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Potassium sensing histidine kinase in *Bacillus subtilis*

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Abstract

The soil-dwelling organism *Bacillus subtilis* is able to form multicellular aggregates known as biofilms. It was recently reported that the process to biofilm formation is activated in response to the presence of various, structurally diverse small molecule natural products. All of these small molecule natural products made pores in the membrane of the bacterium, causing the leakage of potassium cations from the cytoplasm of the cell. The potassium cation leakage was sensed by the membrane histidine kinase KinC, triggering the genetic pathway to the production of the extracellular matrix that holds cells within the biofilm. This chapter presents the methodology used to characterize the leakage of cytoplasmic potassium as the signal that induces biofilm formation in *B. subtilis* via activation of KinC. Development of novel techniques to monitor activation of gene expression in microbial populations led us to discover the differentiation of a subpopulation of cells specialized to produce the matrix that holds all cells together within the biofilm. This phenomenon of cell differentiation was previously missed by conventional techniques used to monitor transcriptional gene expression.

Introduction

Bacterial communities survive in their natural habitats due to their ability to sense environmental changes and respond accordingly. To do this, bacteria have evolved complex sensing apparatuses to monitor external fluctuations, allowing them to adjust their behavior in response to specific cues (Mascher *et al.*, 2006). These cues might come directly from the environment, such as changes in temperature or nutrient limitation, or be small-molecule natural products secreted by neighboring microorganisms.

Small molecules produced by one microorganism can be perceived by other microorganisms cohabitating within the same ecological niche. The small-molecules can serve as signals to activate distinct pathways and trigger changes in gene expression, allowing bacteria to adapt as the environment and other members of the microbial community change. Moreover, these molecules can be produced by members of the bacterial community with the purpose of triggering a self-response, a process known as quorum sensing (Camilli & Bassler, 2006, Ng & Bassler, 2009).

How bacteria respond to the presence of small-molecules varies. Some organisms respond by differentiating, creating a subpopulation of specialized cells that express a certain pattern of genes (Kearns & Losick, 2005). The subpopulation of cells may in turn produce or respond to different signals and serve a distinct function within the community. For example, the soil bacterium *Bacillus subtilis* differentiates several subpopulations of specialized cell types in response to different environmental cues (Lopez *et al.*, 2009b). One of these specialized subpopulations produces the extracellular matrix necessary to hold cells together during the

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process of biofilm formation (Chai *et al.*, 2008). Differentiation of this subpopulation of matrix producers is cued by the activation of a gene regulator termed Spo0A (Lopez *et al.*, 2009a). The active form of Spo0A (Spo0A~P) indirectly activates a regulon of genes that includes the two operons responsible for the production of extracellular matrix, the 15-gene operon *epsA-O* (henceforth simply *eps*) (Branda *et al.*, 2001, Branda *et al.*, 2004), responsible for the production of the extracellular polysaccharide, and the *yqxM-sipW-tasA* operon (henceforth simply *yqxM*), responsible for the production of the matrix-associated protein TasA (Branda *et al.*, 2006, Branda *et al.*, 2004).

Phosphorylation of Spo0A~P to trigger matrix production is driven by the phosphorylative action of two transmembrane sensor histidine kinases, KinC and KinD (Hamon & Lazazzera, 2001, Kobayashi *et al.*, 2008, Jiang *et al.*, 2000, LeDeaux *et al.*, 1995). KinD is a canonical membrane kinase with two transmembrane segments connected by a large extracellular sensor domain, presumably involved in recognizing and binding to an unknown extracellular signal. On the contrary, the membrane kinase KinC shows two transmembrane segments connected by only six amino acids, leaving no room for an extracellular sensor domain. Instead, KinC has a PAS sensor domain localized in the cytoplasmic region of the kinase. PAS domains are signaling modules known to monitor changes in light, redox potential, oxygen, and small ligands in the cytoplasm of the cell (Gu *et al.*, 2000, Taylor & Zhulin, 1999).

Recently, the PAS domain of KinC was described as the domain responsible for sensing the leakage of potassium cations from the cytoplasm of *B. subtilis*, leakage induced by diverse small-molecules able to form pores in the membrane of the bacterium. The leakage of potassium cations serves as the stimulus to activate the sensor kinase and trigger the phosphorylation of Spo0A~P, which in turn, leads to the differentiation of a subpopulation of matrix producers and the formation of biofilm (Lopez *et al.*, 2009a). While the nature of the stimulus is always consistent (potassium leakage caused by the formation of pores in the membrane), the various small-molecules identified that induce matrix production via KinC differ wildly in their molecular structure. Among these molecules are the macrolide polyenes nystatin and amphotericin and the peptide antibiotics gramicidin and valinomycin, all of which are derived from soil-dwelling bacteria. However, perhaps the most interesting small-molecule described to trigger matrix production via KinC is the self-generated lipopeptide, surfactin (Arima *et al.*, 1968, Kluge *et al.*, 1988, Lopez *et al.*, 2009a). Once produced by the community of *B. subtilis*, surfactin causes the leakage of potassium by the formation of pores in the membrane (Sheppard *et al.*, 1991) and that triggers the differentiation of a subpopulation of matrix producers that secretes the extracellular matrix in a new fashion described as the first paracrine signaling molecule in bacteria (Lopez *et al.*, 2009c).

Recognizing the mode of action of a signaling molecule rather than its structure is a remarkable strategy that allows *B. subtilis* to sense a diverse array of signals and to respond not only to self-produced molecules but also to natural products secreted by other soil-dwelling organisms. In this manuscript, we discuss the procedures and protocols used to describe the mechanism of action of the kinase KinC as a membrane kinase that senses potassium leakage from the cytoplasm of the cell. We analyze the methods and techniques used, highlighting particularities and tricks discovered over the course of the study.

Screen for molecules that stimulate KinC sensor kinase

To screen for small-molecule natural products capable of activating biofilm formation in *B. subtilis*, we developed a bioassay to identify one or more substances that, when added to a culture of *B. subtilis* in small concentrations, induce the formation of biofilm. Development of such an assay was remarkably difficult given the intrinsic nature of undomesticated strains of *B. subtilis* to form a biofilm under almost all conditions. The likely reason for constitutive

biofilm production under most conditions is that activation of the cascade to biofilm formation is driven by the phosphorylation of the regulator Spo0A~P by the action of five different kinases, each one of them able to sense different stimuli, most of them unknown (Hamon & Lazazzera, 2001, LeDeaux et al., 1995).

To identify the precise culture conditions under which *B. subtilis* does not produce a biofilm and Spo0A remains dephosphorylated, we cultured *B. subtilis* using different media and various conditions. Following the comparative study, MSgg was chosen as the positive control medium because it was discovered *B. subtilis* always makes biofilm when grown in this medium. MSgg is a defined medium composed of 5 mM potassium phosphate (pH 7), 100 mM Mops (pH 7), 2 mM MgCl₂, 700 M CaCl₂, 50 M MnCl₂, 50 M FeCl₃, 1 M ZnCl₂, 2 M thiamine, 0.5% glycerol, 0.5% glutamate, 50 g/ml tryptophan, and 50 g/ml phenylalanine. MSgg, with glycerol and glutamate as the carbon and nitrogen sources, respectively, is commonly used to induce the process of cell differentiation in *B. subtilis* (Branda et al., 2001). When *B. subtilis* is plated on MSgg and incubated for three days at 30°C, biofilm formation is observed and colonies exhibit several morphological features indicative of the differentiation of distinct cell subpopulations involved in the process of biofilm formation. For example, the production of an extracellular matrix that encases all cells within the biofilm results in the formation of wrinkles on the colony surface (Branda et al., 2004). Similarly, the raising of aerial structures on the surface of the biofilm is indicative of the presence of a subpopulation of sporulating-cells. Spore are localized in the apical area of these aerial structures (Branda et al., 2001, Vlamakis *et al.*, 2008).

In contrast to *B. subtilis* always producing biofilm when grown in MSgg, it was found that *B. subtilis* grown in Luria-Bertani (LB) medium did not show any of the developmental characteristics observed in MSgg, even after extended incubation. *B. subtilis* incubated in LB medium, comprised of 1% tryptone, 0.5% yeast extract, and 0.5% NaCl, developed into colonies composed of a mass of undifferentiated cells, in which cell differentiation was rarely observed according to the occurrence of spores. LB medium proved to be an excellent base medium in which to grow *B. subtilis* in order to screen for small molecules that induce biofilm formation.

To screen each small molecule independently, *B. subtilis* was grown shaken in liquid LB medium rather than stationary on LB agar plates. Upon reaching stationary phase, 1 ml of the shaken culture was used to inoculate each well of a polystyrene 24 well plate. The experimental design allowed each small molecule to be independently tested at many different concentrations because a different concentration of each small molecule could be directly added to a separate well. Surprisingly, simply modifying the protocol to grow *B. subtilis* in LB liquid culture resulted in *B. subtilis* producing a weak biofilm. Without the addition of any small molecule, in liquid LB medium *B. subtilis* produces a weak biofilm or thin floating pellicle. We hypothesize that biofilm development is caused by activation of the master regulator Spo0A~P. Because liquid shaken cultures had reached stationary phase prior to transfer to the 24 well plate, phosphorylation of Spo0A~P had likely already taken place, leading to low level, background biofilm production.

To resolve the issue of producing low background levels of biofilm during the assay, a protocol was developed such that *B. subtilis* cultures were in exponential phase when tested against the library of small molecules. We reasoned that cultures should be grown in exponential phase for prolonged time periods to eliminate any remaining Spo0A~P. In order to maintain cultures in exponential phase, we defined a culture protocol to grow *B. subtilis* at low optical density for almost 12 h. Following the repeated passaging of cultures, cultures did not exhibit any background pellicle formation when incubated standing and undisturbed. We used *B. subtilis*

cultures in exponential phase to assay a battery of small-molecules for their ability to induce *B. subtilis* biofilm formation (detailed protocol provided below).

Protocol to screen for pellicle formation by adding small-molecules

1. Undomesticated *B. subtilis* strain NCIB3610 was cultured overnight in LB medium at 37°C with vigorous agitation.
2. The overnight culture was used to inoculate new LB medium at dilution 1:100. We routinely used 30 µl of culture to inoculate a 15 ml tube containing 3ml of liquid LB. The culture was incubated at 37°C with vigorous agitation until reaching OD₆₀₀ = 0.4.
3. The culture at OD₆₀₀ = 0.4 was used to inoculate new LB medium at dilution 1:100. The new culture was incubated at 37°C with vigorous agitation until reaching OD₆₀₀ = 0.4. The passaging was repeated three additional times, to keep cultures growing in exponential phase for a prolonged period.
4. The final inoculation requires a scale up in volume according to the number of wells that are ultimately needed. A 24 well plate requires 24 ml of LB to be inoculated with 240 µl of culture at OD₆₀₀ = 0.4. When this culture has reached OD₆₀₀ = 0.4, the culture is transferred to the wells (1 ml of culture per well) where a solution of the small-molecule at the desired final concentration was previously placed.
5. 24 well plates were incubated at 30°C with no shaking. To avoid dehydration of the wells, plates were either incubated in a covered container with wet towels at the bottom or wrapped with parafilm.

Several repetitions of each experiment were required to standardize results. Variations due to differential dehydration (positioning of the plate close to the bottom and the wet towels led to less dehydration), disturbances during the incubation (avoid incubation near shakers or platforms in motion that can create vibration), and edge effects within the plate (wells on the side of the plate may experience slight dehydration) may influence pellicle formation.

Using the pellicle formation assay, a pre-selected battery of small-molecules at a variety of concentrations were tested and it was observed that only compounds defined to cause potassium leakage from the cytoplasm of the cell were able to induce pellicle formation (See table 1). Compounds that induced pellicle formation included the polyene nystatin, produced by *Streptomyces noursei*. Nystatin is known to create pores in the membrane that selectively allow the leakage of potassium and other related cations (Bolard, 1986). Nystatin induced pellicle formation in *B. subtilis* when added to the culture at a final concentration of 60 µM. Amphotericin, a natural product produced by *Streptomyces nodosus* and similar in structure and function to nystatin, also induced pellicle formation. The final concentration to induce pellicle formation with amphotericin was also 60 µM. Surfactin is a lipopeptide produced by *B. subtilis* itself and it too induced pellicle formation in our pellicle formation assay when added at a final concentration of 20 µM. Surfactin is known to cause pores in the membrane allowing for the selective leakage of cytoplasmic cations, generally potassium (Sheppard et al., 1991). In addition to these molecules, the antimicrobials valinomycin and gramicidin, produced by some species of *Streptomyces* and *Bacillus*, respectively, induced pellicle formation. Both valinomycin and gramicidin induce potassium leakage from the cell. Particularly interesting is the case of valinomycin. Valinomycin does not make pores in the membrane but rather works as an ionophore that evacuates potassium exclusively (Marrone & Merz, 1995). The concentrations of valinomycin and gramicidin that induced pellicle formation in *B. subtilis* were less than 2 µM.

The molecules discovered to induce biofilm formation in wild type *B. subtilis* failed to induce pellicle formation in cultures of the $\Delta kinC$ -deficient mutant. The mutant lacking KinC was generated by deleting the gene that encodes the kinase, *kinC*, and replacing the gene with an antibiotic resistance cassette. We used the long flanking homology PCR technique to create the DNA fragment to replace the gene *kinC* (Wach, 1996). To create the cassette, we used primers to amplify the upstream and downstream regions of *kinC*, used the appropriate primers to amplify the resistance gene, and then performed a joining PCR generate the cassette. To amplify the upstream and downstream regions of *kinC*, the following primers were used: upforward: 5'-tcttctgtgattaacccgccaaga-3', upreverse: 5'-gaacaacctgcaccattgcaagaagtatttcaattgcatcgctccaa-3', downforward: 5'-ttgatcctttttataacaggaattcttcattgaaagtgaagtgcgaaga-3', downreverse: 5'-tggttaagatattcttcacctgggta-3'. The resulting cassette was inserted into the genome of the domesticated strain of *B. subtilis* 168 by inducing its natural competence (Hardwood & Cutting, 1990). Constructs were subsequently transferred to the undomesticated strain NCIB3610 by SPP1 phage transduction (Yasbin & Young, 1974).

Furthermore, when surplus amounts of potassium were present in the LB medium, the small-molecules also failed to induce pellicle formation in the wild-type strain. Addition of extracellular potassium to levels at or near the cytoplasmic concentration of potassium apparently blocks the efflux of potassium ions from the cytoplasm when the pores are made. Remarkably, the blockage in the signaling to biofilm formation caused by the excess of extracellular potassium results in an overproduction of the signaling molecule surfactin, as described by Fall and co-workers (Fall *et al.*, 2006, Kinsinger *et al.*, 2005, Kinsinger *et al.*, 2003). To cause this inhibition, any given potassium salt can be dissolved in LB medium at a final concentration of 150 mM or more. The medium may need to be buffered to pH=7.4 depending on the potassium salt dissolved. To test that extracellular levels of potassium would inhibit biofilm formation, LB+K⁺ was used as the culture medium for the final passage of *B. subtilis* (step 4 of the protocol) prior to the transfer of the culture to individual wells where the small molecules were tested. The 24 well plates were incubated at 30°C with no shaking over 8 h to induce biofilm formation.

Quantitative analysis of the activation of KinC

Surfactin, nystatin and several other potassium-selective pore formers stimulate the membrane sensor kinase KinC. Stimulation of KinC in turn, leads to the differentiation of a subpopulation of cells responsible for the production of extracellular matrix, which encapsulates and holds cells together within the biofilm. The subpopulation of matrix producers expresses the *eps* operon (Branda *et al.*, 2001, Branda *et al.*, 2004) and the *yqxM* operon responsible for the production of the extracellular exopolysaccharide and the matrix-associated protein TasA (Branda *et al.*, 2006, Branda *et al.*, 2004), respectively.

Hence the activation of KinC was observed by monitoring the activation of the genetic cascade that triggers the differentiation of the subpopulation of matrix producers. The activation of the expression of both the *eps* and *yqxM* operons was used as a direct readout for the activation of the cascade. Transcriptional fusions, of the promoter controlling the expression of each operon to the reporter genes *lacZ* or *yfp*, were created such that the activation of operon expression would simultaneously activate the reporters. The expression of the reporters was observed either by the production of the β -galactosidase enzyme or the yellow fluorescent protein (YFP), respectively. The amount of protein produced can be quantitatively measured either by monitoring enzymatic activity of the β -galactosidase or the fluorescence emitted by the protein YFP. β -galactosidase activity can be measured with a colorimetric assay using the substrate *ortho*-Nitrophenyl- β -galactoside (ONPG). ONPG is normally colorless. However, when β -galactosidase is present, it hydrolyzes the ONPG molecule into galactose and *ortho*-

nitrophenol. The latter compound has a yellow coloration that can be measured. The yellow fluorescent protein (YFP) has the advantage that it can be detected at the single-cell level, either qualitatively by using a microscope equipped with fluorescence detection or quantitatively, by measuring fluorescent cells using flow cytometry.

To construct the appropriate transcriptional fusions, promoters from the desired genes were amplified by using PCR, excluding their natural ribosome-binding site (RBS). The DNA fragments were cloned into the plasmid pDG1661 or pKM008 (Fig. 1) using a multicloning site located upstream of the *lacZ* or *yfp* gene, respectively, and fused to an optimized RBS. Additionally, both vectors have a specific resistance marker downstream of the reporter gene (pDG1661 has a chloramphenicol (cm^R) marker whereas pKM008 has a spectinomycin (spc^R) resistance marker), which permits positive selection when the construct integrates into the genome. Integration is successful by double recombination. DNA fragments of the *amyE* locus flank the cassette harboring the transcriptional fusion with the resistance gene. Prior to the transfer of individual plasmids to *B. subtilis* by natural competence, each plasmid is linearized. Selection for the appropriate resistance will yield only the colonies that have integrated the transcriptional fusion into the *amyE* locus of the genome.

When quantifying gene expression with transcriptional fusions, it is important to consider that not all of the cells are expressing the genes being reported by the fusions. For instance, *eps* and *yqxM* operons are expressed in only the subpopulation of cells specialized as matrix producers. The matrix producing subpopulation represents approximately the 35% of the total cells, meaning that only 35% of the signal will be detected when the whole population is analyzed. Taking accurate measurements of subpopulations becomes an issue when transcriptional fusions are coupled to the expression of enzymes such as β -galactosidase. Quantification of gene expression is given by the ratio of the enzymatic activity to the total number of cells and therefore a correct interpretation of the results may be confounded as dramatic pellicle formation would be correlated with a low increase in the expression of the *eps* and *yqxM* operons (3-fold increase). Because subpopulations exist, it is recommended that gene expression be quantified using transcriptional fusions coupled to the expression of fluorescent proteins. By using fluorescent proteins as reporters, it is possible to track the specific cells that are expressing the reporter and study the activation of the pathway in this specific subpopulation. The single-cell analysis using fluorescent proteins as reporters estimated that cells, which differentiated to become matrix producers, increase their fluorescence more than 10-fold when the signal molecule is added. Monitoring of fluorescence at the single-cell level was done using flow cytometry. The flow cytometer is a cell counter coupled with a fluorescence detector. Samples are measured by counting a predetermined number of cells. A total of 50,000 cells were measured by the flow cytometer for each sample and the number of fluorescent cells and the intensity of fluorescence, when present, were determined.

Several considerations need to be made with regard to the preparation of samples for flow cytometry. First, the flow cytometer requires a strong fluorescent signal for proper detection of the fluorescent cells. To ensure proper detection, the promoter coupled to the fluorescence protein gene must strongly induce the expression of the fluorescence protein when activated. As such, the promoter used in the construction of transcriptional fusions must be carefully selected for use in flow cytometry. Selection of the appropriate reporter led to the use of a transcriptional fusion of the *yqxM* promoter to *yfp* (using the primers PyqxMforward: 5'-tggcgaattctcagagtaaattgattgcttcact-3' and PyqxMreverse: 5'-cctaagcttgtaaactgtaactgatatgacaa-3'). Transcriptional fusions to the promoter of the *eps* operon were not used because the signal obtained with the activation of the promoter was not strong enough to be detected by flow cytometry. Additionally, samples of cells prepared for flow cytometry need to be dispersed as single cells in solution. Obtaining solutions of single cells is extremely difficult when working with any type of biofilm and especially difficult with

B. subtilis' biofilms because the cells grow in chains and are encased within the matrix. The flow cytometer counts and measures single cells. If the samples contain clumps of cells, these clumps will likely be counted by the flow cytometer as a single cell and the variable fluorescent background is likely confuse the detector. To avoid this problem, samples must be mildly sonicated prior flow cytometry. Sonication will disperse the clumps into single cells without causing cell lysis. In order to avoid any alteration of the gene expression due to sonication, samples were fixed with paraformaldehyde before the cells were sonicated. Below is the detailed protocol used to prepare samples for flow cytometry.

1. Cultures were sampled, centrifuged and washed with 1 ml of PBS buffer. Pellicles formed at the top of a well were easily disrupted by repetitive passage through a pipette tip or a needle.
2. Washed samples were centrifuged and fixed with 4% paraformaldehyde. Pellets were dissolved in 500 µl of 4% paraformaldehyde and incubated at room temperature for exactly 7 min.

4% paraformaldehyde was prepared as follows:

10 ml stock 16% paraformaldehyde

26 ml distilled water

4 ml 10X PBS

µl 10 N NaOH

The final solution is filtered through a 0.22 µm filter and aliquoted.

3. After fixation, cells were washed twice with 1 ml of PBS and resuspended in GTE buffer (50 mM glucose, 10 mM EDTA at pH 8, 20 mM Tris-HCl at pH 8). GTE buffer is preferred to PBS as it preserves cells under more optimal osmolar conditions.

Prior to analysis by flow cytometry, cells were subjected to mild sonication under conditions that disrupt the cells from the extracellular matrix but do not lyse cells at detectable levels (Branda et al., 2006). Mild sonication is defined as two series of 12 pulses (output 5, amplitude 0.7 sec). The efficiency of sonication may be confirmed by light microscopy.

In preparation for injection into the flow cytometer, cells were diluted in PBS and directly loaded into a BD LSR II flow cytometer (BD Biosciences) operating a solid-state laser at 488 nm. For each sample, at least 50,000 cells were analyzed. Data containing the fluorescent signals were collected by a 505LP and a 530/30-bp filter, and the photomultiplier voltage was set between 300 and 500 V. Data were captured using FACS Diva software (BD Biosciences) and further analyzed using FlowJo 8.5.2 software (<http://www.flowjo.com>) (Vlamakis et al., 2008).

Flow cytometry was used to compare cultures that had been exposed to a signal molecule, such as surfactin, to cultures that had not been exposed to a signal molecule. Flow cytometry of cultures exposed to a signaling molecule during the pellicle formation assay resulted in the visualization of the subpopulation of cells differentiated as matrix producers. For the mutant lacking the membrane kinase KinC, which failed to sense the presence of surfactin in the pellicle formation assay and as expected, visualization of a subpopulation of matrix producers by flow cytometry was not observed. No subpopulation of matrix producers was observed by flow cytometry following the addition of excess potassium in the form of KCl to the pellicle formation assay (Fig. 2).

Structural analysis of KinC

The activation of KinC leads to biofilm formation. Thus the activation of KinC can be monitored qualitatively using the pellicle formation assay and quantitatively by monitoring the differentiation of matrix producers using flow cytometry. To characterize the sensor kinase and determine how KinC senses potassium leakage, different alleles of the kinase KinC were constructed, each lacking different domains of the protein, in an effort to pinpoint the critical components of the kinase involved in sensing potassium leakage. Various constructs were generated and their ability to complement the mutant deficient in KinC monitored using both the qualitative and quantitative measurement techniques.

KinC is a membrane kinase that has two transmembrane segments connected by six extracellular amino acid residues in the N-terminal region. Following the transmembrane region is an intracellular PAS-PAC domain. PAS domains have been described as able to monitor changes in light, redox potential, oxygen, small ligands, and the overall energy level of a cell (Gu et al., 2000, Taylor & Zhulin, 1999). The PAC motif is proposed to contribute to the folding of the PAS domain. The C-terminal region of KinC harbors the phosphoacceptor and the ATPase domains of the kinase. While the PAS-PAC domain was likely responsible for sensing the leakage of potassium from the cytoplasm of the cell, experiments were conducted to conclusively determine whether the transmembrane domain or the PAS-PAC domain of kinC was the sensory domain that activated the kinase.

In order to determine whether the transmembrane domain or the PAS-PAC domain of kinC was the sensory domain responsible for activating the kinase, the $\Delta kinC$ -deficient mutant was complemented with three different alleles of the kinase KinC: the wild-type version of the protein, an allele lacking the transmembrane region and another allele lacking the PAS-PAC sensor domain (Fig. 3). Each allele was fused to *cfp* to allow their expression in *B. subtilis* to be followed by western blot. Technically, the alleles were designed by amplifying the DNA fragments independently. To do this, the following primers were employed: PASupfw: 5'-aaaagaattcgatcgccgattgagttgag-3', PASuprev: 5'-ctgggattccctcgccagttcagaagctgtttatacttc-3', PASdwfw: 5'-ctggcgaggggaatccag-3', PASdwrev: 5'-ttttgatccgtatacaaacagaagcgag-3', TMRuprev: 5'-accagctgctgtttctcatccattgatattttctcatatgaccacc-3', TMRdwfw: 5'-gatgagaaacagcagctggt-3'. The DNA fragments amplified were combined by joining-PCR as described by Wach *et al.*, for LFH-PCR. The wild type allele of *kinC* was constructed by joining the entire structural *kinC* gene (including its own promoter) to the *cfp* gene. The allele of KinC lacking the transmembrane region was created by joining the kinC promoter with the initial six amino acids upstream of the transmembrane region, the PAS-PAC sensor domain, and the *cfp* gene. Similarly, the allele of KinC lacking the PAS-PAC sensor domain was created by joining the promoter region and the transmembrane region of kinC, the phosphoacceptor and ATPase domains of the kinase, and the *cfp* gene. To successfully construct and express all three different alleles of the protein KinC, it was critical to join the DNA fragments in such a way as to preserve the same open reading frame. Preservation of the open reading frame ensured each allele would be transcribed into a single molecule of mRNA that in turn, would be translated into the desired protein (allele).

The three distinct alleles of KinC were cloned into a modified pKM003 vector (lacking *cfp*) and integrated into the genome of the $\Delta kinC$ -deficient strain by double recombination into the *amyE* locus, as described above. The expression of the different versions of KinC was confirmed by the detection of the CFP protein fused to each allele. To detect CFP, the conventional western blot technique was employed, using monoclonal antibodies (Promega) against CFP (monoclonal antibodies against CFP can also be used to recognize GFP and YFP proteins, since the three proteins share a common epitope). Results indicated that both the expression of the wild type allele and the expression of the allele lacking the transmembrane

region restored the ability of the $\Delta kinC$ -deficient mutant to form biofilm communities in the biofilm-inducing medium MSgg. Also, these two alleles induced the formation of pellicle in the presence of surfactin in the pellicle formation assay. The allele of KinC lacking the PAS-PAC sensor domain was neither able to induce the formation of biofilm in MSgg nor respond to the presence of surfactin in the pellicle formation assay.

To further confirm the results observed during the pellicle formation assay, quantitative measurements were made using flow cytometry. The experiment required the expression of the transcriptional fusion P_{yqxM} -*yfp* but the neutral *amyE* locus used previously to integrate the constructs was occupied in every case by the distinct kinC alleles that complemented the $\Delta kinC$ -deficient strain. An additional neutral locus was required to integrate the transcriptional reporter of the *yqxM* operon. For that purpose the vector pDR183 was used (Doan *et al.*, 2005). Vector pDR183 has a multicloning site next to an erythromycin-resistant marker and the cassette is flanked by DNA fragments of a second neutral *B. subtilis* locus *lacA* (See Fig. 1). The transcriptional fusion P_{yqxM} -*yfp* was transferred from pKM008 to pDR183 by endonuclease digestion and T4 ligase ligation. The vector was linearized and integrated into the *lacA* locus by double recombination using *B. subtilis*' natural competence. Colonies carrying the transcriptional fusion P_{yqxM} -*yfp* within the *lacA* locus were resistant to erythromycin.

The transcriptional fusion P_{yqxM} -*yfp* under the control of the distinct alleles of kinC resulted in the occurrence of a subpopulation of matrix producers only in the presence of the wild-type allele or the allele lacking the transmembrane region, and only when surfactin was added. The allele of KinC lacking the PAS-PAC sensor domain neither responded to surfactin nor differentiated to create a subpopulation of matrix producers.

Monitoring the signals using indirect measurements

Whether the membrane kinase KinC is directly activated by the leakage of potassium cations from the cytoplasm or is indirectly activated remains to be determined. Other cellular responses associated with the leakage of potassium from the cytoplasm were considered as stimuli, leading to the activation of the membrane kinase KinC. For instance, the efflux of potassium cations might be counteracted by the uptake of other cations, to maintain the electrophysiology of the bacterium. Thus, the PAS-PAC domain might possibly sense the uptake or increased concentration of another cation or cations, rather than the loss of potassium ions directly.

In order to identify and study the nature of the signal that activates KinC in detail, several specific experiments were performed. First, we attempted to determine whether the efflux of potassium acts as the signal triggering the kinase KinC. To do this, a potassium-sensitive electrode was used to measure the extracellular concentration of potassium (Katsu *et al.*, 2002, Orlov *et al.*, 2002, Yasuda *et al.*, 2003). By measuring the fluctuation in extracellular potassium following the addition of surfactin, it was possible to calculate the concentration of potassium evacuated from the cytoplasm of the cells.

Several technical problems were encountered while using the potassium-sensitive electrode. The most prominent issue was the tremendous sensitivity of the electrode for potassium cations. The sensitivity of the electrode required measurements to be made in a buffer free of potassium cations. Otherwise, the background obscured detection of any variation in the concentration of extracellular potassium when surfactin was added. However, the complete absence of potassium in the extracellular milieu represents neither natural nor laboratory conditions under which surfactin was previously tested. Therefore, working with a buffer free of potassium cations was unlikely to yield any additional information about the nature of the signal detected by KinC.

Ultimately, cytoplasmic potassium leakage could be measured by the electrode after resuspending cells in a buffer containing 10 mM KCl. Under these conditions, a 1.2 mM increase in the extracellular concentration of potassium was detected an hour after cells were treated with surfactin. Rough calculations, considering only the volume of cell mass vs. volume of resuspension buffer, suggested the cytoplasmic decrease of potassium was 40 mM when surfactin was added. Although the experiments could be both repeated and refined, the fluctuation in cytoplasmic potassium appears to be negligible considering the concentration of potassium in the cytoplasm has been estimated to be between 350–650 mM.

Possibly, the leakage of cytoplasmic potassium may be associated with other internal effects such as pH variation or the uptake of other cations such as calcium. We monitored pH variation in the cytoplasm using pH-sensitive dyes. For instance, 5(6)-Carboxynaphthofluorescein is a pH-sensitive fluorescent dye with λ_{ex} = 512 nm and λ_{em} = 567 nm under acidic or neutral conditions or λ_{ex} = 598 nm and λ_{em} = 668 nm under basic conditions (Butterfield *et al.*, 2009, Song *et al.*, 1997). While exposed to the dye, *B. subtilis* cells were observed to increase cytoplasmic acidity upon treated with surfactin. The result suggests that potassium leakage is probably linked to an uptake of H⁺. Additional experiments need to be completed in order to decipher the nature of the signal that stimulates the activation of the membrane kinase KinC. Thus far, we cannot discard that other factors might be involved.

Applications of the system Signal-Kinase

Characterization of any system which senses and recognizes a known and defined signal is a remarkable molecular tool that may permit us to control desired genetic pathways in order to direct the production of enzymes, antibiotics or developmental processes in *B. subtilis* or other bacteria. In our case, the signaling molecules surfactin or nystatin activate the sensor kinase KinC and in turn, KinC triggers the phosphorylation of its cognate regulator Spo0A~P. The phosphorylation domain of KinC recognizes exclusively its cognate regulator Spo0A as a common feature of all kinases to selectively recognize their cognate regulators at a molecular level. Thus, phosphorylation of any other cognate regulator by the phosphorylative action of KinC is possible by changing the phosphorylation domain, which recognizes Spo0A, to a phosphorylation domain that recognizes another regulator. It is therefore possible to create a kinase chimera, composed of the sensing domain of KinC and a phosphorylation domain that phosphorylates another regulator we are interested to activate.

For instance, motility in *B. subtilis* is inhibited when the regulator DegU is activated by phosphorylation (Amati *et al.*, 2004, Kobayashi, 2007, Verhamme *et al.*, 2007). Phosphorylation of DegU is governed by the action of the kinase DegS (Dahl *et al.*, 1991). Thus, activation of DegS leads to an inhibition of motility. We controlled motility in *B. subtilis* in response to the presence of nystatin by creating a kinase chimera, which was comprised of the transmembrane region and the PAS-PAC sensor domain of KinC and the phosphoacceptor and ATPase domain of the kinase DegS (Fig. 4A). The activation of the chimera required the signaling molecule nystatin. However, instead of inducing biofilm formation, nystatin activated the regulator DegU and in turn, motility was inhibited.

In order for the above chimera to be the exclusive phosphorylator of the regulator DegU, the strain needs to be defective in the native kinase DegS. Without the native DegS, the phosphorylation of DegU will be done wholly by the DegS domain of the kinase chimera and the results will not be confounded. The *degS* gene was deleted by LFH-PCR as explained above. To create the deletion cassette we used the primers: degSupforward: 5'-agccctacaactaccaatagtgcga-3', degSupreverse: 5'-caattcgccctatagtgcgtgtagcatttggaatccatctttgtttt-3', degSdownforward: 5'-ccagcttttgttccttagtgagataggtcttggaacattattatgatt-3', degSdownreverse: 5'-

tcaatttcttcacggttaagtctcct-3'. We complemented the $\Delta degS$ -deficient mutant with the kinase chimera by integrating the cassette in the *amyE* locus by double recombination. The KinC-DegS chimera was constructed by combining a PCR-amplified DNA fragment of the *kinC* gene harboring the natural promoter of the kinase, the transmembrane region and the PAS-PAC sensor domain, to a PCR-amplified fragment of the *degS* gene corresponding to the phosphoacceptor and the ATPase domain. The fragments were linked by joining PCR. Again, the experiments conserved the open reading frame across the resulting DNA fragment such that the expression of the kinase chimera was not affected. The resulting DNA fragment was cloned into the modified pKM003 vector (lacking *yfp*) and integrated into the *amyE* locus of the genome of a $\Delta degS$ -deficient mutant, using the resistance to spectinomycin provided by the cassette.

The resulting chimera was tested for the inhibition of motility in the presence of nystatin. The assay for the inhibition of motility was prepared using the LB medium described by Kearns et. al., to promote motility (Kearns & Losick, 2003). The assay was performed on freshly prepared LB plates with a low agar concentration (1%). We inoculated the plates with different concentrations of nystatin. Three μ l of an overnight culture of the strain harboring the chimera were spotted onto the plate and the plates were then incubated at 30°C for 24 h.

The process of making kinase chimeras is not always straightforward. Some kinases require specific conformational changes to permit the transference of the phosphate group to its cognate regulator. Often, the process of making chimeras changes the particular configuration of the kinase making it difficult or impossible for the activation of the chimera. It is difficult to avoid this problem. The generation of a functioning KinC/DegU chimera required experimentation with various lengths of each separate PCR-amplified fragment.

Regardless of the difficulties inherent in creating modified kinases and chimeras, the signal-kinase systems can be used to control the expression of a desired gene in any given bacterial system. It is possible to construct a transcriptional fusion of a desired gene, placing it under the control of a promoter that is recognized by a cognate regulator of the kinase. To test this principle, the gene *cfp* was expressed in *Listeria monocytogenes*, a gram-positive pathogen that does not have the KinC-Spo0A system. We made a transcriptional fusion of the *cfp* gene to the promoter of the *skf* operon. The *skf* operon is an operon in *B. subtilis*, the transcription of which is directly activated by the regulator Spo0A~P (Hobbs, 2006). We used PCR to construct a cassette that joined *kinC* with its natural promoter, *spo0A* with its natural promoter and the transcriptional fusion P_{skf} -*cfp*. The primers used to amplify the fragments and construct the cassette are: KinC-up: 5'-cactcttcattacgctgcttgccgcattgtcatgcc-3', KinC-dw: 5'-gatcgagcttatgtatacac-3', Spo0A-up: 5'-gtgtatacataagctcgatcgaaattaccgatccaagac-3', Spo0A-dw: 5'-aaaagtcgactactacgattttg caggac-3', Pskf-up: 5'-aagcagcgtatgaagagtc-3', Pskf-upSalI: 5'-aaaagtcgacaagcagcgta atgaagagtg-3', and Pskf-cfp-dw: 5'-aaaacggccgtgatcgaaatagtcataatg-3'. As illustrated in figure 4B, the joining PCR cassette containing *kinC*, *spo0A* and the transcriptional fusion P_{skf} -*cfp* was made by joining the three genes ordered in divergent transcriptional frames. The use of divergent transcriptional frames in the expression of each gene eliminates false activation of the reporter fusion P_{skf} -*cfp* due to possible interference in the expression of the different promoters.

The cassette was cloned in the pPL2 (Lauer et al., 2002) plasmid, using the multicloning site of the vector. The pPL2 plasmid harbors a phage attachment site on the *L. monocytogenes* chromosome, which leads to the integration of the plasmid at the 3' end of an arginine tRNA gene (tRNA^{Arg}). The sequence reconstitutes a complete tRNA^{Arg} gene after the integration of the plasmid. Transformation of *L. monocytogenes* was carried out using electroporation, as previously described for other studies (Lemon et al., 2007). Colonies with the plasmid integrated into the genome can be positively selected by using chloramphenicol 5 μ g/ml.

Cultures of *L. monocytogenes* carrying the construction were grown in TSBYE medium (TSB medium + 1% yeast extract) and dispensed in wells where the signal molecule surfactin had been previously added to a final concentration of 50 µg/ml. Activation of the recombinant KinC led to the phosphorylation of the recombinant Spo0A and thus, activation of the expression of the transcriptional fusion P_{skf} -*cfp*. Fluorescent cells were detected by fluorescence microscopy. We were unable to use flow cytometry for quantitative analysis because the flow cytometer was unable to count *L. monocytogenes* cells due to their smaller size as compared to *B. subtilis*.

Conclusions

We have presented in this chapter the procedures, methods and protocols established in the laboratory to characterize the leakage of cytoplasmic potassium as a signal to induce biofilm formation. The various small molecules identified to cause potassium leakage by making pores in the membrane of *B. subtilis*. (Lopez et al., 2009a) are sensed by the membrane kinase KinC and trigger the differentiation of the subpopulation of cells responsible for the production of the extracellular matrix that holds cells together in a biofilm. Recognizing the mode of action of a signaling molecule rather than the molecular structure allows a bacterium to sense of a large number of signals and to respond not only to self-produced molecules but also to natural products secreted by other organisms in close proximity.

Although it is clear that the PAS-PAC domain of the kinase KinC is responsible for sensing the leakage of potassium cations from the cell, the nature of the signal has yet to be elucidated. Whether the membrane kinase KinC is activated directly by the leakage of potassium cations from the cytoplasm or indirectly, due to variation of another parameter linked to potassium leakage, remains unknown.

The ability to monitor populations at the single-cell level through the use transcriptional fluorescent reporters that could be detected by microscopy techniques or flow cytometry, was critical to the success of our work. The new techniques employed permitted the characterization of the subpopulation of cells responsible for the production of the extracellular matrix (Smits *et al.*, 2005, Vlamakis *et al.*, 2008). This subpopulation represents 35% of the total cell population and showed a 10-fold induction of the genes directly responsible for matrix production. Conventional techniques frequently used to monitor transcriptional changes in gene expression, such as the β -galactosidase assay or other enzymatic activities coupled to colorimetric assays, would fail to detect this effect. This limitation resides in the fact that conventional techniques estimate the relative values of gene expression with respect to the size of the whole cell population, normally considered as a measurement of the optical density OD₆₀₀. Conventional techniques will systematically and drastically underestimate the induction value of gene expression when gene expression only occurs in a subpopulation of cells.

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Figure 1. Plasmids used to integrate the distinct transcriptional fusions in the genome of *B. subtilis* Schematic representation of the three plasmids (pKM008, pDR183 and pDG1661) used to construct the transcriptional fusions for integration into the neutral loci *amyE* (in the case of pKM008 and pDG1661) and *lacA* (in the case of pDR183), in the genome of *B. subtilis*. Selected promoters were fused to the yellow fluorescent protein gene (*yfp*), in the case of pKM008 and to the β -galactosidase gene (*lacZ*), in the case of pDG1661. When required, constructions were transferred to the *lacA* locus by subcloning into the pDR183 vector. Restriction sites are indicated by the name of the endonucleases that recognize their respective sequences. Integration of these vectors into the neutral loci of the genome was performed by double recombination. The vectors carry different antibiotic resistance makers: *amp^R* = ampicillin resistance marker; *spc^R* = spectinomycin resistance marker; *erm^R* = erythromycin resistance marker; *cm^R* = chloramphenicol resistance marker. The plasmids and the genes are not to scale.

Figure 2. Flow cytometry analysis of WT cells harboring the transcriptional fusion P_{yqxM} - yfp
 The profiles illustrate cultures of untreated cells and cells in the presence of the small molecule inducer surfactin (20 μ M). The control profile is represented by untreated cells that do not harbor any yfp gene and a single population of cells with very low relative fluorescence is observed. Cells harboring the P_{yqxM} - yfp fusion also show a single population, similar to the control, in the absence of surfactin (blue peak). When surfactin was added (green peaks), subpopulations of cells with high relative fluorescence were observed as the shoulder to the right of the primary peak. This subpopulation represents approximately the 35% of the total cells. Addition of surfactin to the $\Delta kinC$ -defective mutant does not induce the differentiation of the subpopulation (red graph). Complementation of the $\Delta kinC$ -defective mutant with a wild type copy of $kinC$ resulted in the restoration of the subpopulation of cells expressing P_{yqxM} - yfp (purple graph). If the allele of $kinC$ does not have the PAS-PAC sensor domain, it cannot complement the $\Delta kinC$ -defective mutant (dark green graph), contrasting to the allele lacking the transmembrane region (pale green graph). This figure is credited to (Lopez et al., 2009a).

Figure 3. Scheme of joining PCR used to generate the different alleles of KinC

The gene *kinC* is represented as a rectangle in which the coding regions of the domains are marked. Primers are illustrated and named. Amplified DNA fragments are represented by dashed lines. In addition to the wild type allele, a subsequent joining PCR yields two modified alleles, one lacking the transmembrane region and another lacking the PAS-PAC sensor domain of the kinase KinC.

A

B

Figure 4.

(A) Scheme of joining PCRs used to generate the kinase chimera KinC-DegS. Both genes *kinC* and *degS* are represented as rectangles in which the coding regions of the domains are marked. PCR amplification of the regions of the genes required to generate the chimera are represented as dashed lines. Primers required for amplification are illustrated as arrows. The regions coding for the transmembrane domain and the PAS-PAC sensor domain of *kinC* was amplified and joined to the kinase domain of *degS* by the joining PCR as represented in the figure. (B) Construction of the cassette *KinC-Spo0A-P_{skf}-cfp* and integration into the genome of *Listeria monocytogenes*. The multicloning site (MCS) of the vector pPL2 was used to clone the joining PCR *KinC-Spo0A-P_{skf}-cfp*. This plasmid integrates into the genome of *L. monocytogenes* due to the PSA prophage integrase gene (*PAS int*) and the PSA prophage attachment site in tRNA^{Arg} (*PSA attPP'*). Selection is given by the chloramphenicol resistance

marker (cm^R). The plasmid harbors two different cm^R genes in order to be selectable in gram-positive strains (Gm- cm^R) and gram-negative strains (Gm+ cm^R).

Table 1
Battery of small-molecules tested to induced biofilm formation in *B. subtilis*, using the pellicle formation assay

The name of each compound tested is provided in the left column. The function of each molecule is detailed in the center column and the ability of each compound to induce pellicle formation is given in the right column. This table is credited to (Lopez et al., 2009a).

Molecule	Function	Biofilm Induction
Nystatin	Cation-selective pore formers	Yes
Amphotericin		
Gramicidin		
Surfactin	Potassium-selective pore former	
Valinomycin	Potassium-selective ionophore	
CCCP	Proton-selective ionophore	No
Polymyxin	Cell wall synthesis inhibitors or membrane disaggregators	
Bacitracin		
Filipin		
Vancomycin		
Aculeacin		
Microcystin		
Nisin	Unselective pore formers	
Iturin		
Syringomycin		
Nonactin	Ammonium-selective pore former	
Triton X-100	Detergents	
Nonidet P-40		
Tween 20		
Sodium dodecyl sulfate		
Novobiocin	Structurally-related compounds	
Stigmatellin		
Antimycin		