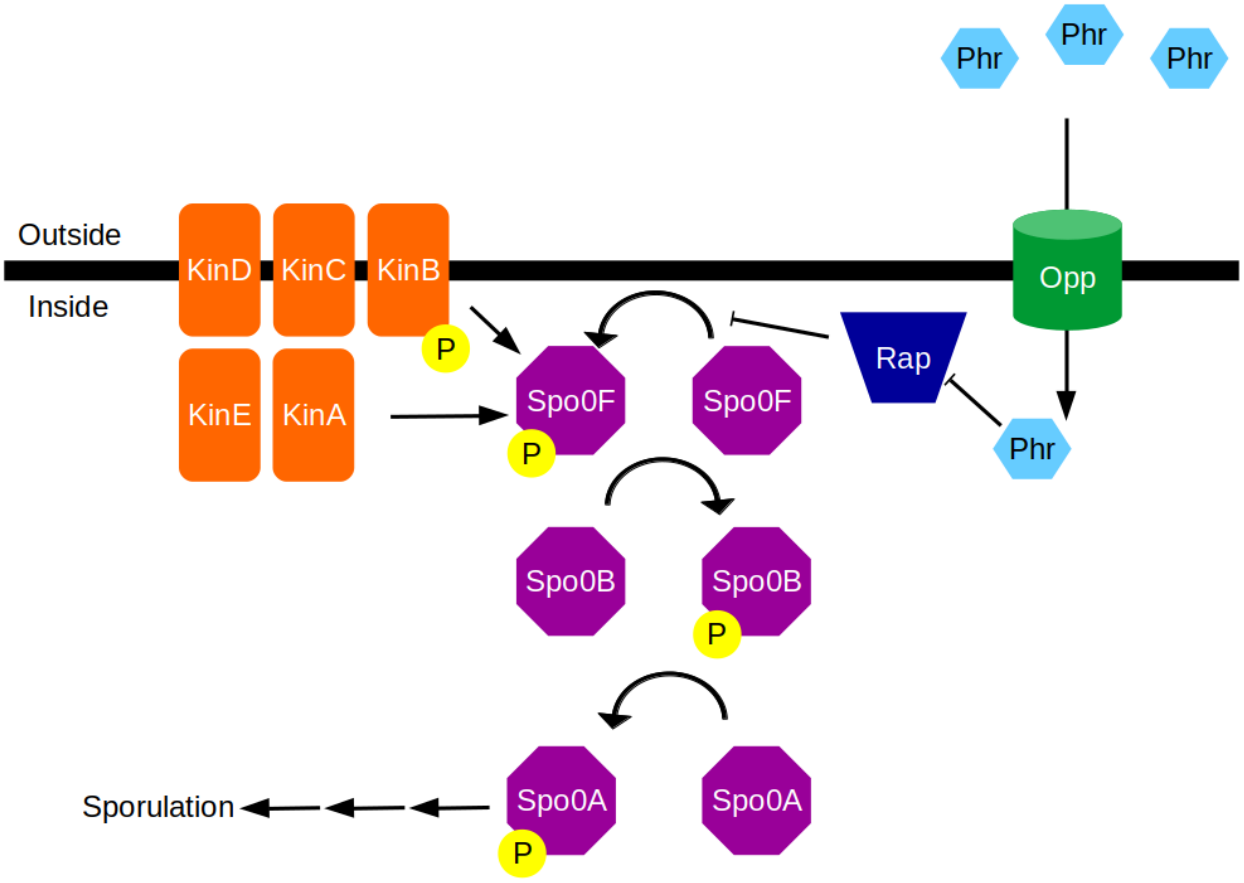

Figure 1.9. Regulation of early sporulation in *B. subtilis*. Sporulation is initiated through the sensing of undetermined environmental signals by membrane-bound (KinB/C/D) and cytosolic kinases (KinA/E). These kinases transfer phosphate groups to Spo0F, which initiates a phosphotransfer relay. Spo0A~P is the master transcriptional activator for early sporulation. Early sporulation is subject to anti-activation by various Rap proteins, which are Spo0F~P phosphatases. Rap proteins are inhibited by Phr peptide signaling molecules, which are imported into the cell via Opp.

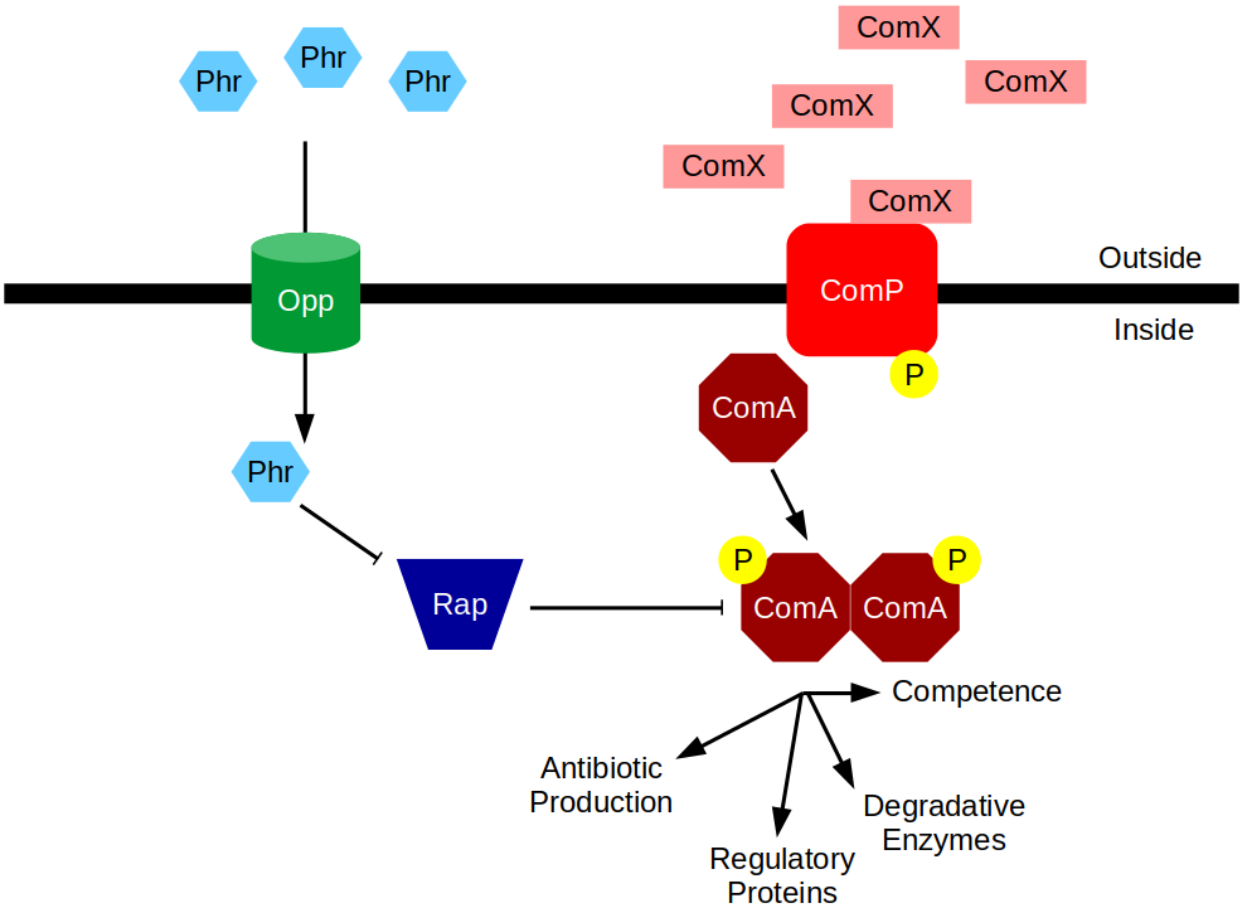

Figure 1.10. Regulation of early genetic competence in *B. subtilis*. Population density is sensed by the histidine kinase, ComP, by monitoring the concentration of the ComX signaling peptide. ComP undergoes autophosphorylation after sensing ComX. ComA, the master transcriptional activator for early genetic competence, is phosphorylated by ComP. ComA~P initiates genetic competence, and the production of antibiotics, degradative enzymes, and other regulatory proteins, including ComS. ComA~P is sterically hindered from binding DNA by Rap anti-activator proteins. Rap proteins are regulated via quorum sensing by means of Phr peptides, which bind Rap proteins, inhibiting their activity.

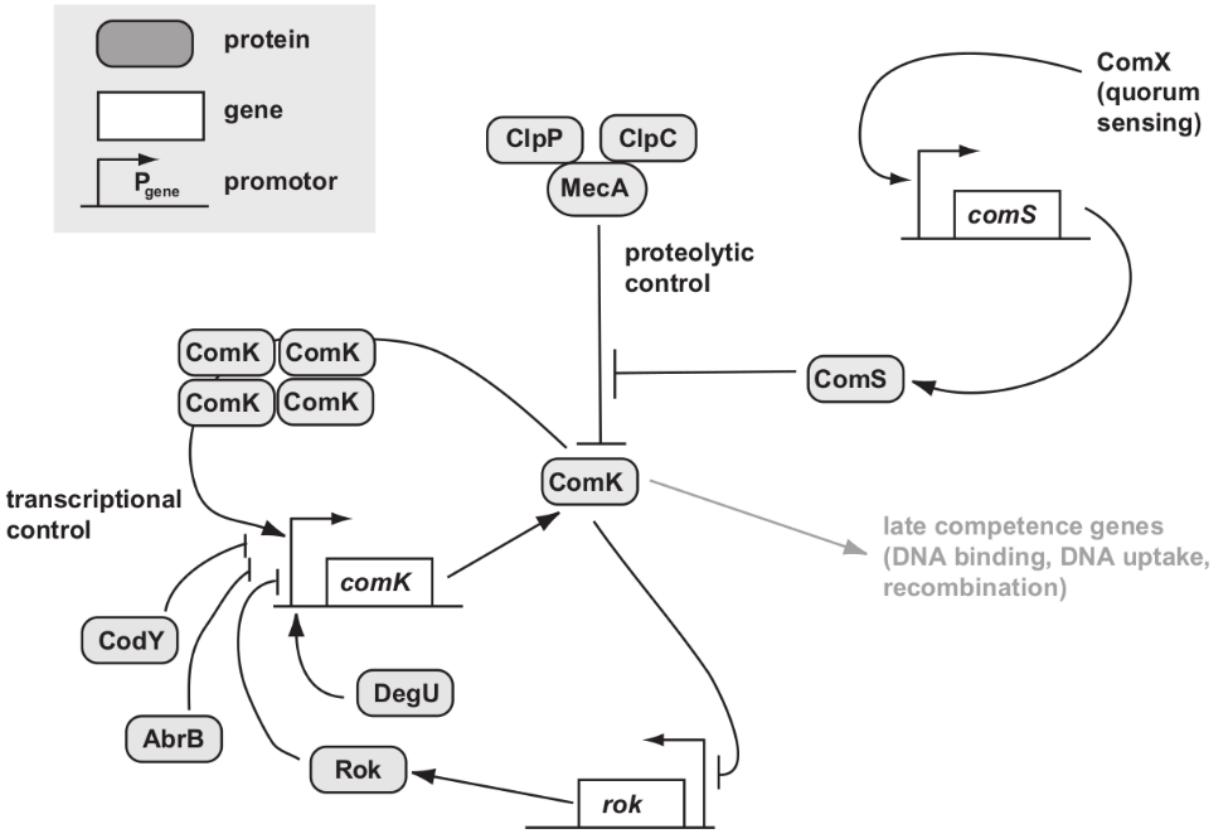

Figure 1.11. Regulation on ComK in *B. subtilis*. ComK activity is regulated by at least five different transcription factors (CodY, AbrB, Rok, DegU, and ComK itself). Late genetic competence is also regulated by proteolysis via ClpCP. ComS, which is under the control of the early genetic competence quorum sensing pathway, blocks proteolysis of ComK. (Leisner, *et al.*, 2007)

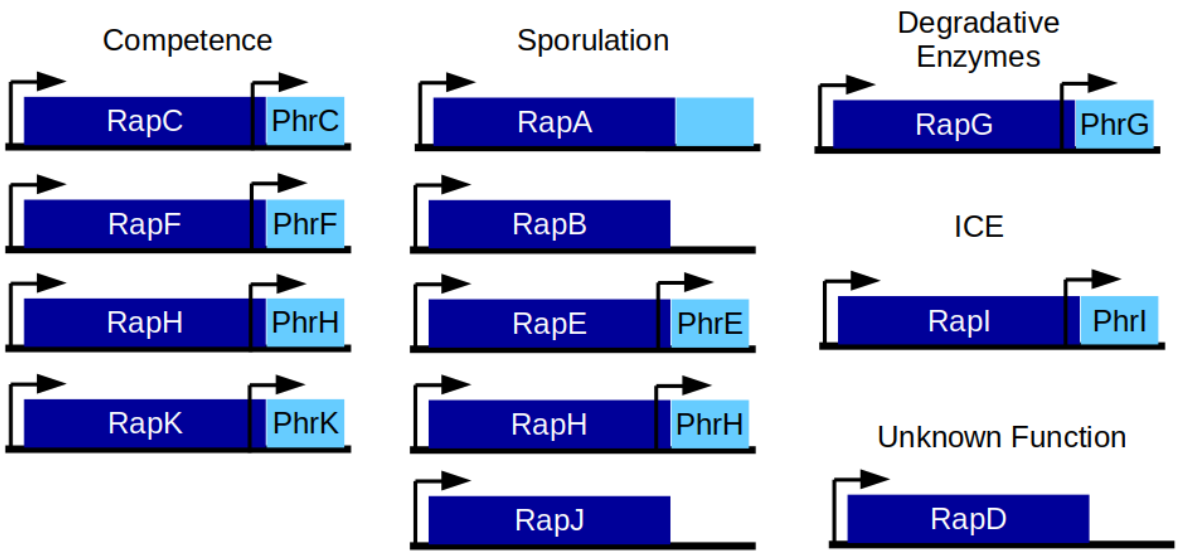

Figure 1.12. Genetic arrangement of genomic *rap-phr* gene pairs in *B. subtilis*. Genomic Rap proteins have been shown to regulate genetic competence, sporulation, degradative enzyme production, ICE mobilization. Many *rap* genes are paired with the gene for a cognate Phr peptide, such as with RapC-PhrC. *Phr* genes may or may not have a promoter internal to the upstream *rap* gene. Other Rap proteins, such as RapB, lack downstream *phr* gene.

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CHAPTER 2

ISOLATION AND CHARACTERIZATION OF CONSTITUTIVE MUTANTS OF COMA

2.1 Introduction

In *Bacillus subtilis*, genetic competence is under the control of multiple quorum sensing systems (Lopez, Vlamakis, and Kolter, 2009). Early genetic competence is initiated by the ComX-ComP-ComA pathway (See Figure 1.9). ComX is an 11-amino acid peptide pheromone modified with an isoprenyl group (Okada *et al.*, 2005). This pheromone accumulates extracellularly and is sensed by ComP, a membrane-bound histidine kinase. After binding ComX, ComP becomes autophosphorylated, and transfers the phosphoryl group to the transcription factor ComA, the master regulator for early genetic competence genes (Nakano, Xia, and Zuber, 1991). The ComA regulon includes genes involved with genetic competence, production of degradative enzymes and antibiotics (Comella and Grossman, 2005). Late genetic competence is controlled by the transcription factor ComK that issubjected to complex regulation (See Figure 1.10). Specifically, at least five different transcription factors (AbrB, CodY, DegU, Rok, and ComK) serve to regulate the expression of ComK at the level of transcription (Hamoen, *et al.*, 2003). ComK is also regulated post-transcriptionally by proteolytic control by ClpCP/MecA (Turgay, *et al.*, 1998). ComK directly activates the transcription of late genetic competence genes involved in DNA binding, uptake, and recombination (Ogura, *et al.*, 2002). This complex regulation of ComK results in bimodal transcription activation of late genetic competence genes within a population, and a subpopulation of cells enters the competence state (Hamoen, *et al.*, 2003).

The early genetic competence, ComX-ComP-ComA pathway is directly linked to the late genetic competence ComK pathway via the regulatory protein ComS (D'Souza, Nakano, and Zuber, 1994). ComS is encoded by a small gene within the *srfA* operon, responsible for the production of surfactin (Nakano, *et al.,* 1991; D'Souza, Nakano, and Zuber, 1994). ComS relieves proteolytic control on ComK (Turgay, *et al.*, 1998). This relief is achieved when ComS binds to MecA, preventing the latter protein from targeting ComK for degradation by the protease, ClpCP (Turgay, *et al.,* 1998).

Previous work has shown that ComA exists in solution as a dimer (Griffith and Grossman, 2008). The ComA binding site is a degenerate tripartite site consisting of three recognition elements (RE). RE1 and RE2 form an inverted repeat separated by four nucleotides with consensus sequence TTGCGGnnnnCCGCAA (Figure 2.1, Griffith and Grossman, 2008). RE3 is found 6-8 or 17-18 nucleotides downstream of RE2 and shares the consensus sequence of RE1. Known ComA-dependent promoters lack a fourth recognizable recognition element, suggesting that the upstream ComA monomer may be available for interaction with RNA polymerase (Figure 2.1).

All three recognition elements and the degeneracy of the recognition element sequences are required for density-dependent transcription of early competence genes. At low cell density, when the concentration of extracellular cell-cell signaling molecules is low, ComA is largely inactive, resulting in low transcription activation of target genes (Figure

2.2). At high cell density, the concentration of signaling molecules is high, and ComA is active, resulting in a density-dependent activation of target genes (Figure 2.2).

2.2 Results

2.2.1 ComA-dependent transcription within a population of cells has a Gaussian distribution

Kuipers and Dubnau demonstrated that late-competence gene expression under control of ComK is bimodal, with only a small fraction of cells achieving competence (Berka, *et al.*, 2002, Figure 2.3C). As previously mentioned, this distribution of expression is achieved as the result of complex regulation of ComK involving at least five transcription factors, and by proteolytic degradation via ClpCP. Early competence gene expression under the control of ComA is coordinated with population density (Comella and Grossman, 2005). Transcriptional activation of *comS*, encoded in the *srfA* operon, is the first step in modulating the activity of ComK with population density. Thus, we wanted to know if early-competence gene expression is also bimodal, or if it follows a more Gaussian distribution.

To determine the distribution of ComA-dependent expression, we performed fluorescent microscopy on reporter strains at low and high cell density. We constructed P*comGA-mCherry* to monitor late-competence gene expression under the control of ComK, and P*srfA-CFP* to monitor ComA-dependent gene expression. At low-cell density (OD_{600} ~0.2), we found normalized intensity to range from 0.3 AU (arbitrary units) to 326.7 AU,

with a mean and median intensity of 94.9 AU and 86.1 AU, respectively (Figure 2.3A). At high-cell density (OD_{600} ~1.0), we found normalized intensity to range from 10.0 AU to 1012.5 AU, with a mean and median intensity of 222.6 AU and 212.8 AU, respectively (Figure 2.3B). An overlaid normal distribution with the same mean and standard deviation shows the distribution of gene expression at both low and high density to be Gaussian (Figure 2.3A and 2.3B). This suggests that the bimodality of ComK-dependent expression is not due to upstream bimodality of early-competence expression genes by ComA, but is due to the complex regulation of ComK (Hamoen, *et al.*, 2003).

2.2.2 ComA protein levels are constant throughout growth

The density-dependence of ComA-dependent gene expression may be due to modulation of protein levels throughout growth, or through activation by the cognate kinase, ComP, or a combination of the two. We wanted to see if ComA protein abundance played a role in the density-dependence and normal distribution of early competence gene expression. To do this, we performed Western blots using anti-ComA antibody on lysates of strain JH642 grown to different culture densities. We were able to detect ComA protein throughout the growth cycle (OD_{600} ~0.2-5.0) (Figure 2.4A). As a control, we included a *comA* deletion strain, for which we did not detect any ComA protein (Figure 2.4A). Using known amounts of purified $Nhis_6$-ComA as standards, we quantified the results of our Western blots. We found that the level of ComA remains relatively constant throughout the growth cycle at approximately 1,500 dimers of ComA protein per cell. Although there appears to be a slight increase in ComA with population density, there is no statistical

difference between the abundance of ComA throughout the growth cycle (Figure 2.4B). Taken together, this suggests that activation of ComA by its histidine kinase, ComP, and additonal regulation by Rap-Phr pairs are the factors responsible for the density dependent gene expression by ComA.

2.2.3 Plate screen to isolate ComA mutants that bypass activation by ComP

In order to further investigate the role of ComP in ComA activation, we sought to generate constitutive mutants of ComA that bypass regulation by its cognate kinase, ComP. We utilized error-prone PCR to mutate *comA* (Materials and Methods). These *comA* mutants were cloned into a derivative of the plasmid pGEMcat, containing *comP* disrupted by an MLS^R resistance cassette upstream of *comA*, and *yuxO* and *mrpG-E* downstream, along with a $spec^R$ resistance cassette. The resulting recombinant strains contained a deletion of *comP*(MLS^R), a mutated *comA*, and a selectable $spec^R$ cassette (Figure 2.5B). On solid LB media with X-gal, our P*srf-lacA* reporter strain produces blue, Lac+ phenotype, colonies (Figure 2.5A, first row). A *ΔcomP* reporter strain wild-type for *comA* produces white, Lac- phenotype colonies (Figure 2.5A, second row). The transformed libraries were plated on LB agar plates containing appropriate antibiotics for selection, along with X-gal. Mutant versions of ComA, capable of transcribing the P*srfA* reporter in the absence of ComP also resulted in blue, Lac+ phenotype colonies (Figure 2.5A, third row). We selected these colonies, and confirmed the Lac+ phenotype with isolation streak plating. Genomic DNA was isolated from colonies for use in

amplification and sequencing of the *comA* gene. Using these methods, we optimized our screen for the generation of single mutants of *comA*.

2.2.4 Results of genetic screen

From our screen, we isolated 36 single mutants, 11 double mutants, and 2 triple mutants of ComA (Table 2.1). Twenty-nine of the single mutations were found to be located in the response regulatory domain of ComA (Figure 2.6B, black bars). Five single mutants in the DNA binding domain were also isolated (Figure 2.6B, hatched bars). The remaining four single mutants were located in the linker region (Figure 2.6B, gray bars). All double and one triple mutant isolated from the screen contained at least one mutation at a site of single mutant also isolated in the screen. The last triple mutant E124K/E164K/E173K contains the single mutations E164K and E173K, also isolated as single mutants.

To begin characterizing the mutants isolated from our screen, β-galactosidase assays were used to monitor ComA-dependent transcription of *PsrfA-lacZ*. ComA-dependent transcription is dependent on population density. Early in growth, ComA activity is low (Figure 2.6A, squares). As population density increases, so does ComA-dependent transcription. We chose to normalize mutant activity to the peak of ComA activity, occurring between about OD_{600} 1.0 to 2.0, as 100 percent. A control, a *comP* deletion strain (EMR56) produces little to no β-galactosidase activity throughout the growth cycle (Figure 2.6A, triangles).

At high cell density, seven mutants (A12V, E15V, V82M, Y86A/C/S, and I113L) were identified to have β-galactosidase activity not significantly different from WT at high cell density. Some mutants, including N57K, were able to make it through our genetic screen, despite not showing high activity in liquid culture. As a general trend, mutants in the response regulatory domain were found to have higher β-galactosidase activity in a *comP* deletion background than those single mutants isolated in the linker or DNA binding domains. None of the mutants found in the linker or DNA binding domain exceeded 50% of WT activity. This is interesting, as it implies that the mutants isolated from our screen are affecting the activation of ComA, at some step other than DNA binding.

2.2.5 Activities of ComA constitutive mutants at high and low culture density

As shown in Figure 2.3, the levels of ComA protein remain constant throughout the growth cycle. Thus, one may expect constitutive mutants of ComA to produce transcriptional activity independent of cell density. We investigated this possibility using the β-galacotsidease assays. We looked at normalized percent activation as before, choosing to characterize mutants with near WT levels of activation at high cell densities (A12V, E15V, T17I, T19I, M65I, V82L, Y86A, Y91F, and I113L). WT ComA, which has activity responsive to cell density, produces approximately 25% of maximal transcriptional activity at an OD_{600} of 0.2 (Figure 2.7). Several of these ComA mutants produce very high activity at low cell density, indicating a loss of density-dependent regulation. For example, ComA(T19I) produces transcription at low cell density of only 50% of WT maximal activity, that is not significantly different than its own maximal

activity (Figure 2.7). ComA A12V, E15V, and V82L, on the other hand, produce nearly 75% of WT activity, even at low cell density. ComA T17I, Y86A, and Y91F produced WT-like low activity at low cell density. Additionally, M65I and I113L produce around 50% of maximal activity at low cell density (Figure 2.7). Taken together, these data suggest that some of the constitutive mutants of ComA isolated from our genetic screen still exhibit density-dependent activity, while others have lost the density-dependent regulation. Moreover, of the ComA^{cons} mutants that exhibit density-dependent regulation, the activity of these mutants is higher at low cell density in a *comP*-deleted background compared to wild type ComA in a *comP+* background.

2.2.6 Regulation of ComA^{cons} mutants by ComP

ComA is known to be activated via phosphorylation by its cognate kinase, ComP. This phosphorylation occurs on the aspartic acid residue at position 55 (Weinrauch, Guillen and Dubnau,1989). The mutants isolated from our genetic screen are able to activate transcription of target genes in the absence of ComP, so we would expect minimal regulation by the histidine kinase. In order to look at regulation of our mutants by ComP, we monitored activation of transcription of P*srf-lacZ* by measuring β-galactosidase activity. For each mutant, we created versions with an additional, alanine substitution at the critical aspartic acid residue at position 55 (D55A). All of these double mutants were transcriptionally inactive (data not shown). To further investigate the role of ComP, we compared β-galactosidase activity from our mutants in *comP+* and *ΔcomP* backgrounds. In a *ΔcomP* background, WT ComA produces about 20% of the transcriptional activity of

WT. Thus, we can say that regulation of WT ComA is ComP-dependent. We chose to classify all mutants that showed a significant increase in activity in the presence of ComP to be similarly dependent on ComP regulation. Interestingly, all but three tested mutants (A12V, V82L, and I113L) were found to be ComP-dependent (Figure 2.8A). We also looked at the role of ComP in the absence of the anti-activator, RapC, the other known major regulator of ComA. In *ΔrapC* strains, we see an increase in WT ComA activity. In a *rapC* deletion, WT ComA produces an additional 30% transcriptional activity. We found no commensurate increase in ComA activity in a *ΔcomP/ΔrapC* double mutant. We found five mutants to still be significantly regulated by ComP in the absence of RapC: E15V, T17I, T19I, M65I and Y91F (Figure 2.8B).

2.2.7 Regulation of ComA^{cons} mutants by RapC

RapC is an anti-activator of ComA (Core and Perego, 2003). The anti-activator protein is able to repress ComA-activity by preventing ComA-DNA binding. RapC is in turn inactivated by its cognate Phr peptide (Core and Perego, 2003). Thus, at high cell densities, when the concentration of Phr peptides is high, RapC is inactive, RapC repression of ComA activity is relieved. We wanted to see how RapC affected transcriptional activation by our ComA^{cons} mutants. We again conducted β-galactosidase assays, this time in *rapC+* and *ΔrapC* backgrounds. As described above, deletion of RapC relieves anti-activation of ComA, resulting in an increase in transcriptional activity of about 30% in a WT ComA strain (Figure 2.8C). We found all but three mutants (E15V, A12V, and V82L) to follow this trend in strains wild-type for *comP*. The remaining

mutants were classified as RapC-dependent. Interestingly, some mutants showed greater than WT relief of anti-activation. For example, Y91F saw an increase of transcriptional activity of around 60% upon the removal of RapC. Likewise, I113L produces an extra 40% activity. We also looked at our mutants in a *ΔcomP* strain to determine the role of RapC in that background. We found only four mutants to be RapC-dependent in a *ΔcomP* background: E15V, M65I, Y86A, and I113L (Figure 2.8D). Taken together, these results suggest different mechanisms of action for our mutants in providing constitutive transcriptional activity.

2.2.8 ComA^{cons} mutants bind DNA similarly to WT ComA

To determine the ability of our ComA^{cons} mutants to bind DNA, we performed gel mobility shift assays using a minimal consensus binding site template. In our previous work, we have shown that ComA binds target DNA containing a minimal tripartite DNA binding site, and produces a shift in DNA mobility between 2 and 20 uM (data not shown). We find little to no shift at 2 uM, intermediate species at 7 uM, and a maximal shift by 20uM. At this time, we are unaware of the stoichiometry of ComA and DNA at these intermediate shifted complexes. Surprisingly, the 9 highly active mutants that showed up in our genetic screen do not show increased binding affinity for DNA at these β concentrations (data not shown). This was unexpected, so we investigated the possibility that our mutants would show increased binding ability at lower, perhaps more physiological concentrations. In gel shift assays performed with protein concentrations

between 0.3 and 2.8 uM, we again saw our mutants exhibit binding affinity similar to WT ComA (Figure 2.9).

2.2.9 ComA^{cons} mutants differentially bind DNA in the presence of acetyl phosphate

Some evidence exists in the literature that ComA may be phosphorylated by small molecule phosphodonors, including acetyl phosphate. Roggiani and Dubnau have shown increased ComA-DNA binding in the presence of ComA pre-phosphorylated with acetyl phosphate. Thus, we attempted gel shift assays with pre-phosphorylation with acetyl phosphate (Materials and Methods, Roggiani and Dubnau 1993). At the higher protein concentrations used previously, we did not observe increased DNA binding by either WT ComA, or any of our constitutive mutants (data not shown). Interestingly, one mutant showed increased binding ability at low concentrations in the 0.3 to 2.8 uM range. The mutant E15V shows dramatically increased binding ability 0.9 and 2.8 uM as compared to both unphosphorylated protein, and phosphorylated WT ComA (Figure 2.9).

2.2.10 Molecular modeling provides insights into ComA activation

In collaboration with Sergey Savinov in the Biochemistry Department at UMass-Amherst, we have produced a model of ComA activation based on related crystal structures. Specifically, we have utilized active and inactive form structures of a transcription factor involved in *Staphylococcus aureus* vancomycin resistance: VraR and a known response regulator from *Streptococcus pneumoniae*, Spr1814. This modeling suggests a hinge mechanism of activation (Figure 2.10). In an inactive state, the space

between alpha helices 1 and 5 is closed, while that between 3 and 4 is open. Upon activation by phosphorylation, this conformation switches to open between helices 1 and 5, and closed between 3 and 4. The opening of a hydrophobic pocket between helices 1 and 5 promotes dimerization. The substitutions isolated from our screen correlate with these changes, by generally increasing volume and hydrophobicity in the region between alpha helices 1 and 5, and decreasing volume in the region around helices 3 and 4.

Additionally, we made predictions about whether our constitutive mutations were likely to stabilize the active ComA dimer, or destabilize the inactive ComA monomer. Residue A12 in ComA is a key residue involved in response regulator dimerization. In other response regulators, this is a larger, hydrophobic residue (e.g. methionine). This residue from one monomer is inserted into a hydrophobic pocket between alpha helices 1 and 5 of a second monomer to anchor dimerization. In the case of our A12V substitution, dimerization may be promoted from replacing the small alanine residue with a larger valine. Residue 17 participates in contact with A12, so the threonine to isoleucine substitution found in our T17I mutant may promote dimerization by increasing the hydrophobicity in this area.

2.2.11 Combination mutations of ComA yield increased activity

Using insights provided by the computational model of ComA activation, we sought to produce mutants with two activating mutations. We rationally chose to pair T17I with either M65I or I113L. The T17I mutation lies in the interface between alpha helices 1 and

5, increasing hydrophobicity in that region. The leucine substitution at position 113 is also an activating mutation found in that same region. This pair of mutations may further promote dimerization. The M65I mutation lies within alpha helix 4, and may contribute to a decrease in volume on that surface. The T17I/M65I pair of mutations therefore may stabilize both sides of the hinge mechanism in an active conformation. Additionally, we produced these strains in a *ΔcomP* background. We used these strains in β-galactosidase assays to monitor transcriptional activity at various cell densities, and normalized activity to WT at maximal activity as 100% (Figure 2.11). As above, WT ComA produces around 20% of maximal activity at an OD of around 0.2. Single mutants show relatively low activity at low cell density, and increased activity at high cell density. M65I and I113L produce maximal β-galactosidase activity approaching WT ComA. The double mutants chosen produce greatly increased transcritpional activity. Additionally, the combination mutants show increased activity at low cell density. The T17I/M65I mutant produces roughly 110% activity at low cell density, and about 130% activity at high cell density. The T17I/I113L mutant produces 115% activation at low cell density and 155% activation at high cell density. Interestingly, despite greatly negating any density-dependent effects, the combinations of mutants chosen still require phosphorylation – triple mutants including D55A are transcriptionally inactive (data not shown).

2.3 Discussion

In this work, we used a genetic screen to isolate activating mutants of ComA, the master transcriptional activator for the early genetic competence pathway, in the absence of its

cognate histidine kinase, ComP. We used *in vivo* assays to characterize these mutants for their ability to be regulated by ComP and RapC, the main anti-activator of ComA. In collaboration with Sergey Savinov, we produced a computational model of ComA activation. Initial steps in validating this model were taken by creating combination mutants that were informed by the suggested hinge mechanism. We also sought to explain the necessity of phosphorylation by ComP by conducting biochemical assays with and without acetyl-phosphate.

2.3.1 Regulation of ComA^{cons} mutants

Two activating mutations appeared to relieve regulation by both ComP and RapC *in vivo*: A12V and V82L. Our model suggests that A12V may promote ComA dimerization by providing a bulkier residue to fit into a binding pocket between alpha helices 1 and 5 of ComA. The V82L mutation lies adjacent to the phosphorylated aspartic acid at position 55 and in the region of the helix 3-helix 4 pocket. Perhaps it affects coordination of the magnesium ion, which is often bound near the residue of phosphorylation. ComA(I113L) is also insensitive to regulation by ComP. As discussed above, this may increase dimerization.

Other mutants remain subject to regulation by phosphorylation and anti-activation in some or all genetic backgrounds tested here. It is difficult to assign roles to these mutations. For example, the pair of tyrosine residues at positions 86 and 91 could be important for the interaction of inactive ComA monomers, stabilizing the inactive dimer.

However, our mutants at these residues have varying effects on regulation. Classifying the effects of the isolated ComA^{cons} mutants would benefit from an analysis of the ComA dimerization interface by x-ray crystallography and/or hydrogen-deuterium exchange experiments.

Additionally, we found none of our ComA^{cons} mutants to have a substantially increased DNA binding ability as compared to WT ComA *in vitro*. One logical next experiment would be to repeat these electrophoretic mobility shift experiments with a less optimized binding template. Our screen was conducted using a WT promoter region for *srfA*, but our electrophoretic mobility shift assays were performed on a minimal consensus binding site template. Perhaps our ComA mutants exhibit greater affinity than WT ComA for sub-optimal promoters. Some ComA-dependent promoters contain an additional, upstream tripartite binding site. It is possible that our ComA mutants facilitate an interaction between ComA dimers bound at both sites, bending DNA in a way that better recruits RNA polymerase. This possibility could be tested by conducting *in vivo* assays with a promoter-*lacZ* fusion for RapA, which lacks this upstream site, and by performing electrophoretic mobility shift assays using a template with two sets of binding sites. Yet another potential explanation of our results is that the activating mutations of ComA that we have found allow for a more efficient recruitment of RNAP, with or without an additional, upstream binding site. This possibility could be explored by conducting *in vitro* transcription assays.

2.3.2 Acetyl-phosphate and ComA activation

While it is clear that phosphorylation by ComP accounts for the greatest amount of activation of ComA during the development of competence, previous work by Dubnau and others suggest a role for acetyl-phosphate in the activation of ComA (Roggiani and Dubnau, 1993; Kim, *et al.*, 2001). We were only able to show an effect in DNA binding upon the addition of acetyl-phosphate for a single ComA^{cons} mutant, E15V. Furthermore, we were not able to replicate an increase in DNA binding with acetyl-phosphate for WT ComA. Taken together, this suggests that our mutants do not likely bypass activation by ComP due to an increased affinity for acetyl-phosphate. To further investigate this possibility, however, an experiment could be performed in a *pta*/*ackA/comP* triple mutant, defective for the ability to produce acetyl-phosphate to test for ComA^{cons}' ability to activate ComA-dependent transcription from early genetic competence promoters. One other consideration here is that the work by Roggiani and Dubnau was conducted using a partially-purified cell extract, and not, as we have done here, purified, histidine-tagged ComA. The possibility, therefore, exists that some factor remains in the cell extract that promotes ComA-DNA binding in the presence of acetyl-phosphate, whether by phosphorylation or some other mechanism.

Ultimately, it is likely that ComA activity depends on some combination of these factors. The constitutive mutants in our screen likely provide a small benefit in one or more of

these directions. Some mutations are suggested to either stabilize the active conformation, or destabilize the inactive conformation by altering charge and volume in different regions of the receiver domain, which may provide a slight shift in the equilibrium of ComA-DNA binding to the bound state. Other mutations, like E15V likely promote increased phosphorylation by small molecule phosphodonors. These small shifts, below the detection limit of our assays, may provide just enough enhanced binding to drive transcription. While sufficient to drive transcription, these mutations are not enough to overcome the requirement that ComA remain able to be phosphorylated at residue 55. Taken together, the data suggests that regulation of ComA by ComP and by Rap-Phr pairs is quite important. This complex regulation makes sense at the population level, as many cell fates in *B. subtilis* appear to be mutually exclusive. The decision to become genetically competent precludes a cell from pursuing another developmental program. Commitment to developmental diversity provides the community with more avenues for survival and proliferation.

2.4 Materials and Methods

2.4.1 Growth media

Routine cloning and growth of both *B. subtilis* and *E. coli* was conducted using LB agar plates. Liquid cultures of *E. coli* were grown in LB medium. Liquid cultures of *B. subtilis* were grown in LB medium, or S7 defined minimal medium slats (Vasantha and Freese, 1980) containing 50 mM 4-morpholinepropanesulfonic acid instead of 100 mM ($S7_{50}$) and supplemented with 1% glucose, 0.1% glutamate, tryptophan (40 ug/mL),

phenylalanine (40 ug/mL) and threonine (200 ug/mL), where appropriate. Where appropriate, the following concentrations of antibiotics were used: ampicillin (100 μg/ml), neomycin (2.5 μg/ml), tetracycline (8-10 μg/ml), phleomycin (5 μg/ml), chloramphenicol (5 μg/ml), spectinomycin (100 μg/ml), and erythromycin (0.5 μg/ml) and lincomycin (12.5 μg/ml) together to select for macrolidelincosamide-streptogramin B (MLS) resistance.

2.4.2 Plasmids, strains, and alleles

Strains DH5α and AG1111 (a MC1061 derivative with F'(*lac*I^q) *lacZM15 Tn10*) of *E. coli* were used for routine cloning. *B. subtili*s strains in this study were derived from strain JH642 (*trpC2 pheA1*) (Perego, et *al.*, 1988).

2.4.3 pBAD-his_6-tagged proteins

N-terminal hexa-histidine fusion proteins were created by PCR amplification of specific genes from *B. subtlis* genomic DNA. Forward primers contain 15-17 bp of gene-specific sequence down stream of the following sequence: 5'-GCTTAGTGGGTACC**AAGGAGA**TATACAT*ATG*catcaccatcaccatcac-3'. Underlined bases contain a *KpnI* restriction site; bold characters signify an optimal ribosome binding sequence; italics the initiation codon; lower case letters the his_6-tag. Each reverse primer contains the following sequence: 5'-TGCTACGAGCATGC**TTA**-3' and 15-17bp of gene-specific sequence following the termination codon. The underlined text represents a *SphI* restriction site, and the bold text denotes the termination codon. *KpnI* and *SphI* restriction

enzymes (New England Biolabs) were used to digest each PCR product prior to ligation into either pBAD-CM33 or pBAD-Ap18 plasmid, also digested with both restriction enzyme. Ligations were transformed into strain AG111 and plated onto LB solid medium containing the appropriate antibiotic. Sequences of clones were confirmed by DNA sequencing.

2.4.4 Oligonucleotides

All oligonucleotides used in this study were obtained as synthesized and desalted by Integrated DNA Technologies.

2.4.5 Computational analyses

ImageJ was used to normalize fluorescent intensity to cell size. LibreOffice Calc and Microsoft Excel, along with the suite of statistical tools available from http://www.in-silico.net were used to perform all statistical analyses. Single sample student *t*-tests were performed, using a *p*-value of 0.05 as a cut-off for statistical significance.

2.4.6 Growth conditions and β-galactosidase assays

ComA activity was monitored by growing reporter strains as light lawns on Spizizen minimal media plates which were used to inoculate shaker flasks containing $S7_{50}$ minimal medium to a final OD_{600} of ~0.02, as described in Griffith and Grossman, 2008.

Liquid cultures were grown in shaker flasks at 37ºC with vigorous aeration. Half-milliliter samples were removed and placed in a 2.2 mL 96-well polypropylene block. Polypropylene blocks were stored at -20ºC between time points and later until β-galactosidase activity was assayed. An additional aliquot was saved for determining OD_{600}. β-galactosidase assays were performed as previously described (Griffith and Grossman, 2008). Briefly, polypropylene blocks were thawed to room temperature. Cells in each well were permeabilized by adding 20 uL of toluene, followed by vigorous pipetting up and down using a multi-channel pipettor. 200 uL of permeabilized cells were transferred to a second block containing 800 uL of Z-buffer (Miller, 1972), and were again mixed vigorously. 100 uL of permeabilized cells diluted in Z-buffer were transferred to a 96-well microtiter plate. The assay was initiated with the addition of 20 µl freshly prepared Ortho-nitrophenyl-β-D-galactopyranoside (4 mg/ml) and terminated with the addition of 40 µl 1 M Na_2CO_3. A_{420} was determined using a SpectraMax plate reader (Molecular Dynamics) and data analysis was performed using Microsoft Excel. β-galactosidase specific activity was calculated as follows: 1000 x [(ΔA_{420}/min/ml)/OD_{600} of culture].

2.4.7 Protein expression and purification

Proteins were expressed and purified as described previously (Boguslawski, Hill and Griffith, 2015). A fresh overnight culture of strain DH5α containing the appropriate plasmid (Table 1) was diluted 1:100 into LB (Fisher) containing the appropriate antibiotic. Cultures were grown to OD_{600} ~0.5 at 37ºC with vigorous aeration. L-

arabinose (Sigma) was added to a final concentration of 0.2% to induce expression from pBAD plasmids. Cultures over-expressing his_6-tagged ComA and ComA mutants were induced for 5-6 hrs at 37°C. Cells were harvested after induction by centrifugation at 5,000g for 10 min at 4°C and cell pellets were stored at -20°C until further use.

His_6-tagged proteins were purified by standard Ni-NTA chromatography as previously described (Griffith and Grossman, 2008). The cell pellet from 1L of culture was thawed on ice, resuspended in 10 ml sonication buffer (10 mM Tris pH 8, 0.3 M NaCl, 5% glycerol, 5 mM imidazole, 5 mM β-mercaptoethanol, and 5 mM $MgCl_2$), and cells were lysed by sonication using a Branson sonifier (10 cycles of 20 sec on and 40 sec off, at setting 6). The culture was cleared by centrifugation at 12,000 rpm for 30 min at 4°C, and the cell extract was passed over 2 ml of Ni-NTA (Qiagen). After 10 washes with 25 ml of sonication buffer, his_6-tagged proteins were eluted from the column in 10 ml sonication buffer with increasing concentrations of imidazole (25 mM, 50 mM, 120 mM, 200 mM, and 300 mM). Fractions were analyzed for purity by SDS-PAGE followed by Coomassie staining. Fractions with the greatest purity were pooled and dialyzed at 4°C against 3 buffer changes of 2L dialysis buffer (10 mM Tris pH 8, 0.3 M NaCl, 5% glycerol, 10 mM β-mercaptoethanol, and 5 mM $MgCl_2$). Dialyzed proteins were concentrated to 5-15 mg/ml using a Centricon-10 (Amicon), His_6-tagged proteins ComA and ComA mutants were stored 4°C in dialysis buffer and remained stable for several months without significant loss of activity (data not shown). The remaining his_6-tagged proteins were stored at -20°C in dialysis buffer containing 40% glycerol. Protein

concentrations were determined by Bradford assay using bovine serum albumin as protein standard. Protein preparations were estimated to be >95% pure as determined by SDS-PAGE followed by Coomassie staining (data not shown).

2.4.8 Gel mobility shift assays

DNA corresponding to the minimal optimal ComA binding sequence was prepared by annealing two complementary oligonucleotides containing the following sequence: 5'-tcaTTGCGGcatcCCGCAAgaaactTTGCGGtc -3'where the bases in uppercase represent Recognition Elements 1-3. DNA templates contain bases 5' –TCA preceding the ComA binding sequence and bases TC- 3' following it and are underlined. One of the oligonucleotides from each pair was labeled on its 5' end using γ-^{32}P-ATP (Perkin Elmer) and T4 polynucleotide kinase (NEB). The kinase reaction was terminated by incubation at 70^{o}C for 20 min. A 1.3-fold molar excess of the complimentary oligonucleotide was added to the mixture and heated to 95^{o}C for 5 min, followed by slow cooling to room temperature to facilitate annealing of the oligonucleotides. Duplex DNA was purified away from the unincorporated label using a G-25 Centrispin 10 column (Princeton Separations).

In vitro binding reactions contained 13 mM Tris pH 8, 50 mM EPPS pH 8.5, 20 mM $MgCl_2$, 0.1 mM EDTA, 100 mM KCl, 3 mM DTT, and 10% glycerol in a 20 µl final volume. Radiolabeled DNA (5 nM) was added to the binding reaction along with the appropriate amount of his_6-tagged Rap and ComA. Protein-DNA complexes were

allowed to equilibrate at 24ºC for 30 min, prior to the addition of 5 µl of 5X agarose gel loading dye. Samples were loaded into the wells of a 10% polyacrylamide gel containing 5% glycerol and electrophoresed into the gel at 300 V. Once the loading dye had entered the gel, the voltage was reduced to 120 V and gels were run for 5-6 hr at 4ºC. Gels were dried and analyzed using a Typhoon PhosphorImager (Molecular Dynamics).

2.5 Tables and Figures

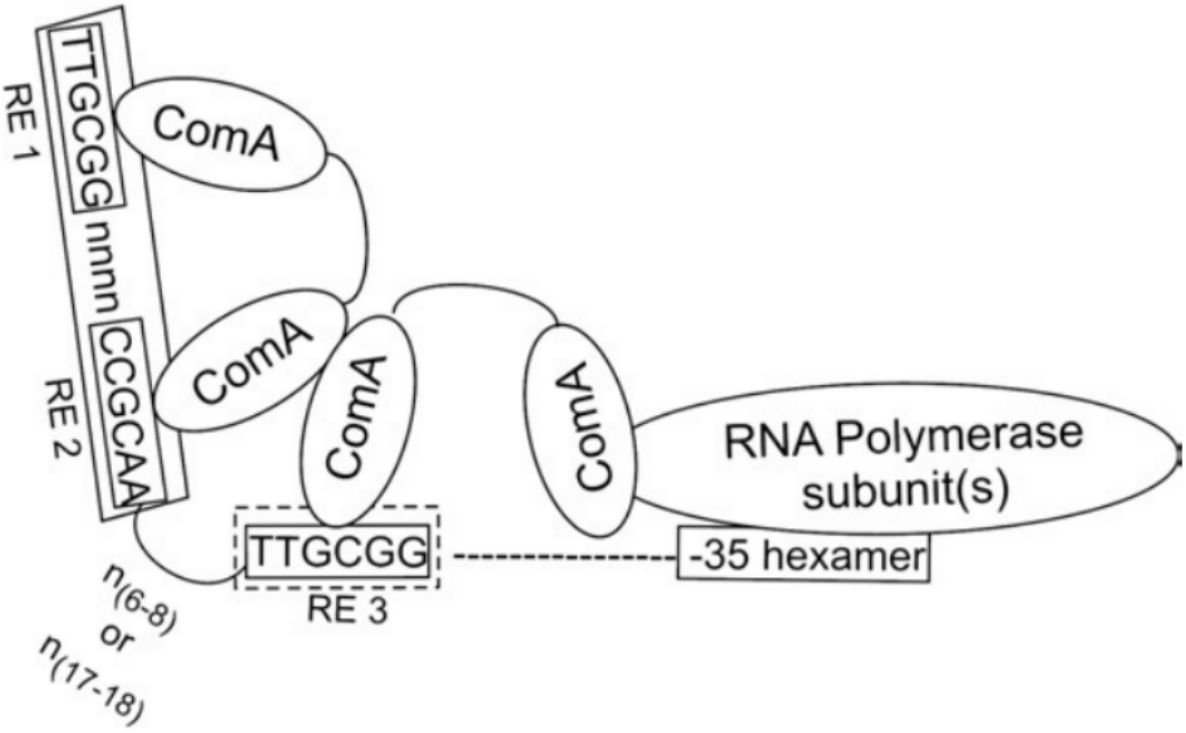

Figure 2.1. Proposed model for ComA binding. The proposed model for ComA binding consists of a dimer of dimers binding a degenerate tripartite binding site. Recognition Elements 1 and 2 (RE1, RE2) comprise an inverted repeat separated by 4 nucleotides. RE3, which must be 6-8 or 17-18 nucleotides downstream is proposed to bind one ComA monomer of a second dimer, leaving the remaining monomer free to interact with RNA polymerase (Griffith and Grossman, 2008).

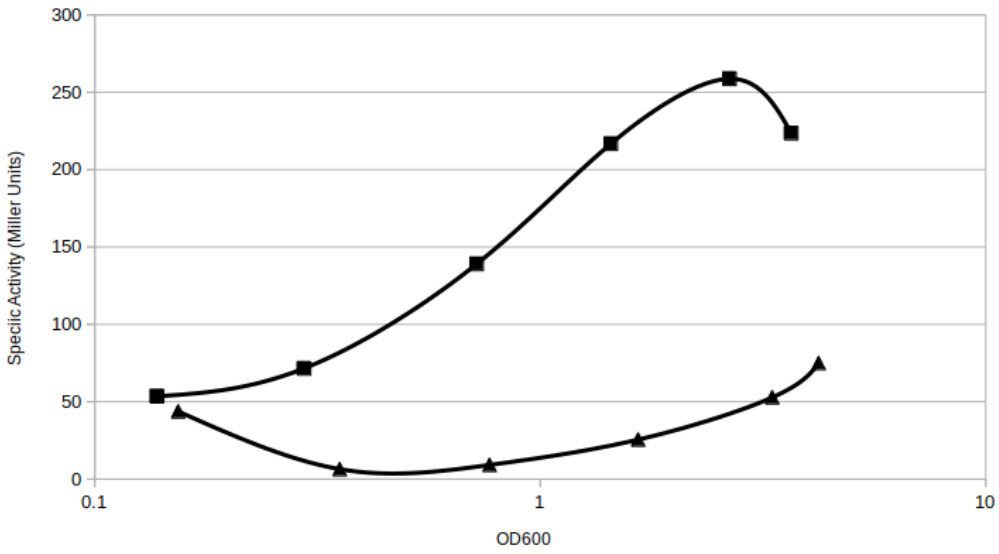

Figure 2.2. Density-dependent response of ComA-dependent β-galactosidase activity. Strains containing the P*srfA-lacZ* reporter were grown in S750 media, with aliquots taken starting at an OD600 of 0.2, and every hour for a total of six samples used to determine β-galactosidase activity. Strain KG74 (WT ComA, squares) and strain EMR56 (*ΔcomP*, triangles); experiment was performed in at least triplicate. Single representative experiment shown.

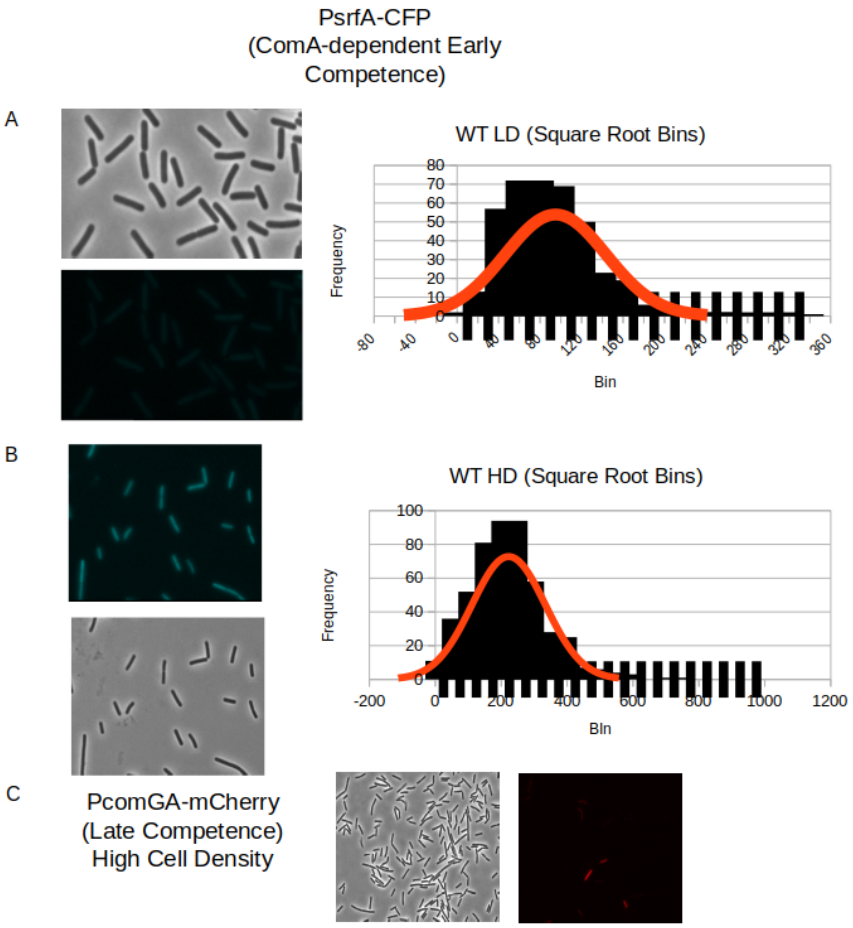

Figure 2.3. Gene expression from early and late competence pathway promoters.

Reporter strains for PsrfA-CFP (EMR161) and PcomGA-mCherry (EMR) were grown in $S7_{50}$ media. Samples were taken and observed under fluorescent light microscopy. Experiments were performed in triplicate. Micrographs of representative experiments are shown. Fluorescent intensity was normalized to area using ImageJ and MicrobeJ. Bin number was determined according to the square root method to create a histogram of the normalized data. A standard curve with the same mean intensity and standard deviation was created and overlaid using Microsoft Excel.

A. PsrfA-CFP at low cell density (OD_{600} = 0.2).

B. PsrfA-CFP at high cell density (OD_{600} = 1.0).

C. PcomGA-mCherry at high cell density (OD_{600} = 1.0).

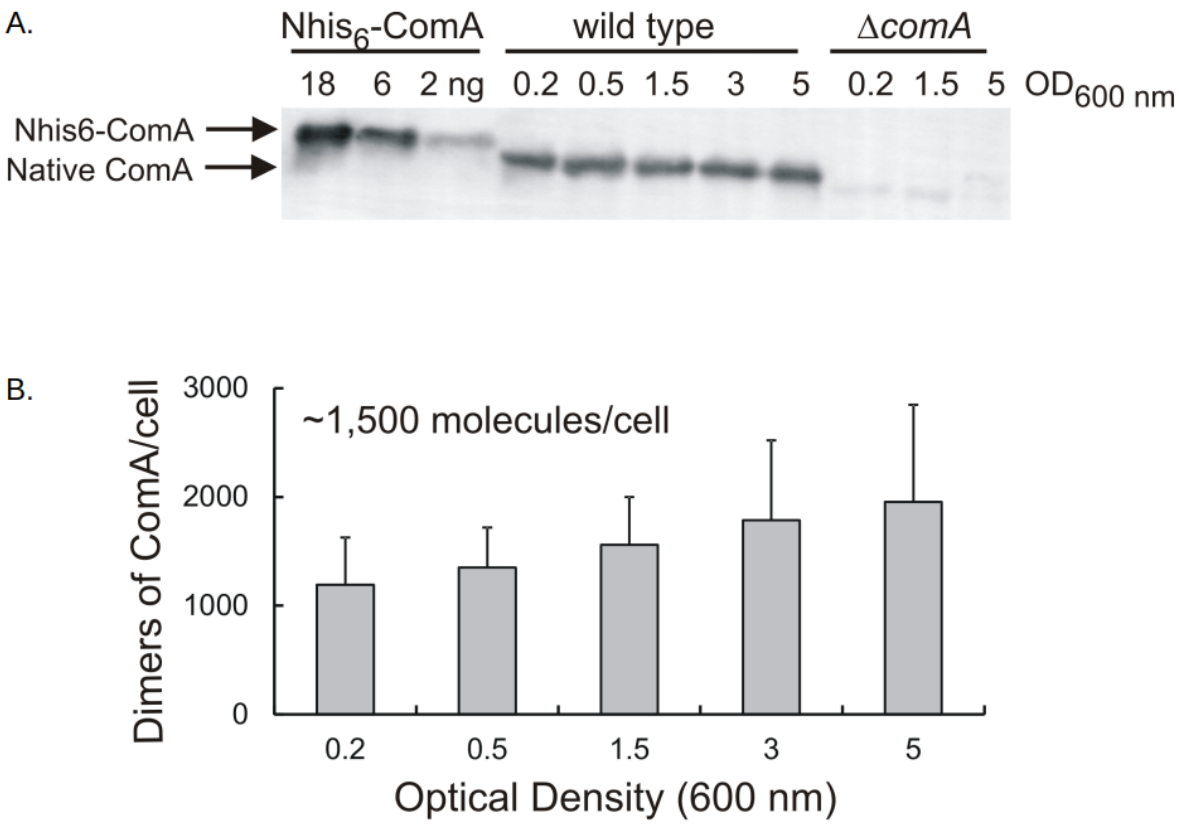

Figure 2.4. ComA abundance throughout growth.
A. WT (JH642) and delta-ComA (KG) *B. subtilis* were grown in , and samples taken at various culture densities. Western blots were performed using anti-ComA antibody on cell lysate. 2 to 18 ng of purified $Nhis_6$-ComA was used as a standard. Experiments were performed in triplicate, and a representative blot is shown.
B. Western blots of JH642 were quantitated using ImageQuant. Standard error is shown.

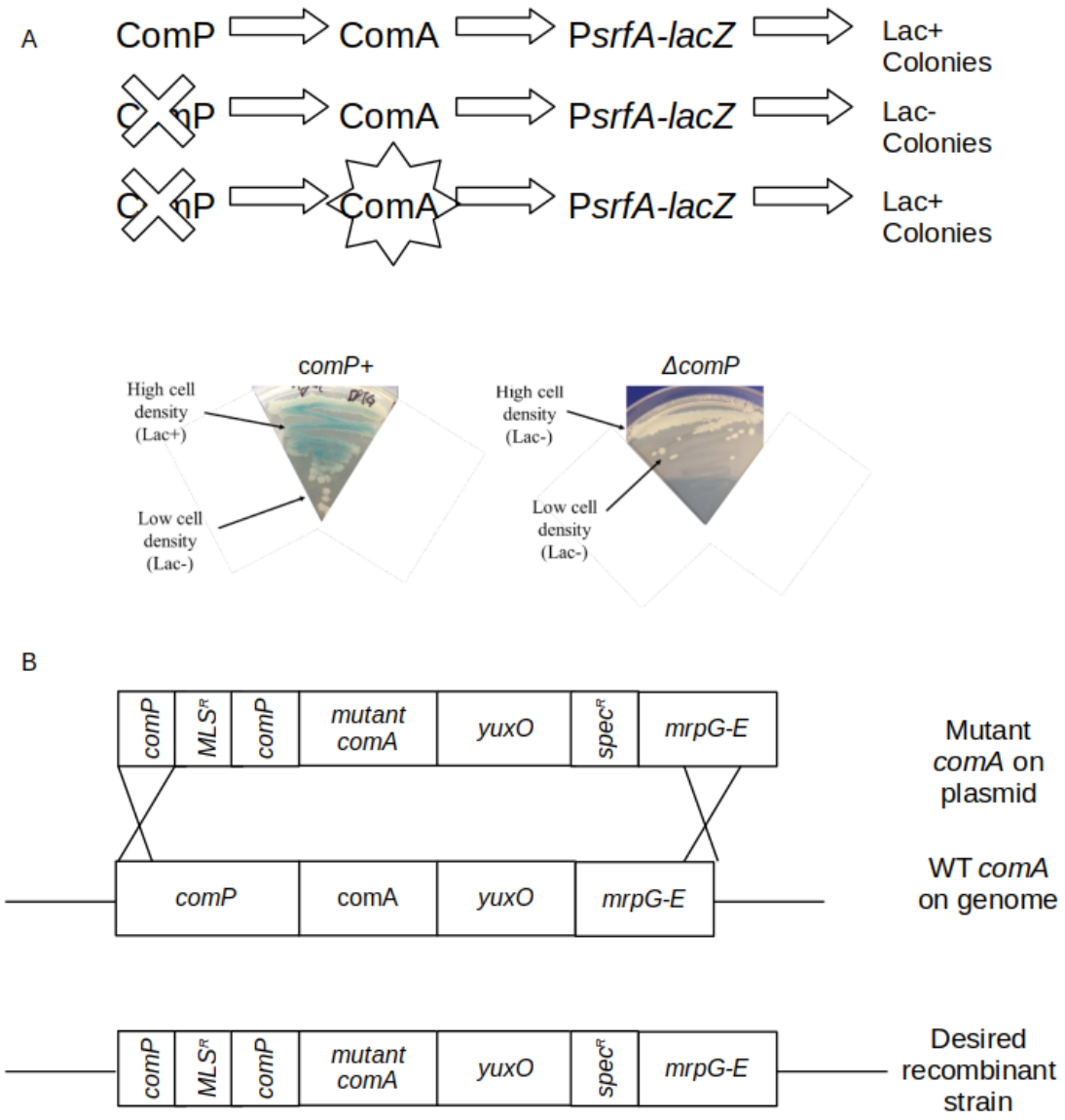

Figure 2.5. Genetic screen to isolate constitutive mutants of *comA*.
A. Plate test to isolate mutants. A *PsrfA-lacZ* reporter strain on LB+X-gal produces blue (Lac+) colonies at high cell density, and white colonies at low cell density. A *ΔcomP* + reporter strain produces white (Lac-) colonies at any cell density. We selected Lac+ colonies from our transformed libraries in a *ΔcomP* background reporter.
B. Generation of ComA mutant libraries. Error-prone PCR mutagenesis was used to generate plasmids with mutant *comA*, along with *spec*^{R} and *ΔcomP.* These plasmids were transformed into P*srfA-lacZ* reporter strains that were wild type at *comP* and *comA*.

Table 2.1. List of mutants of ComA isolated from genetic screen

Single Mutations		Double Mutations	Triple Mutations
A12V	Y91F	A12V, G64E	T19A, S47P, T137A
M14L	H101R	T17I, D50N	E124K, E164K, E173K
E15V	T107A	T17I, I192T	
T17I	K112R	S48P, G60L	
S33G	I113L	M65I, M136I	
Q43K	I113T	M65I, P143L	
L53I	I117T	K69E, I117V	
L56M	I117V	K69N, E164K	
N57K	Y118H	S105N, I113F	
G60C	E164G	K112R, D30G	
M65I	E164K	K112R, L154F	
M65V	F169C		
Q70P	F169S		
V82L	E173A		
V82M	E173K		
Y86A	L180F		
Y86C	S188G		
Y86S	T202M		

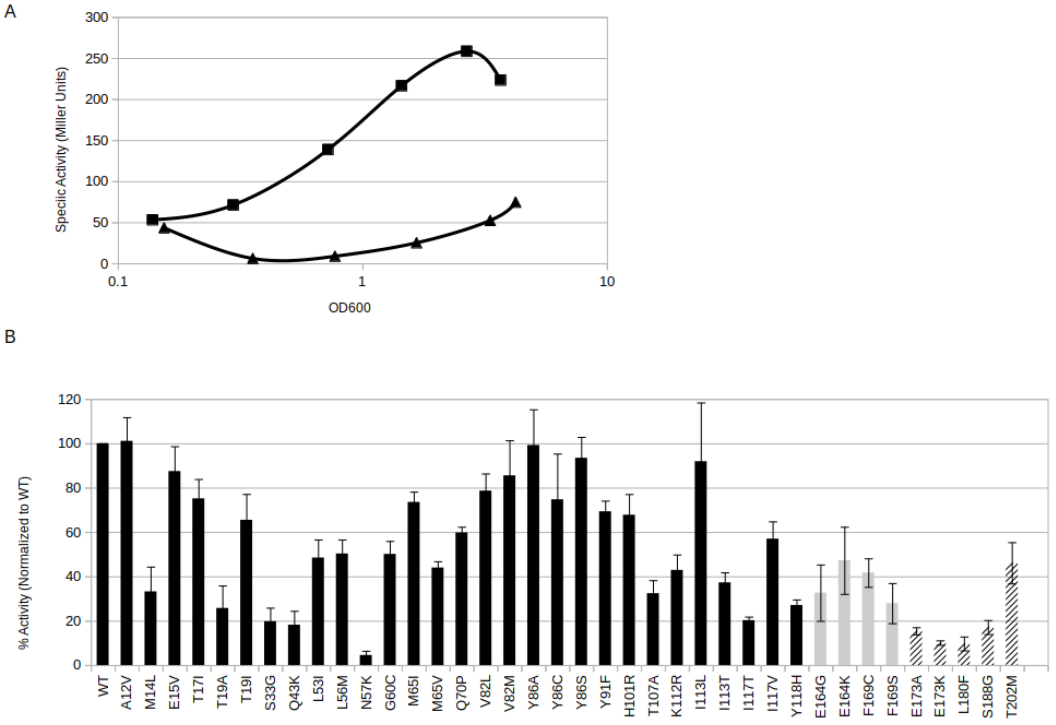

Figure 2.6. β-galactosidase activity of single mutants isolated from genetic screen to isolate mutants of ComA that bypass regulation by cognate histidine kinase, ComP. Strains containing the P*srfA-lacZ* reporter were grown in S750 media, with aliquots taken starting at an OD600 of 0.2, and every hour for a total of six samples used to determine β-galactosidase activity.
A. Strain KG74 (WT ComA, squares) and strain EMR56 (*ΔcomP*, triangles); experiment was performed in at least triplicate. Single representative experiment shown.
B. β-galactosidase activity was determined for all single mutants of *comA* and normalized to strain KG74 (wild type). Asterisks represent P-values > 0.01. Mutants represented with black bars are found in the Response Regulatory Domain, gray bars in the linker, and hatched bars in the DNA Binding Domain.

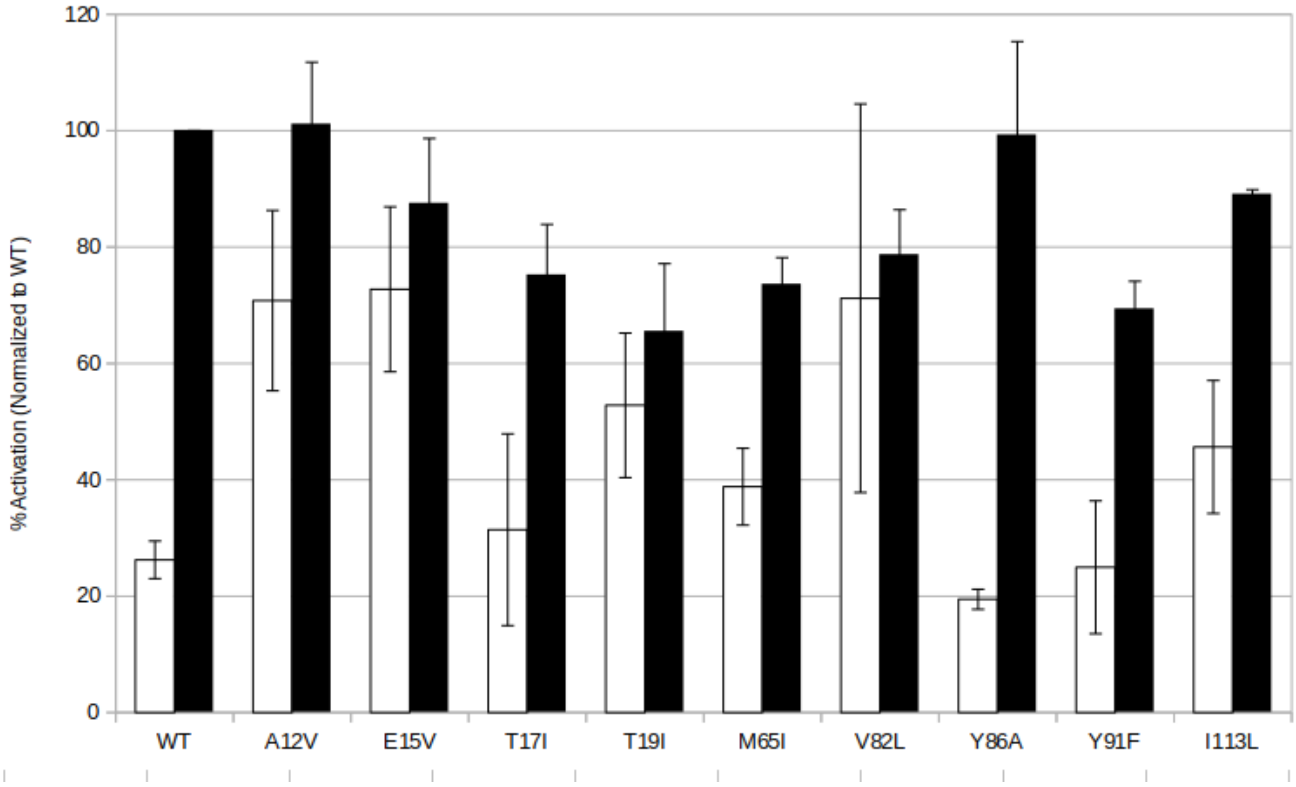

Figure 2.7. β-galactosidase activity of select ComA^{cons} mutants at high and low culture density. Strains containing the P*srfA-lacZ* reporter were grown in S750 media, with aliquots taken starting at an OD600 of 0.2, and every hour for a total of six samples used to determine β-galactosidase activity. Activity was normalized to KG74 at OD600 ~ 1.0. White bars represent percent activation at OD600 = 0.2. Black bars show percent activation at OD600 = 1.0.

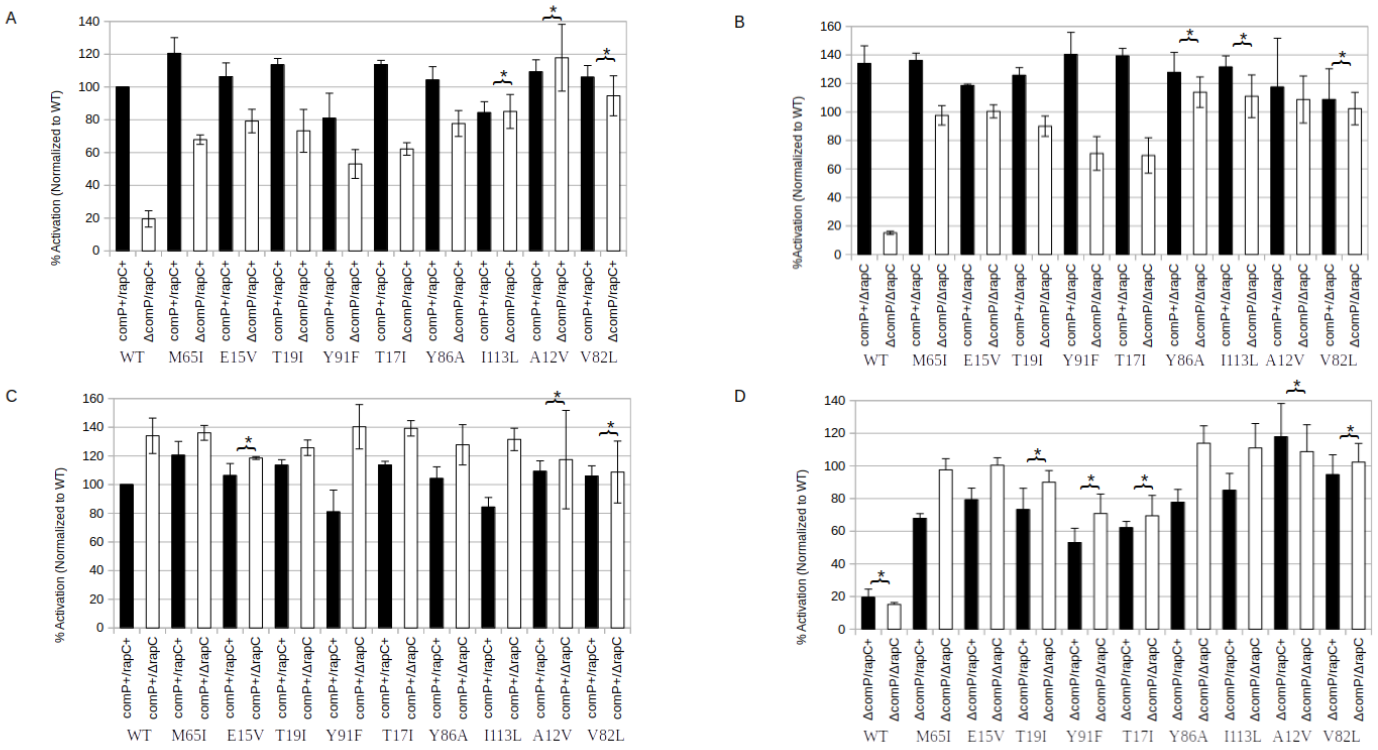

Figure 2.8. Roles of ComP and RapC in regulation of ComA^{cons} mutants.
Strains containing the *PsrfA-lacZ* reporter were grown in S750 media. Aliquots were saved beginning at OD600 = 0.2, and then every hour for six total samples. β-galactosidase activity was determined, and activity was normalized to strain KG74. Experiments were performed in triplicate. Asterisks represent P-values > 0.05.
A. All strains are *rapC+*. Black bars are indicated in *comP+* background; white bars are *ΔcomP*.
B. All strains are *ΔrapC.* Black bars are indicated in *comP+* background; white bars are *ΔcomP*.
C. All strains are *comP+*. Black bars are indicated in a *rapC+* background; white bars are *ΔrapC*.
D. All strains are *ΔcomP.* Black bars are indicated in a *rapC+* background; white bars are *ΔrapC*.

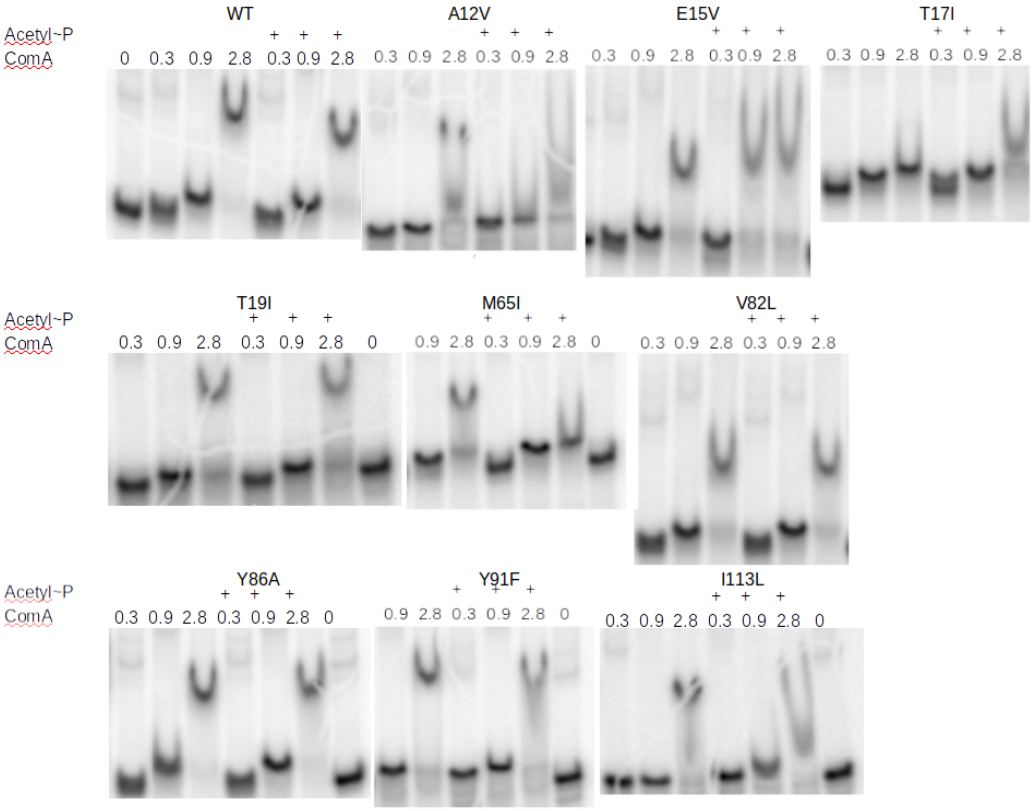

Figure 2.9. DNA Binding of Selected ComA mutants. Gel mobility shift assays were conducted using purified ComA proteins and a DNA template containing a minimal ComA binding sequence (tcaTTGCGGatcCCGCAAgaaactTTGCGGtc). Purified his6-tagged ComA proteins were incubated with 5 nM ^{32}P-labeled double stranded DNA template, reaction buffer, and acetyl phosphate, where appropriate. Proteins and template were allowed to equilibrate at room temperature for 30 minutes prior to separation on 12% native acrylamide gels.

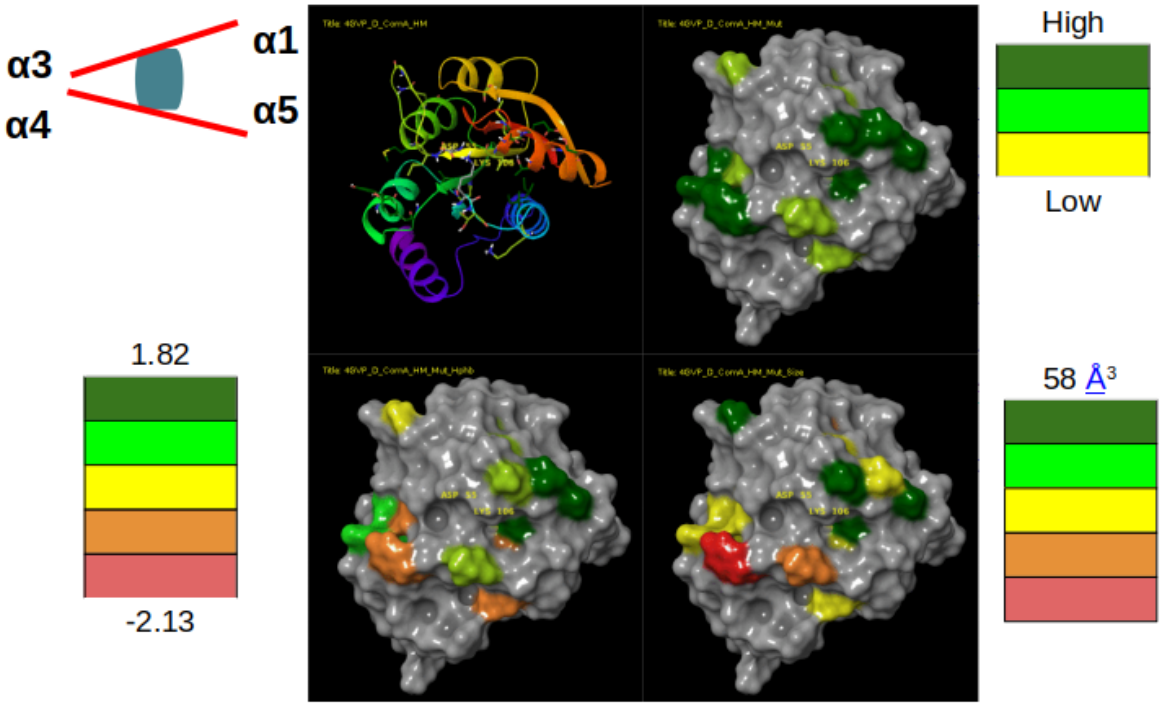

Figure 2.10. Molecular modeling of ComA activation.
A. Ribbon structure model of active form of ComA.
B. Surface model of active form of ComA. Positions of mutants from the genetic screen are color coded from yellow (low activity) to dark green (high activity).
C. Surface model of active form of ComA. Positions of mutants from the genetic screen are color coded from red (negative change in hydrophobicity) to dark green (positive change in hydrophobicity).

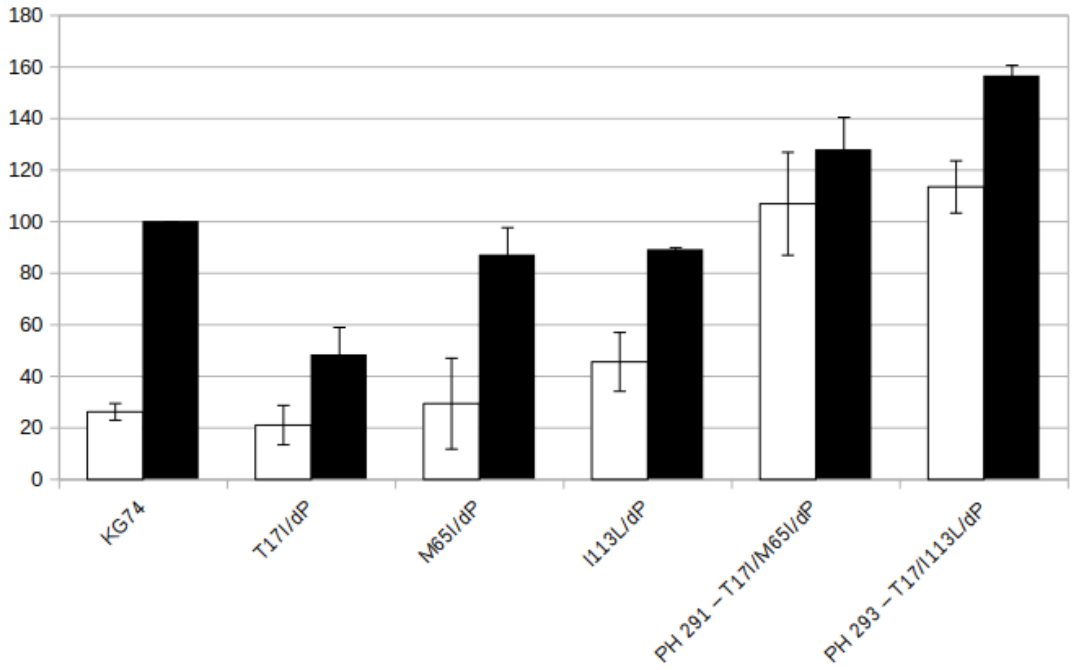

Figure 2.11. Effects of double constitutive mutations. Strains containing the P*srfA-lacZ* reporter were grown in S750 media. Aliquots were saved starting at OD600 = 0.2, and then every hour for six total samples. β-galactosidase activity was determined, and activity was normalized to KG74. Experiments were performed in triplicate.

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CHAPTER 3

IDENTIFICATION OF NOVEL ROLES OF RAP PROTEIN REGULATION

3.1 Introduction

The activity of many quorum sensing pathways in *B. subtilis* are controlled by *rap-phr* regulator pairs. Rap proteins derive their name from their initial discovered function, **R**egulators of **A**spartate **P**hosphatases, that modulate the activity of phosphorylated targets (Perego and Hoch, 1996). These proteins are founding members of the RRNPP (Rap, Rgg, NprR, PlcR, PrgX) group of proteins, that sense peptide signaling molecules in a number of other bacteria, including *Enterococcus faecalis*, *Bacillus cereus*, *Streptococcus* species, and *B. subtilis* (Do and Kumaraswami, 2016). RRNPP proteins are characterized by a C-terminal domain consisting of multiple tetratricopeptide repeats (TPRs). TPRs are ubiquitously conserved among living organisms in a wide variety of proteins. These motifs are important for protein-protein and protein-peptide interaction and are critical for many cellular processes, including gene regulation, mitosis, protein import, pathogenesis, and quorum sensing (D'Andrea and Regan. 2003). Proteins of the RRNPP group sense peptide pheromones with their C-terminal TPR domains (Do and Kumaraswami, 2016). While many RRNPP proteins tend to be transcriptional activators when bound to their peptide pheromones, Rap proteins are typically anti-activators that inhibit the activity of positive regulators (Lopez, Vlamakis and Kolter, 2008). Rap proteins are inhibited by short peptide pheromones called Phrs (Perego and Brannigan, 2001). The genes for Phr peptides are often oriented directly downstream, or even overlapping with the cognate *rap* gene (Figure 1.8).

Rap proteins, the founding member of the RRNPP family, serve as a model for understanding the function of several important regulatory proteins. In *B. subtilis*, there are 10 *rap* genes encoded on the chromosome (Kunst, *et al.*, 1997). Additional *rap* genes are found on mobile genetic elements, including plasmids, bacteriophages, and conjugative elements (McLoon, *et al.*, 2011; Auchtung, *et al.*, 2005). Regulation of the quorum response is complex, often involving redundant signaling pathways with multiple layers of control. For example, we found that a single Rap protein, e.g. Rap60 from the plasmid pTA1060, can influence multiple developmental processes by directly modulating the activities of several separate regulatory proteins (Boguslawski, Hill and Griffith, 2015). Additionally, the activities of multiple Rap proteins often converge to modulate the activity of a single regulatory protein. For example, RapC, RapF, and RapG all function to regulate the activity of the early competence regulator, ComA (Core and Perego, 2003; Comella and Grossman, 2005; this work).

Rap proteins were initially discovered for their role in regulating the developmental process of sporulation (Pergo, *et al.*, 1994). The *B. subtilis* Rap proteins known to regulate sporulation include RapA, B, E, H, J, and the plasmid-borne Rap60 (Perego, *et al.*, 1994; Fawcet, *et al.*, 2000; Smits, *et al*., 2007, Parashar, *et al.*, 2011, Boguslawski, Hill and Griffith, 2015). Rap proteins modulate sporulation by acting as phosphatases of Spo0F~P, an early protein in the sporulation phosphotransfer relay system (Figure 1.11). Rap60 has an additional role in modulating sporulation by interfering with KinA

autophosphorylation, resulting in disruption of the sporulation phosphor-relay system (Boguslawski, Hill and Griffith, 2015).

Other Rap proteins (Rap C, D, F, G, H, I, K, P and 60) are anti-activators of genetic competence (Core and Perego, 2003; Ogura and Fujita, 2007; Bongiorni, *et al.*, 2005; Hayashi, *et al.*, 2006; Smits, *et al.*, 2007; Parashar, *et al*., 2013a; Auchtung, *et al*., 2006, Boguslawski, Hill and Griffith, 2015). These Rap proteins can be divided into at least two classes based on their mechanism of anti-activation of the trancriptional activator ComA (Figure 3.1). Class I Rap proteins, including RapC, RapF, and RapH (Table 3.1), inhibit ComA dimerization, thus preventing ComA from accessing promoter DNA (Figure 3.1, middle). Class II Rap proteins (e.g. Rap60, Table 3.1) form ternary complexes with ComA and DNA (Figure 3.1, bottom). We presume this ternary structure interferes with ComA access to the transcriptional machinery. A potential third class of ComA anti-activation by Rap proteins also exists and will be discussed later.

Still other Rap proteins are known to regulate additional processes, including the production of extracellular proteases, motility, biofilm formation, and horizontal gene transfer. The two component system DegSU is involved in cell motility and biofilm formation. Specifically, at low levels of phosphorylation, DegU activates swarming motility. At intermediate levels of DegU~P, genes involved in biofilm production are activated. High levels of DegU~P activate genes involved in the production of exoproteases, while genes involved in motility, and biofilms are repressed (Verhamme, *et*

al., 2007). RapG inhibits expression of DegU-dependent expression of genes involved in the production of exoproteases, including *aprE,* the gene for the serine protease, subtisilin. (Ogura, *et al*., 2003). This inhibition is the result of RapG interfering with DegU binding to DNA (Ogura, *et al.*, 2003).

Rap proteins are also involved in horizontal gene transfer. Specifically,RapI regulates the expression, excision, and transfer of the mobile conjugative element ICE*Bs1* (Auchtung, *et al*., 2005.). While most Rap proteins inhibit cellular processes, RapI plays a role in the excision of ICE*Bs1*, most likely by affecting the anti-repressor, ImmA (Bose, *et al.*, 2008).

A second plasmid-borne Rap protein, RapP, negatively regulates biofilm formation by acting on Spo0F and also ComA, through an as of yet unknown target (Parashar, *et al.*, 2013b; Bendori, *et al.*, 2015). Lab strains of *B. subtilis*, including strain JH642, which we have used for our experiments have become defective for biofilm formation through the process of domestication. On the other hand, the undomesticated strain NCIB 3610 produces colonies with a complex architecture and robust pellicles in liquid culture (Figure 3.2). This complex colony architecture is in part regulated by RapP, present on plasmid pBS32 (Konkol, *et al.* 2013). Plasmid cured strains, including strain DS2569 produce hyper-rugose colonies and form robust pellicles (Konkol, *et al.*, 2013; Boguslawski, Hill and Griffith, 2015). Laboratory strains with defects in biofilm

development tend to produce flat, featureless colonies and weak pellicles (Konkol, *et al.*, 2013; Figure 3.2).

Several Rap proteins have been shown to regulate more than one biological process. One such example is RapH, which is known to regulate both genetic competence and sporulation (Smits, *et al.*, 2007). Other Rap proteins with more than one known function include RapG, RapH, RapI and RapP (Table 3.1). The plasmid-borne Rap proteins, Rap60 and RapP, have been shown to regulate genetic competence, sporulation, and biofilm production.

The basis for these multiple functions have been elucidated at the molecular level. Specifically, structural data from the Neiditch group shows that Rap proteins bind ComA and Spo0F on separate surfaces (Figure 3.3). RapF specifically interacts with residues of the ComA DNA binding domain and interferes with binding to DNA (Parashar, *et al.*, 2011). RapH binds Spo0F via a non-overlapping surface, approximately 49 Angstroms from where RapH binds ComA. These distinct surfaces suggest the possibility that known Rap proteins may regulate additional processes than have already been identified. Here, we explore Rap protein regulation of multiple biological processes in greater detail.

3.2 Results

Rap proteins modulate a variety of biological processes by directly affecting specific regulators. Extracellular Phr peptides coordinate these biological processes with

population density by inhibiting the activity of Rap proteins. We seek to expand upon the roles of Rap proteins in the regulation of known biological processes. To accomplish this goal, it was necessary to bypass existing regulation of Rap proteins by Phr peptides in order to observe their ability to regulate known processes. We accomplished this by expressing only the Rap protein without its cognate Phr peptide and observed its effects on transcription utilizing reporter strains to monitor activity for competence, sporulation and motility (Figure 3.4A).

3.2.1 Identification of new Rap protein regulators of genetic competence

Seven of the endogenous Rap proteins (RapC, D, F, G,H, I, and K) regulate genetic competence (Table 3.1). Additionally, two Rap proteins present on mobile genetic elements (Rap60 and RapP) also regulate competence (Table 3.1). As some Rap proteins have been shown to regulate more than one cellular process, including RapH that regulates both genetic competence and sporulation, we sought to test additional Rap proteins for their ability to regulate competence. We created reporter strains using P*srf-lacZ* fusions to monitor transcripional activation of the early genetic competence pathway. Constructs to singly over-express each Rap protein were introduced into the reporter strain. These constructs lacked the genes for expressing cognate Phr pepties. We monitored the effect of ComA-dependent transcription of P*srf-lacZ* by measurement of β-galactosidase activity. For these assays, we used the minimal concentration of inducer to express each Rap protein that was required to achieve maximal repression of β-galactosidase activity from the reporter.

ComA-dependent transcription is typically low at low cell density, and peaks at a cell density between OD_{600} 1 and 2 (Figure 3.4A). β-galactosidase activity was normalized to ComA-dependent activity of all over-expression constructs to that of a vector-only construct at the point of maximal activity (Figure 3.4A). RapC, RapD, RapG, RapH and RapK have been demonstrated previously in the literature to function as anti-activators of ComA, as shown by a reduction of β-galactosidase activity from the P*srfA-lacZ* promotor (Figure 3.4B). Three additional Rap proteins (RapB, E and J) were found to exhibit novel activity by inhibiting ComA activity (Figure 3.4B). RapE and RapJ appear to be weak inhibitors of ComA, reducing activity by 65% and 60%, respectively. Expression of RapB resulted in an 85% decrease in ComA activity. RapA, which has not been prevously shown to function as an anti-activator, served as a control. Over-expression of RapA had no effect on ComA activity. Taken together, these results suggest that we have identified three Rap proteins (B, E and J) that when over-expressed inhibit ComA-dependent transcription of P*srfA-lacZ* to different extents.

3.2.2 Identification of new Rap protein regulators of sporulation

Five endogenous Rap proteins (RapA, B, E, H, and J) have been previously demonstrated to function as anti-activators of sporulation (Table 3.1). We sought to determine if other Rap proteins could modulate sporulation. We created reporter strains using P*spoIIA-lacZ* fusions to monitor transcriptional activation of early sporulation. As above, we introduced constructs to singly over-express each Rap protein in the absence of its

cognate *phr* gene. We monitored Spo0A-dependent transcription of P*spoIIA-lacZ* by measurement of β-galactosidase activity. The minimal concentration of inducer was used for Rap expression that achieved maximal repression of β-galactosidase activity.

Over-expression of RapA, B, and J resulted in significant decreases in Spo0A-dependent β-galactosidase activity from the P*spoIIA-lacZ* promoter (Figure 3.4C). This is consistent with previous results (Perego, *et al.*, 1994; Parashar, *et al.*, 2011). RapC and RapD, anti-activators of ComA, but which have no known function on sporulation, function as negative controls. Over-expression of RapC and RapD had no effect on β-galactosidase activity produced from P*spoIIA-lacZ*. On the other hand, we found RapG to be a novel regulator of sporulation, resulting in a 100-fold decrease in β-galactosidase activity as compared to vector only (Figure 3.4C).

3.2.3 Identification of new Rap protein regulators of motility and biofilm formation

Activity of DegU~P is regulated by RapG (Table 3.1). RapG can also regulate genetic competence (Figure 3.3A). We tested several remaining endogenous Rap proteins, and plasmid-borne Rap60 for their ability to additionally regulate motility. We created a reporter strain using a P*hag-lacZ* fusion to monitor transcriptonal activation of the motility pathway. Into this reporter strain, we introduced constructs to singly over-express each Rap protein in the absence of the cognate *phr* gene. We monitored the effect of DegU-dependent transcriptional activation of P*hag-lacZ* by measurement of β-galactosidase activity. As with the assays described above, the minimal concentration of

inducer was used to express each Rap protein in order to achieve maximal repression of the reporter.

We found RapB to inhibit DegU, as shown by a reduction in β-galactosidase activity from the P*hag-lacZ* promoter (Figure 3.4D). *RapA* over-expression resulted in a 40% increase in β-galactosidase activity as compared to vector-only. This activity was both reproducible, and statistically significant ($P < 0.0001$). Interestingly, over-expression of *rapG* had no effect on β-galactosidase activity. RapG has been shown to regulate exoprotease production, but not specifically motility. Perhaps regulation of *hag* is too far downstream from the activity of RapG, and *fla/che* would be a better target for testing. Further studies are required to determine precisely where in the motility pathway RapA and RapB function.

3.2.4 Identification of amino acids important for Rap60 activity

Structural and biochemical studies from the Neiditch laboratory provide insight into how Rap proteins interact with ComA and Spo0F to regulate their activities. Specifically, the RapH-Spo0F co-crystal structure revealed amino acids present in the α-helix 3 and the carboxyl terminal tetratricopeptide repeat (TPR) sequences of RapH important for interacting with Spo0F (Parashar, *et al.*, 2011, Figure 3.5A). Similarly, the co-crystal structure of RapF in complex with the DNA binding domain of ComA identified several amino acids present in the amino terminus of RapF important for interacting with ComA (Baker and Neidtich, 2011, Figure 3.5A). Not surprisingly, these amino acids are highly

conserved among Rap proteins that regulate genetic competence and sporulation (Singh, *et al*., 2013, Figure 3.5A). To determine if Rap60 utilizes similar residues, single alanine substitutions were tested for their effects on the activities of Spo0A and ComA using P*spoIIA-lacZ* and P*srfA-lacZ* fusions, respectively. In addition, mutants of Rap60 were purified and tested for Spo0F~P phosphatase activity and binding to ComA *in vitro*.

A highly conserved glutamine, present in helix 3, constitutes the catalytic residue of RapH responsible for dephosphorylating Spo0F~P (Parashar, *et al.*, 2011, Figure 3.5A). Not surprisingly, substitutions to alanine or asparagine at the equivalent position 46 of Rap60 abolished Spo0F~P phosphatase activity (Figure 3.5D) resulting in increased transcription of P*spoIIA-lacZ* (Figure 3.5B, black bars). Interestingly, the catalytically defective phosphatase mutants still inhibited KinA autophosphorylation similar to wild type Rap60 (Figure 3.5E). This might explain why we observe a 20% decrease in β-galactosidase activity from cultures over-expressing Rap60(Q46A) or Rap60(Q46N) compared to vector (Figure 3.5B, black bars). Inhibition of KinA autophosphorylation would result in less Spo0A~P in the cell and a decrease in transcription of early sporulation genes. Rap60(Q46N) had no effect on ComA activity as judged by ternary complex formation (Figure 3.5C) and the regulation of *srfA* transcription (Figure 3.5B, gray bars). This suggests that the overall structure of Rap60 is not affected by these substitutions. Taken together, these data indicate that, like RapH, Rap60 utilizes two very distinct regions of the protein to regulate the activities of Spo0F and ComA. Moreover, different regions of Rap60 appear to be important for regulating the phosphorylation of

Spo0F and KinA, further expanding the role of Rap60 in modulating the sporulation response.

Six amino acids present in RapF comprise the ComA binding interface (Baker and Neidtich, 2011, and Figure 3.5A, marked as C). Alanine substitutions at 4 out of the 6 equivalent positions were constructed in Rap60 and their effects on Rap60 activity was determined. Amino acid D70 of Rap60 was omitted from our mutational analysis since a glutamic acid to alanine substitution at the equivalent position in RapF (E71A) had no effect on RapF binding to ComA (Baker and Neidtich, 2011). Similarly, Q78 of the RapF-ComA binding pocket was also omitted from our analysis since it is difficult to discern the equivalent amino acid in Rap60 due to gaps in the alignment and a general lack of conservation among different Rap proteins in this region (Figure 3.5A). Instead, we focused our mutagenesis on positions 23, 26, 27, and 66 of Rap60.

The proline at position 27 of RapF (equivalent to R26 in Rap60) is particularly important because it is predicted to redirect α-helix 2 to form the ComA binding pocket (Baker and Neiditch, 2011). Interestingly, a substitution to alanine or proline at the equivalent position 26 of Rap60 had little effect on Rap60 binding to ComA (Figure 3.5C) and the regulation of *srfA* transcription (Figure 3.5B, gray bars). An alanine substitution at position 27 of Rap60 significantly enhanced the activity of Rap60 resulting in a 40-fold reduction in β-galactosidase activity compared to wild type Rap60 (Figure 3.5B, gray bar). While purified his_6-Rap60(N27A) exhibited a modest increase in binding to ComA

(Figure 3.5C), the magnitude of binding is inconsistent with the 40-fold reduction in ComA activity observed *in vivo*. This discrepancy is likely attributed to partial activity of purified his_6-Rap60(N27A) used in the *in vitro* studies due to extreme insolubility of the mutant protein during the purification process (data not shown). Consistent with this idea, purified his_6-Rap60(N27A) also exhibited decreased Spo0F~P phosphatase activity compared to wild type Rap60 (Figure 3.5D), despite inhibiting *spoIIA* transcription to wild type levels (Figure 3.5B, black bars).

Single alanine substitutions at positions 23 (F to A) and 66 (L to A) of Rap60 disrupted ternary complex formation (Figure 3.5C) resulting in increased β-galactosidase from P*srfA*-*lacZ*, similar to strain KB22 containing an empty vector (Figure 3.5B, gray bars annotated with an "X"). These amino acids likely make direct contacts with ComA without adversely affecting the overall structure of the protein since Rap60(F23A) and Rap60(L66A) both retain the Spo0F~P phosphatase activity (Figure 3.5D) and inhibit *spoIIA* transcription (Figure 3.5B, black bars). Taken together, Rap60 appears to utilize a small subset of the equivalent amino acids that comprise the RapF-ComA interface to regulate the activity of ComA.

3.3 Discussion

In this work, we characterized endogenous Rap proteins for their ability to regulate transcriptional activation in the early genetic competence, early sporulation, and motility pathways. Previous work in the Kuipers, Perego, and Neiditch groups, as well as the

work described herein, have shown some Rap proteins to have specificities for multiple regulatory proteins (Smits, *et al.*, 2007; Parashar, *et al.*, 2013b; Boguslawski, Hill and Griffith, 2015). We expanded upon the known Rap proteins exhibiting multiple specificities to include RapA, RapB, RapE and RapJ. We also moved forward work in classifying the mechanisms of action of Rap protein regulators of ComA by site-directed mutagenesis and biochemical analyses. Using this approach, we were able to precisely define the interaction surfaces of Rap60 important for differentially modulating genetic competence development and sporulation.

While this work contributed to the classification of Rap60 as a Class II Rap protein for the regulation of competence, Raps D, G, K and P remain unclassified. The RapF-ComA interface is comprised of RapF residues at positions 23, 26, 27, 66, 70 and 77 (Baker and Neidtich, 2011). An alignment of Rap protein amino acid sequences shows that RapC shares 100% identity with RapF at these residues (Figure 3.6A) RapH shares identity at 4 out of 6 residues. Raps D, G, K and P share either no identity, or a single identical amino acid at these residues with characterized Class I or Class II Rap proteins. We predict that these Rap proteins may perform anti-activation by a yet undiscovered third mechanism, or perhaps utilize different surfaces to act by a Class I or Class II mechanism.

3.3.1 Identification of novel Rap protein regulators of developmental processes

In addition to previously known Rap protein regulators of early genetic competence, we found Raps A, B, E, and J, when over-expressed inhibit ComA-dependent transcription of

early genetic competence genes. Similarly, over-expression of RapG inhibited Spo0A-depenent transcription of early sporulation genes. Over-expression of RapA and RapB inhibited and increased transcription of motility genes, respectively.

Although beyond the scope of this work, it would be interesting to determine if additional *rap* genes found on mobile genetic elements are capable of modulating additional biological processes. Specifically, RapI, found within the *B. subtilis* genome on ICE*Bs1* should be assayed for inhibition of early sporulation genes. Additionally, Rap20 could be assayed for activity regulating both competence and sporulation, and RapXO1 from *Bacillus anthracis* could be assayed for regulation of genetic competence. Furthermore, RapG could be purified along with proteins of the sporulation phosphorelay system to precisely determine its role in regulating sporulation. The effects of RapA and RapB on motility should be confirmed by functional assay, including assays for swarming motility. Moreover, the role of RapA and RapB in the regulation of DegU activity can be determined using similar *in vitro* analyses as described in this work for ComA (genetic competence) and Spo0A (sporulation) activity.

This work raises the question of why bacteria would regulate complex biological processes with population density through Rap-Phr pairs? The ability to uptake DNA, either foreign, or from other *Bacillus* species, offers the cell several advantages. One proposed advantage to becoming competent is the ability to utilize exogenous DNA as a source of food (Redfield, 1993). This is a plausible benefit, as development of genetic

competence in *B. subtilis* is regulated by nutritional mechanisms, in addition to cell density (Serror and Sonenshein, 1996). Another potential advantage is the ability to utilize homologous DNA as a template for DNA repair. We know this can occur, as transformation has been shown to increase survivorship of *B. subtilis* in the face of a challenge by UV light (Michod, *et al.*, 1988). A third benefit of the ability to uptake DNA is the chance to try out new genes to compete in a different environmental niche. In fact, *B. subtilis* and *Bacillus licheniformis* have been demonstrated to spontaneously exchange DNA in the soil (Duncan, *et al.*, 1989). This exchange of genetic material could, undoubtedly, benefit the cell if genes whose protein products provided a selective advantage over competing organism were integrated into its genome. It makes sense to have these processes regulated with population density. As more cells accumulate in the population, cells eventually die and release their cellular contents, including DNA. Regulating the genetic competence machinery with population density helps to ensure there is extracellular DNA present for uptake.

The formation of endospores and dissemination of mobile genetic elements allow cells to preserve their genetic material. Choosing to become an endospore grants the cell protection from a number of environmental conditions. For example, *Bacillus* endospores are resistant to UV radiation, dessication, and heat (Nicholson, *et al.*, 2000). Thus, the endospore may survive to germinate under better conditions and pass its genetic information onto daughter cells. ICEs have been shown to contain genes granting benefits such as antibiotic resistance and metabolic flexibility, in addition to genes encoding

proteins for their own maintenance and dissemination via conjugation (Burrus and Waldor, 2004). Plasmids and phage offer similar traits. Both endospores and mobile genetic elements benefit by regulating their activity in response to population density. If a population reaches a high density, cells will be at a higher risk of being in an environment deficient in resources. Sufficient population density also ensures mobile genetic elements find a new host to propagate their lineage.

Biofilm formation and cellular motility are inversely related processes. Cells that become motile may move to colonize new locations with greater resources. Biofilm formation provides yet another set of beneficial characteristics. Cells that produce biofilms provide additional benefits to the whole community by protecting it from environmental assaults. Being in a biofilm also allows for the sharing of resources and the easier conductance of cell-cell signaling. Cell-cell signaling helps the population to coordinate regulation of these complex processes in a way that maximizes chances of survival. Thus, this population density-dependent response allows biofilm formation and exoprotease production to occur at high cell density, while low cell density is reserved for cellular motility This inverse relationship is logical. Cells at low density would not best be able to take advantage of the communal benefits of a biofilm.

A complete characterization of Rap protein function could help us to further understand the broad range of biological processes controlled by cell-cell signaling. The density-dependent processes described above are exquisitely regulated in both time and space, so

that only a fraction of cells in a population are expressing a given biological program at any one time. *Rap-phr* quorum sensing pairs play a key role in this orchestration. This fact is supported by the existence of so many of these genes - 11 on the *B. subtilis* chromosome, and many more on mobile genetic elements. Moreover, some *rap*-phr pairs are shown to exhibit multiple specificities in their regulation. We have expanded upon this subset of Rap proteins here. What remains to be uncovered are the functional effects of the transcriptional regulation we have shown above. Growth conditions contribute greatly to cell fate in *B. subtilis*. Transcriptional regulators involved in this differentiation also control transcription of *rap-phr* pairs. These condition-dependent changes in gene activation not only suggest the need to assay the physiological relevance of our findings, but also speak again to the complex regulation of the quorum response. Further exploration of the functions of *rap-phr* pairs could help us to understand the nature of the processes they regulate, and uncover novel mechanisms of regulation by RRNPP family members, as described in our work above.

In summary, the work presented here expands upon both mechanisms and roles of regulatory proteins involved in the control of quorum sensing pathways in *B. subtilis*. The bulk of the work presented seeks to explain the mechanism behind ComA activation. Toward this end, we conducted a genetic screen with the aim of isolating constitutive mutants of ComA, capable of bypassing regulation by its cognate histidine kinase, ComP. We were able to isolate 38 single ComA^{cons} mutants, and worked to characterize 9 of these with the greatest transcriptional activity, both *in vivo* and *in vitro*. Our work found

that many of these mutants still exhibited density-dependent regulation. Furthermore, most of these mutants exhibit no greater affinity for DNA than wild type ComA, even in the presence of small molecule phosphodonors. Unexpectedly, none of the ComA^{cons} mutants tested here were transcriptionally active with an additional alanine substitution at position 55, the aspartic acid phosphorylated by ComP during ComA activation. Our isolated mutants were, however, consistent with a computational model of ComA activation arrived at by our collaborator, Sergey Savinov. In a first step at validating this model, we created combinations of constitutive mutations that resulted in increased transcriptional activity, but that did not bypass the requirement for an aspartic acid present at residue 55. Taken together, this work speaks to the importance of phosphorylation in ComA activation, and hints at the the importance of small effects in ComA's ability to activate transcription. Our work also helped to define Rap60 as a new class of ComA anti-activator. Previous work from our laboratory group showed Rap60 to negatively regulate ComA-dependent transcription. That work also found that Rap60 does not prevent ComA-DNA binding, as with previously characterized anti-activators of ComA, such as RapC and RapF. Here, we have shown that Rap60 utilizes an overlapping, but distinct interaction surface with ComA, as compared to RapF.

In addition to our work on mechanisms of regulation of quorum sensing, we have also expanded upon the identified roles of known regulators of the quorum response. We assayed endogenous Rap proteins for their ability to regulate transcription in three pathways: genetic competence, sporulation and motility. This allowed us to identify new

transcriptional regulation of the early genetic competence pathway by RapB, Rap E and RapJ. We also found RapG to regulate transcription of early sporulation genes, and RapA and RapB to regulate late motility genes. These findings, both mechanistic and role-related, surrounding the complex regulation of the quorum response in *B. subtilis* are consistent with the benefit derived from maintaining developmental diversity within a population.

3.4 Materials and Methods

3.4.1 Growth media

Liquid cultures of *B. subtilis* were grown in LB, Difco nutrient broth sporulation medium (Harwood and Cutting, 1990), MSgg medium (Branda et al., 2001), or S7 defined minimal medium salts (Vasantha and Freese, 1980) containing 50 mM 4-morpholinepropanesulfonic acid instead of 100 mM ($S7_{50}$) and supplemented with 1% glucose, 0.1% glutamate, tryptophan (40 µg/ml), phenylalanine (40 µg/ml), and threonine (200 µg/ml), where appropriate. *B. subtilis* was grown on solid medium plates containing Spizizen salts (Harwood and Cutting, 1990) supplemented with 1% glucose, 0.1% glutamate, and amino acids, where appropriate. LB agar plates were used for routine cloning and growth of *B. subtilis* and *E. coli*. The following concentrations of antibiotics were used: ampicillin (100 µg/ml), neomycin (2.5 µg/ml), tetracycline (8-10 µg/ml), phleomycin (5 µg/ml), chloramphenicol (5 µg/ml), spectinomycin (100 µg/ml), and erythromycin (0.5 µg/ml) and lincomycin (12.5 µg/ml) together to select for macrolidelincosamide-streptogramin B (MLS) resistance.

3.4.2 Plasmids, strains, and alleles

Escherichia coli strains DH5α and AG1111 (a MC1061 derivative with F'(*lac*I^{q}) *lacZM15 Tn10*) were used for routine cloning. *B. subtilis* strains were derived from the parental strain JH642 (*trpC2 pheA1*) (Perego et al., 1988) or strain DS2569 which is cured of the plasmid pBS32 and is a prototrophic derivative of strain NCIB3610 (Konkol et al., 2013).

3.4.3 Promoter-*lacZ* fusions

Promoter fusions to *lacZ* were constructed and integrated into the *B. subtilis* chromosome as in Boguslawski, Hill and Griffith, 2015.

3.4.4 Pspank-*rap* and *phr*

Constructs were engineered to express of various Rap proteins from the inducible Pspank promoter and P(hy)spank promoter and integrated into the *B. subtilis* chromosome at the *amyE* locus. Primers were used to amplify by PCR the gene encoding r*ap60*, *phr60*, and *rap60-phr60* from pTA1060 plasmid DNA and endogenous Rap proteins from JH642 genomic DNA. Each forward primer contains the sequence 5'-GCTTAGTGAAGCTT**TAAGGAGG**TATACAT*ATG*-3' with a *HindIII* restriction site underlined, an optimal ribosome binding sequence in bold, the initiation codon in italics, and 15-17 bp of gene-specific sequence immediately downstream of the ATG beginning with amino acid 2 of each gene. The reverse primers have the sequence 5'-

TGCTACGAGCATGC**TTA**-3’ with a *SphI* restriction site underlined, the termination codon in bold, and 15-17 bp of gene-specific sequence preceding the termination codon. PCR products were digested with *HindIII* and *SphI* restriction enzymes and ligated into pDR110 (kind gift from D. Rudner), which was also digested with the same two restriction enzymes. Ligation reactions were transformed into strain DH5α and plated on LB solid medium with ampicillin. Correct clones were verified by DNA sequencing. Plasmid DNA was linearized by digestion with *NcoI* restriction enzyme, transformed into *B. subtilis* strain JH642, and plated on LB solid medium with spectinomycin. Single amino acid substitutions were created in Pspank-*rap60* by PCR SOE (Horton et al., 1990), using the same procedure as described above.

3.4.5 Modified pTA1060

Plasmid pTA1060 DNA was isolated from *B. amyloliquefaciens* strain IFO3022 using the alkaline lysis method according to the manufacturer (Qiagen) with a single modification. Cell pellets were resuspended in P1 buffer with the addition of lysozyme (3 mg/ml), and allowed to incubate for 20 min at 37ºC. Several modifications were made to plasmid pTA1060 to make it more suitable to genetic manipulation. First, a medium copy pBR322 origin of replication and a gene encoding ampicillin resistance were cloned into pTA1060 for selection and plasmid maintenance in *E. coli*. Primers were used to amplify a 2.8 KB region of plasmid pBR322 DNA containing the origin of replication and the gene encoding ampicillin resistance. Unique restriction endonuclease sites were engineered into each primer flanking the pBR322 cassette. The PCR product and plasmid pTA1060

were digested with *NcoI* restriction enzyme and ligated with T4 ligase. The ligation mixture was transformed into strain DH5α and plated onto LB solid medium supplemented with ampicillin. Plasmid DNA was isolated and was used in a second step to introduce an erythromycin resistance gene for selection of pTA1060 in *B. subtilis*. Primers were used to amplify a 1.2 KB fragment containing the erythromycin resistance gene from plasmid pHP13. The restriction enzyme sites *XhoI* and *SpeI* were engineered into the primers for directional cloning into the modified pTA1060 plasmid. An erythromycin resistant colony was selected and the plasmid was confirmed by restriction digests. These modifications were introduced into the intergenic region of pTA1060 between *orf7* and the *mob* gene flanked by transcriptional terminators to prevent read through transcription into surrounding plasmid genes. The modified plasmid was named pTA1060*::erm*, but is referred to in the text simply as pTA1060.

Plasmid pTA1060-Δ*rap60*-Δ*phr60* was created by digesting the modified pTA1060 plasmid with *NaeI* restriction enzyme. *NaeI* recognition sites are present in the middle of *rap60* and ~250 bp downstream of the *phr60* coding sequence. The fragment corresponding to pTA1060 was gel purified from the *rap60-phr60* fragment and re-ligated with T4 ligase. Plasmid pTA1060-Δ*phr60* was created by introducing two tandem termination codons early in the coding sequence of *phr60* by add-on PCR.

3.4.6 pBAD-his$_6$-tagged proteins

Constructs to over-express N-terminal hexa-histidine fusion proteins were created by PCR amplification of the genes of interest from *B. subtilis* genomic DNA. Rap60 was amplified from the modified pTA1060 plasmid. Each forward primer contains the sequence 5'-GCTTAGTGGGTACC**AAGGAGA**TATACAT*ATG*catcaccatcaccatcac-3' with a *KpnI* restriction site underlined, an optimal ribosome binding sequence in bold, the initiation codon in italics, the his_6-tag in lowercase, and 15-17 bp of gene-specific sequence immediately downstream of the his_6-tag beginning with amino acid 2 of each gene. The reverse primers contain the sequence 5'-TGCTACGAGCATGC**TTA**-3' with a *SphI* restriction site underlined, the termination codon in bold, and 15-17 bp of gene-specific sequence preceding the termination codon. PCR products were digested with *KpnI* and *SphI* restriction enzymes and ligated into pBAD-Cm33 or pBAD-Ap18, which was also digested with the same two restriction enzymes. Ligations were transformed into strain DH5α and plated on LB solid medium with the appropriate antibiotic. Correct clones were confirmed by DNA sequencing. Amino acid substitutions were created in pBADCm33-Nhis_6-Rap60 by PCR SOE, using the same procedure as described above.

3.4.7 pET-his_6-SUMO-tagged proteins

Constructs to over-express Nhis_6-SUMO fusion proteins were created by amplifying *spo0F*, *spo0B*, and *comA* from *B. subtilis* genomic DNA. Each forward primer contains the sequence 5'-GCTTAGTGACC**GGTGGT**-3' where the *AgeI* restriction site is underlined, two codons corresponding to glycine are in bold (required for efficient cleavage by his_6-Ulp1), and 15-17 bp of gene-specific sequence immediately downstream

of the last glycine beginning with amino acid 2 of each gene. The downstream primer contains the sequence 5'-TGCTACGAGCGGGCCGC**TTA** with a *NotI* restriction site underlined, the termination codon in bold, and 15-17 bp of gene-specific sequence preceding the termination codon. PCR products were digested with *AgeI* and *NotI* restriction enzymes, and ligated into pET-his$_6$-SUMO (Lee et al., 2008) that was digested with the same two enzymes. Ligations were transformed into strain DH5α and plated on LB solid medium with ampicillin. The correct clones were confirmed by DNA sequencing.

3.4.8 Oligonucleotides and peptides

All oligonucleotides used in this study were synthesized and desalted by Integrated DNA Technologies. Sequences are available upon request. Peptides used in this study were synthesized using solid phase peptide synthesis and purified using reverse phase HPLC by AnaSpec, Inc. Peptide concentrations were determined by the manufacturer and found to be >95% pure for full-length protein using mass spectrometry (AnaSpec, Inc).

3.4.9 Computational analyses

Statistical analyses were performed using Microsoft Excel and the suite of statistical tools from www.in-silico.net. Single sample student *t*-tests were performed and a *p*-value of 0.01 was used as a cut-off for statistical significance. Alignments of the primary amino acid sequences of the Rap proteins were made using the ClustalW program from the Galaxy Project (www.usegalaxy.org). Standard default program settings were used.

3.4.10 Colony morphology and pellicle formation

Overnight cultures were inoculated in LB at 30ºC. A 10 µl aliquot was spotted onto MSgg plates that were poured fresh the day before and placed in a laminar flow hood for 20 min prior to inoculating with bacteria. Inoculated plates were left at 24ºC for 3 days prior to being photographed with a Hamatsu camera using a UVP gel documentation system. To assess pellicle formation, 10 µl of fresh overnight cultures were added to 3 ml of liquid MSgg medium in 6 well microtiter plates (Corning). The microtiter plates were left undisturbed at 24ºC for 3 days prior to being photographed as described above.

3.4.11 Growth conditions and β-galactosidase assays

Spo0A activity was monitored in reporter strains by inoculating single colonies from an LB plate into shaker flasks containing DSM medium to measure sporulation genes. To monitor ComA activity, overnight cultures of reporter strains were grown as light lawns on Spizizen minimal medium plates and used to inoculate shaker flasks containing $S7_{50}$ minimal medium (ComA activity) at a final OD_{600} ~0.02 as described in Griffith and Grossman, 2008.

Liquid cultures were grown in shaker flasks at 37ºC with vigorous aeration. One ml aliquots were removed and placed in a 2.2 ml 96-well polypropylene block, which was stored at -20ºC until time to assay β-galactosidase activity. A second aliquot was taken to determine OD_{600}. β-galactosidase assays were performed as previously described (Griffith

and Grossman, 2008). Briefly, cells were prepared by thawing to room temperature, adding 20 µl of toluene to each well, and permeabilizing cells directly in the block by vigorous pipetting up and down using a multi-channel pipettor. The assay was initiated with the addition of 20 µl freshly prepared Ortho-nitrophenyl-β-D-galactopyranoside (4 mg/ml) and terminated with the addition of 40 µl 1 M Na_2CO_3. Cell debris was pelleted in a microtiter plate by centrifugation at 3000g for 10 min. A 100 µl aliquot of each supernatant was transferred to a new plate using a multichannel pipettor. A_{420} was determined using a SpectraMax plate reader (Molecular Dynamics) and data analysis was performed using Microsoft Excel. β-galactosidase specific activity was calculated as follows: 1000 x [(ΔA_{420}/min/ml)/OD_{600} of culture].

3.4.12 Protein expression and purification

A fresh overnight culture of strain DH5α containing the appropriate plasmid was diluted 1:100 into LB (Fisher) containing the appropriate antibiotic. Cultures were grown to OD_{600} ~0.5 at 37ºC with vigorous aeration. L-arabinose (Sigma) was added to a final concentration of 0.2% to induce expression from pBAD plasmids and 1 mM IPTG was used to induce expression from pET vectors. Cultures over-expressing his_6-tagged KinA and Rap60 were induced for 48 hrs at 25ºC and 15ºC, respectively. All other cultures containing his_6-tagged constructs were induced for 5-6 hrs at 37ºC. Cells were harvested after induction by centrifugation at 5,000g for 10 min at 4ºC and cell pellets were stored at -20ºC until further use.

His_6-tagged proteins were purified by standard Ni-NTA chromatography as previously described (Griffith and Grossman, 2008). The cell pellet from 1L of culture was thawed on ice, resuspended in 10 ml sonication buffer (10 mM Tris pH 8, 0.3 M NaCl, 5% glycerol, 5 mM imidazole, 5 mM β-mercaptoethanol, and 5 mM $MgCl_2$), and cells were lysed by sonication using a Branson sonifier (10 cycles of 20 sec on and 40 sec off, at setting 6). The culture was cleared by centrifugation at 12,000 rpm for 30 min at 4ºC, and the cell extract was passed over 2 ml of Ni-NTA (Qiagen). After 10 washes with 25 ml of sonication buffer, his_6-tagged proteins were eluted from the column in 10 ml sonication buffer with increasing concentrations of imidazole (25 mM, 50 mM, 120 mM, 200 mM, and 300 mM). For his_6-Rap60 and his_6-KinA, two additional washes in sonication buffer containing 25 mM and 50 mM imidazole were performed prior to elution in buffer containing 120 mM, 200 mM, and 300 mM imidazole. Fractions were analyzed for purity by SDS-PAGE followed by Coomassie staining. Fractions with the greatest purity were pooled and dialyzed at 4ºC against 3 buffer changes of 2L dialysis buffer (10 mM Tris pH 8, 0.3 M NaCl, 5% glycerol, 10 mM β-mercaptoethanol, and 5 mM $MgCl_2$). Dialyzed proteins were concentrated to 5-15 mg/ml using a Centricon-10 (Amicon), with the exception of $Nhis_6$-Rap60 which remained soluble ≤ 1 mg/ml. His_6-Ulp1 and his_6-RapB were stored at -20ºC in dialysis buffer. His_6-tagged proteins Rap60, KinA, and RapC were stored 4ºC in dialysis buffer and remained stable for several months without significant loss of activity (data not shown). The remaining his_6-tagged proteins were stored at -20ºC in dialysis buffer containing 40% glycerol. Protein concentrations were determined by Bradford assay using bovine serum albumin as protein standard. Protein preparations

were estimated to be >95% pure as determined by SDS-PAGE followed by Coomassie staining (data not shown).

3.4.13 *In vitro* cleavage with Nhis$_6$-Ulp1

Spo0F, Spo0B, and ComA were purified as N-terminal his$_6$-SUMO fusion proteins, similar to the methods described above. His$_6$-Ulp1 protease was also purified by Ni-NTA as previously described (Lee et al., 2008). Purified his$_6$-Ulp1 was added to the pooled his$_6$-SUMO-tagged protein fractions in a 1:10 ratio (Ulp1 to his$_6$-SUMO-tagged proteins) and dialyzed against 3 buffer changes of 1L dialysis buffer at 4^{o}C (see above). Ni-affinity chromatography was used to remove the his$_6$-tagged proteins away from the native proteins. Briefly, the imidazole concentration of each sample was adjusted to 10 mM prior to incubation with 0.3 ml Ni-NTA (Qiagen) at 4^{o}C for 1 hr. The flow through contained only native Spo0F, Spo0B, or ComA and no his$_6$-tagged proteins were present as shown by SDS-PAGE followed by Coomassie staining (data not shown). Native proteins were concentrated using a Centricon-10 device (Amicon). Protein concentrations were determined by Bradford assay.

3.4.14 Gel mobility shift assays

DNA corresponding to the minimal optimal ComA binding sequence was prepared by annealing two complementary oligonucleotides containing the following sequence: 5'-tcaTTGCGGcatcCCGCAAgaaactTTGCGGtc -3'where the bases in uppercase represent Recognition Elements 1-3. DNA templates contain bases 5' –TCA preceding the ComA

binding sequence and bases TC- 3' following it and are underlined. One of the oligonucleotides from each pair was labeled on its 5' end using γ-^{32}P-ATP (Perkin Elmer) and T4 polynucleotide kinase (NEB). The kinase reaction was terminated by incubation at 70°C for 20 min. A 1.3-fold molar excess of the complimentary oligonucleotide was added to the mixture and heated to 95°C for 5 min, followed by slow cooling to room temperature to facilitate annealing of the oligonucleotides. Duplex DNA was purified away from the unincorporated label using a G-25 Centrispin 10 column (Princeton Separations).

In vitro binding reactions contained 13 mM Tris pH 8, 50 mM EPPS pH 8.5, 20 mM $MgCl_2$, 0.1 mM EDTA, 100 mM KCl, 3 mM DTT, and 10% glycerol in a 20 µl final volume. Radiolabeled DNA (5 nM) was added to the binding reaction along with the appropriate amount of his_6-tagged Rap and ComA, and 300 µM purified hexapeptide, where appropriate. Protein-DNA complexes were allowed to equilibrate at 24°C for 30 min, prior to the addition of 5 µl of 5X agarose gel loading dye. Samples were loaded into the wells of a 10% polyacrylamide gel containing 5% glycerol and electrophoresed into the gel at 300 V. Once the loading dye had entered the gel, the voltage was reduced to 120 V and gels were run for 5-6 hr at 4°C. Gels were dried and analyzed using a Typhoon PhosphorImager (Molecular Dynamics). The amount of free DNA and DNA in complex with ComA and Rap60 was determined using ImageQuant software (Molecular Dynamics) and Microsoft Excel.

3.4.15 Dephosphorylation of native Spo0F~P and Spo0B~P

To determine if Rap proteins act as phosphatases of Spo0F and Spo0B in the absence of other phosphorelay proteins, native Spo0F or Spo0B was first radiolabeled with γ-^{32}P-ATP in the presence of his_6-tagged phophorelay proteins for 1 hr at 24ºC. The his_6-tagged proteins were purified away from the native proteins using Ni-NTA chromatography. Briefly, 20 mM imidazole was added to the reaction mixture prior to incubation with 100 µl Ni-NTA (Qiagen). The tube was gently agitated for 30 sec. Native Spo0F and Spo0B were each recovered after centrifugation at 500 rpm for 30 sec to pellet the his_6-tagged proteins bound to the Ni-NTA resin. Approximately 50% of native Spo0B and 10% of native Spo0F was recovered from the chromatography step and no his_6-tagged proteins were recovered as shown by SDS-PAGE followed by autoradiography (data not shown). The concentration of his_6-Rap was adjusted to maintain a 1:2 molar ratio of Spo0F or Spo0B to his_6-tagged Rap protein. Aliquots were removed at the specified times and subjected to SDS-PAGE. Gels were analyzed as described above.

3.5 Tables and Figures

Table 3.1. Known functions of *Bacillus* Rap Proteins

Rap Protein	Process(es) Regulated	Target(s) of Rap	Reference(s)
Genomically-Encoded Rap Proteins			
RapA	Sporulation	Spo0F~P	Perego, *et al.*, 1994; Perego and Hoch, 1996; Fawcet, *et al.*, 2000
RapB	Sporulation	Spo0F~P	Perego, *et al.*, 1994; Perego, 1997; Fawcet, *et al.*, 2000
RapC	Competence	ComA	Core and Perego, 2003; Comella and Grossman, 2005; Solomon, *et al.*, 2007
RapD	Competence		Ogura and Fujita, 2007
RapE	Sporulation	Spo0F~P	Fawcet, *et al.*, 2000; Jiang, *et al.*, 2000
RapF	Competence	ComA	Bongiorni, *et al.*, 2005; Comella and Grossman, 2005; Auchtung, *et al.*, 2006; Baker and Neiditch, 2011
RapG	Degradative Enzyme Production	DegU	Kunst, *et al.*, 1997; Mader, *et al.*, 2002; Hayashi, *et al.*, 2006; Ogura, *et al.*, 2006
RapH	Sporulation, Competence	Spo0F~P, ComA	Kunst, *et al.*, 1997; Hayashi, *et al.*, 2006; Ogura, *et al.*, 2006; Smits, *et al.*, 2007; Parashar, *et al.*, 2011
RapI	Competence, ICE*Bs1* gene expression, excision, and transfer		Auchtung, *et al.*, 2005; Parashar, *et al.*, 2013a; Singh, *et al*, 2013
RapJ	Sporulation	Spo0F~P	Parashar, *et al.*, 2011
RapK	Competence		Auchtung, *et al.*, 2006
Rap Proteins on Mobile Genetic Elements			
RapP	Sporulation, Competence, Biofilm Formation	Spo0F~P	Parashar, *et al.*, 2013b; Bendori, *et al.*, 2015
Rap20	Conjugation		Singh, *et al.*, 2013
Rap60	Sporulation, Competence, Cannibalism, Biofilm Formation	ComA, Spo0F~P	Boguslawski, *et al.*, 2015
RapXO1 (BXA0205)	Sporulation	Spo0F~P	Bongiorni, *et al.*, 2006

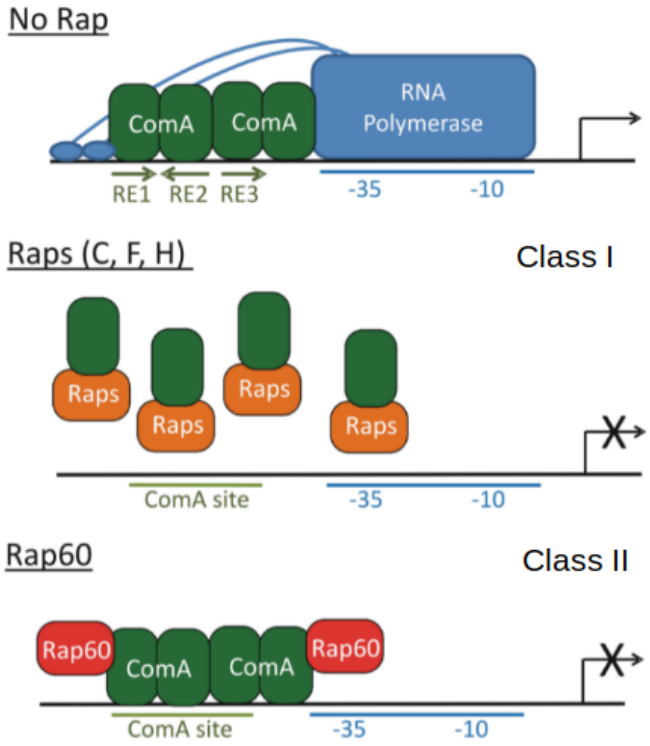

Figure 3.1. Proposed mechanisms of Rap protein regulation of ComA. In the absence of Rap protein, ComA recognizes and binds a tripartite binding sequence upstream of the promoter element recognized by RNA polymerase. Rap proteins C, F, and H bind ComA, and prevent the transcription factor from accessing DNA (Class I). Rap60 does not prevent ComA from binding DNA, but still prevents ComA-dependent transcriptional activity (Class II). Adapted from (Boguslawski, *et al.*, 2015).

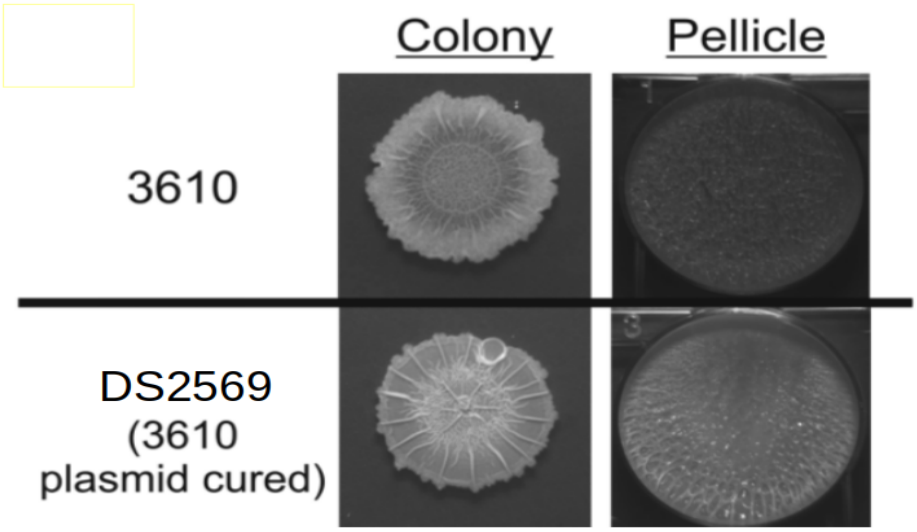

Figure 3.2. Colony morphology of Wild Type and plasmid cured strains of *B. subtilis*. Colony morphology was determined by inoculating 10 uL of an overnight culture onto Msgg solid medium and incubating plates at 24C for 3 days. Pellicle formation was determined by inoculating 10 uL of an overnight culture into 3 mL of liquid Msgg medium in 6-well microtiter plates, followed by incubation at 24C for 3 days (Boguslawski, Hill and Griffith, 2015)

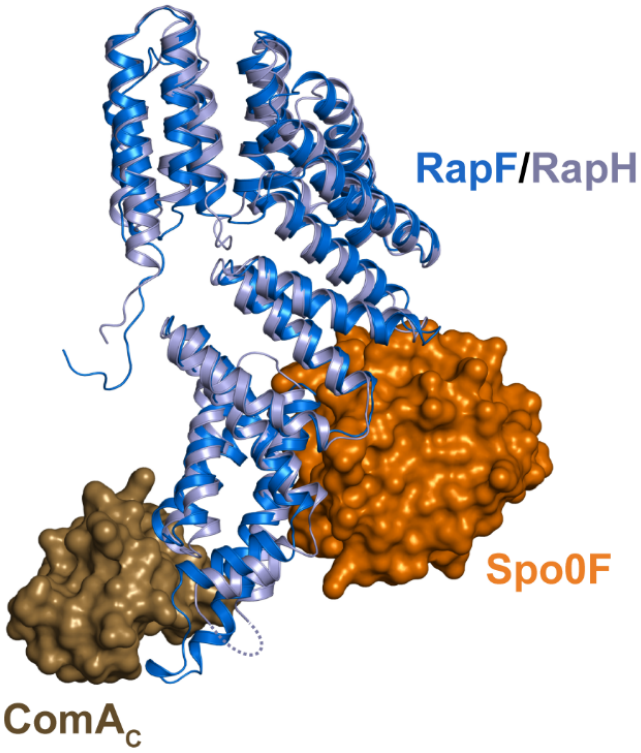

Figure 3.3. Rap proteins bind ComA and Spo0F via different surfaces. Structures of RapF in complex with the DNA binding domain of ComA ($ComA_c$) and RapH in complex with Spo0F. (Parashar, *et al.*, 2011).

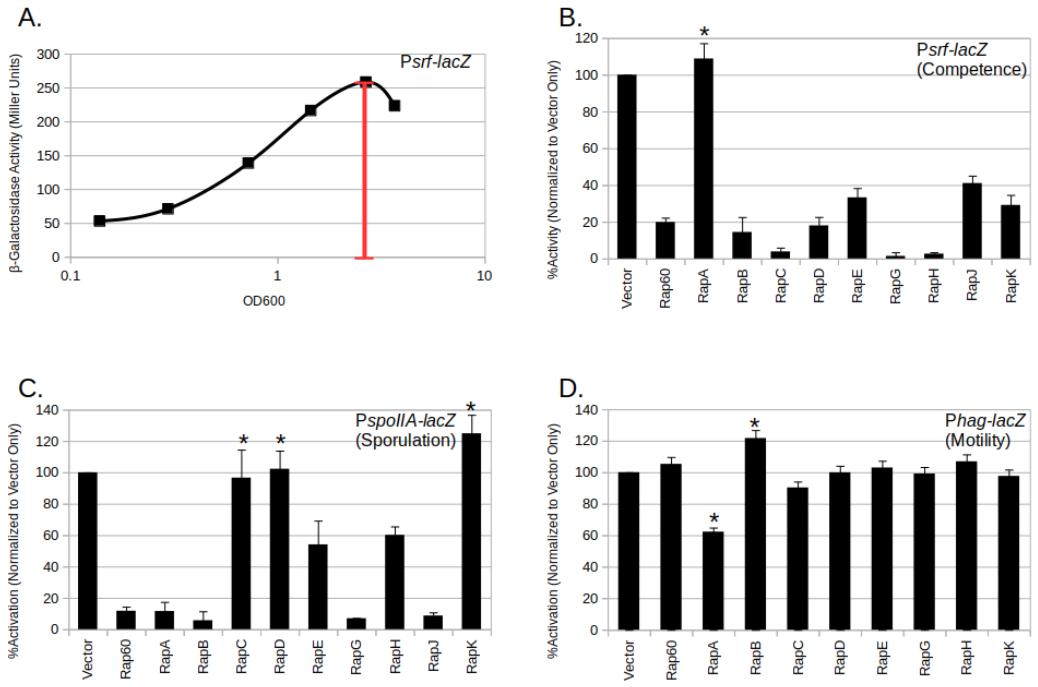

Figure 3.4. Rap proteins differentially regulate different quorum sensing pathways.

A. Representative β-galactosidase assay. Cultures containing promoter-*lacZ* were grown in the appropriate medium with maximally-repressive IPTG (typically 0.1mM). Aliquots were removed at the specified time and β-galactosidase activity was determined. The maximal activity of each culture (red bar, typically around OD_{600} ~1-2) was normalized to reporter strains containing empty vector.

B. Effect of Rap proteins on ComA activity. Cultures contained the *Psrf-lacZ* fusion and Pspank-vector, Pspank-*rap* or Pspank(hy)-*rap*. Experiments were performed in triplicate with standard error shown. All Rap proteins were statistically different from vector-only ($P < 0.0001$), except for RapA, designated with an asterisk (*).

C. Effect of Rap proteins on Spo0A activity. Cultures contained the *PspoIIA-lacZ* fusion and Pspank-vector, Pspank-*rap* or Pspank(hy)-*rap*. Experiments were performed in triplicate with standard error shown. Rap proteins were statistically different from vector-only ($P < 0.01$), except for those designated with an asterisk (*).

D. Effect of Rap proteins on DegU activity. Cultures contained the *Phag-lacZ* fusion and Pspank-vector, Pspank-*rap* or Pspank(hy)-*rap*. Experiments were performed in triplicate with standard error shown. All Rap proteins were not statistically different from vector-only,except for RapA and RapB, designated with an asterisk (*, $P < 0.001$).

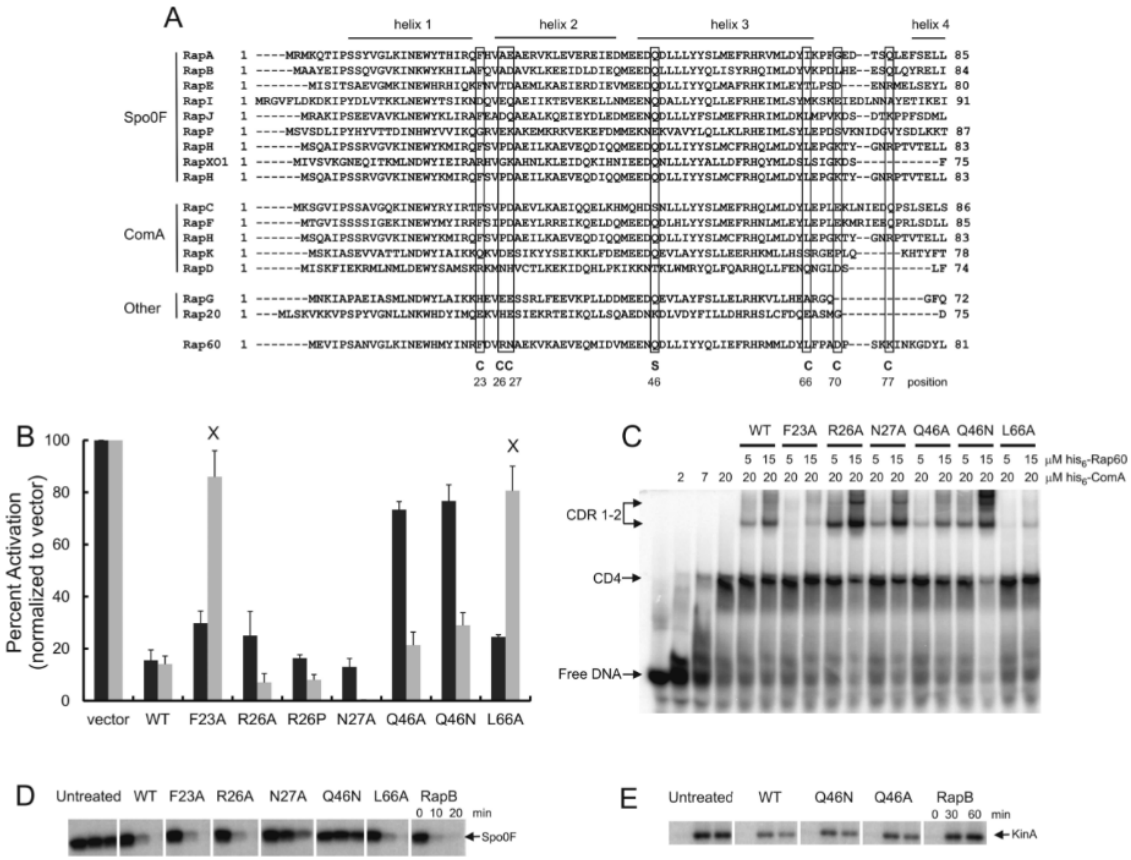

Figure 3.5. Rap 60 Mutants differentially regulate Spo0F and ComA.
A. Alignment of the N-terminal region of Rap proteins from *B. subtilis*. Regions corresponding to the alpha-helices in RapH are indicated on top. Amino acids that comprise the ComA interface in the RapF-ComA crystal structure (Baker and Neiditch, 2011) are indicated with a 'C'. The catalytic Gln47 of RapH, important for dephosphorylation of Spo0F~P, is shown with an 'S'.
B. Cultures containing P*srfA-lacZ* or P*spoIIA-lacZ* fusions along with mutations of Pspank-*rap60* were grown in the appropriate medium with 0.1 mM IPTG. Aliquots were removed at the specified time and β-galactosidase activity was determined. The maximal activity of each culture (typically around OD_{600} ~1-2) was normalized to reporter strains containing empty vector. P*spoIIA-lacZ* (black bars) and P*srfA-lacZ* (gray bars). Experiments were performed in triplicated, and the percent standard error is shown. The transcriptional effects of all the Rap60 mutants were statistically different from vector (*P*-value < 0.01) with the exception of Rap60(F23A) and Rap60(L66A) for *srfA* transcription (denoted by an 'X').

C. Gel mobility shift assaywith DNA containing the ComA binding sequence, purified ComA and mutants of Rap60. Binding conditions are as described in *Materials and methods*. ComA-DNA binary complexes (CD) and ComA-DNA-Rap ternary complexes (CDR).
D. Spo0F~P dephosphorylation by Rap60 mutants.
E. KinA autophosporylation in the presence of Rap60(Q46N) and Rap60(Q46A).

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