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A Life in *Bacillus subtilis* Signal Transduction

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Abstract

This is a tale of how technology drove the discovery of the molecular basis for signal transduction in the initiation of sporulation in *Bacillus subtilis* and in bacterial two-component systems. It progresses from genetics to cloning and sequencing to biochemistry to structural biology to an understanding of how proteins evolve interaction specificity and to identification of interaction surfaces by statistical physics. This is about how the people in my laboratory accomplished this feat; without them little would have been done.

Contents

PROLOGUE

The Early Years

Brookings, South Dakota, where I was born, is a place of religion, fundamentalist institutions, and moral certitude. “It is a great place to raise kids,” they say. My mother was a nurse born to first-generation potato famine Irish. My father was derived from Pennsylvania Dutch ancestors whose Hessian forefathers came to the United States in 1754 indentured to the King of England to fight in the French and Indian War. He was a seed salesman for the local grain elevator after joining the Army, invading Mexico looking for Pancho Villa with Blackjack Pershing and fighting in World War I. Out of this pairing came my sister, Rita, who once got a B+ to ruin her all-A record in high school and college, my brother, George, who did research in photosynthesis and taught thermodynamics at the University of Rochester, and me. We all graduated from South Dakota State College because it was in Brookings and inexpensive, my father’s major requirement. However my father did offer, perversely, to send me to St. John’s University in the hope I would become a priest. Father-son communication was not our strong suit!

There was no hint I would end up doing anything worthwhile; I was not a great student. I stuttered badly, or well, depending on your point of view. I could not answer questions in class from teachers. I could not call home because calling required speaking my telephone number, 39W, to an operator (the phone system in Brookings—no dials, no buttons—was one step up from two tin cans connected by a string).

Academics provided a potential way out of town from my various jobs as Texaco bulk truck driver, gas station attendant, and used car detail man. My first exposure to scientific experimentation came at the Agricultural Biochemistry Experiment Station. There I worked doing Kjeldahl protein, fat, and crude fiber analyses on what a sheep ate and what it eliminated. The conclusions from this cutting-edge research were never revealed to me.

I acquired an interest in microbiology from an excellent professor, E.C. Berry. His interest in the subject and, perhaps more importantly, his interest in helping students led me to a fascinating lifetime profession. Dr. Berry also guided me to graduate school at the University of Illinois through his friend I.C. (Gunny) Gunsalus, whose children I had grown up with in Brookings.

At Urbana, Illinois, I was a student of Ralph DeMoss. I purified and characterized the enzyme tryptophanase from *Bacillus alvei* and isolated mutants in the tryptophan pathway, among other things. Near the end of my time there Ralph suggested I try to obtain a National Institutes of Health (NIH) fellowship to study with Costa Anagnostopoulos in Paris. European NIH fellowships were a scarce commodity and no one was more surprised than I that I actually got one—$5,000 a year to live in Paris!

Learning Genetics

In France I began a lifelong love affair with *Bacillus subtilis*. John Spizizen discovered DNA-mediated transformation in one of Charley Yanofsky's *B. subtilis* strains (74). Costa Anagnostopoulos, in John's laboratory at Western Reserve University, worked out the criteria for efficient, reproducible transformation (3). Costa returned to Paris, where I joined him as an NIH postdoctoral fellow at the Centre de Génétique Moléculaire, Gif-sur-Yvette. There I gained experience in the genetics of *B. subtilis* using transformation and PBS-1 transduction. Costa was a well-read intellectual who never forgot a book. He remained a close friend for years. He schooled me in the politics running science and professorships in France and elsewhere. It had little impact at the time, as I was more interested in racing my Triumph TR4A with the Ferraris and Porsches on the Champs-Elysées at night. What possessed me to leave there? After two years, in 1967, I returned to the United States and was fortunate to join John Spizizen as a postdoctoral fellow at The Scripps Clinic and Research Foundation in La Jolla, California.

IDENTIFYING SIGNAL TRANSDUCTION PROTEINS IN SPORULATION

The crux of the problem in differentiation is understanding how signaling networks in a cell determine the fate of the cell. The decision in sporulation is whether to continue growing and dividing or to stop and begin expressing genes leading to a spore.

John had been isolating mutants in *B. subtilis* sporulation and had a large collection of *spo0* mutants that were essentially locked in growth and unable to enter the earliest stage of sporulation, stage 0. I undertook the characterization of this class of mutants using transformation and transduction to determine how many genes were involved in the shift from growth to sporulation (25). I was not alone in this effort, as Pierre Schaeffer at the Université de Paris, Orsay, and his students had already established a solid basis for sporulation genetics. Needless to say that group

regarded me an interloper! Joel Mandelstam, Oxford University, was also spawning friends and competitors, e.g., Patrick Piggot and Jeff Errington. Salvador Luria came to La Jolla during this period and told John that his boys had proven that the initiation of sporulation was due to proteolysis of an RNA polymerase subunit (38). While depressed by this revelation, I wondered what all these *spo0* mutants were about if you only needed to cleave RNA polymerase. I soldiered on with genetics of stage 0 sporulation genes, ultimately revealing at least eight individual *spo0* loci for such mutants (29). This turned out to be an underestimate when we applied a different genetic trick, described below, to find genes affecting the initiation of sporulation.

By 1976 some of the theories for sporulation initiation, such as the episome hypothesis and cleavage of RNA polymerase, had been discarded (26). It was clear from the work of Piggot and the Schaeffer crowd, among several others, that there were a large number of genes involved in constructing the spore and they could be morphologically ordered in a sequence from stage II to stage IV (32, 65). In my case the *spo0* mutants had distinct phenotypes, but not much else could be made of that information with regard to a pathway; we had no knowledge of the products encoded by these stage 0 genes.

What now? We kept doing what we knew how to do. We continued genetic mapping of genes for various biochemical pathways, citric acid cycle genes (27, 68), aromatic amino acid biosynthesis genes (30), chemotaxis genes (50), and genes for small acid-soluble spore proteins (7), among others, for many years.

My journey into mammalian cells began because technology did not exist to proceed further with *spo0* mutants. I was fascinated by somatic cell hybrids between human and mouse cells; so I set up the tissue culture system for their production. These hybrids allowed us to map several human lymphocyte receptors with HLA genes (8, 28, 51) as well as to study alkaline phosphatase from human tumor cell lines in such hybrids (89). We also ventured into studies of other mammalian genes of interest (1, 2). Despite the intellectual stimulation research in new areas of science provided, I finally realized that I had strayed from signal transduction in sporulation, and I was uneasy about depending on others for reagents.

GENE CLONING AND DNA SEQUENCING CHANGED EVERYTHING

This combination of technologies formed the basis for research that greatly advanced our understanding of the proteins encoded in genes defined by *spo0* mutants.

Sporulation genes had been found in cloned DNA (41). This stimulated Eugenio Ferrari and Dennis Henner to construct a large fragment library of *B. subtilis* DNA in the lambda phage Charon 4A, in which they found both *spo0A* and *spo0B* genes as well as many other genes (13). We started to clone everything in sight, including other *spo0* genes (14, 15); the levansucrase *sacB* gene, which became an important selection system for *Escherichia coli* genetics (21); the *Bacillus thuringiensis* insecticidal protoxin gene (22); and others.

Maxam and Gilbert sequencing arrived in the laboratory in 1983 in the form of Kathleen Trach, who began sequencing our cloned DNA for *spo0* genes using meter-long polyacrylamide gels (16, 17). Everyone in the lab became a recorder of sequences read from these gels and was excited to find where the genes started and ended, locate ribosome binding sites and terminators, and determine what proteins were encoded there. It was a heady time that is lost to present-day investigators who outsource cloning, sequencing, and interpretation.

In our craze to clone and sequence genes we were carried away by the technology and became sought-after experts by colleagues at Scripps. We cloned and sequenced the cDNA of rat liver dihydropteridine reductase with my colleague John Whiteley (72). We did the same for beef heart mitochondrial nicotinamide nucleotide transhydrogenase and determined its signal sequence from

mRNA sequencing (97, 98). Again I was off on another tangent unrelated to *spo0* genes; I had to focus!

The *Bacillus* Conference

There was a great deal of activity in the genetics and molecular biology of *B. subtilis* in the 1980s that required a common forum for information exchange. A.T. Ganesan (Stanford University) and I began the International Conference on Genetics and Biotechnology of Bacilli at Stanford University in 1981. After his untimely death, Marta Perego and I continued to organize this biennial conference until 2015 with industrial support, mainly from Genencor, but various pharmaceutical and enzyme companies also contributed to its success. Ultimately it was moved to Italy, reflecting the fact that *Bacillus* research remained strong in Europe (not to mention the quality of the food and wine) while such research in the United States had dwindled to a few laboratories.

Sequencing the *B. subtilis* Genome

In the early years our genetic contributions were driven by a desire to complete the genetic map of *B. subtilis*, which was shown to be circular in Raymond Dedonder's laboratory (37). In 1986, I induced Raymond, then Director General of the Institut Pasteur, Paris, to respond to a request for proposals from the NIH for international cooperation in sequencing bacterial chromosomes. We brought together a US-European consortium of investigators to sequence the *B. subtilis* chromosome and applied for the grant. The proposal received high marks by the study section and was weeks away from funding when the new Genomics Institute director, Jim Watson, stopped the funding. He came to San Diego and talked to me about it. I listened to his message. Despite this setback, European and Japanese investigators successfully completed sequencing the genome (36). What these people accomplished in several years could now be done in an hour or less. This great advance in sequencing speed was the vision that Jim Watson told me he held when we met. In retrospect, he did me a favor.

THE BEGINNINGS OF TWO-COMPONENT SIGNAL TRANSDUCTION AND THE DISCOVERY OF THE PHOSPHORELAY

Two-component signal transduction consisting of a signal-activated kinase that phosphorylates a response regulator is a major mechanism by which microbes and plants respond to signals from their internal or external environment.

1985 was a banner year for understanding *spo0* gene products and two-component signal transduction. We sequenced the *spo0A* gene, which revealed its protein product to be sequence related to the OmpR protein of *E. coli*. Moreover, the sequencing of *spo0A* mutants convinced us that "the Spo0A protein interacts directly with the transcription machinery to effect the initiation of sporulation" (18). Subsequently, we collaborated with the Piggot laboratory and sequenced the *spo0F* gene, revealing protein sequence homology with Spo0A and the *E. coli* proteins OmpR and SfrA in the amino terminal half of these proteins (85). Sequences of regulatory proteins such as CheY also were homologous to these proteins (76). These response regulator proteins consisted of either a single domain such as Spo0F or CheY or the amino terminal domain of a two-domain protein such as Spo0A. The widespread use of a common protein domain across species had to be the basis for some conserved means of regulation, but the mechanism by which it operated was still not apparent.

Alex Ninfa and Boris Magasanik made an important breakthrough in two-component signal transduction by discovering that response regulator proteins were subject to phosphorylation by a

specific kinase protein to which they were genetically paired. They suggested that the phosphorylated form of the response regulator domain of a transcription factor was the active form responsible for the initiation of transcription. Furthermore, they postulated that the ATP-dependent kinase was responsible for phosphorylation and dephosphorylation of the transcription factor in response to additional signals, in their case nitrogen availability (46). They later showed that this molecular mechanism was the same for CheY phosphorylation by the CheA kinase (47), and other laboratories found similar results for different systems, thus establishing phosphorylation as a key factor in signal transduction regulation. While we knew Spo0A was a transcription factor, the nagging question remained as to how all of our Spo0 proteins—Spo0F, Spo0B, Spo0E, Spo0H, and others—fit together in a phosphorylation scheme for the initiation of sporulation.

It was becoming clear that proteins with a response regulator domain such as Spo0F and Spo0A commonly were associated genetically with a kinase. Probable kinase genes were not found next to any *spo0* gene, but a gene for a *spoII* locus exhibited this homology and its protein, now named KinA, was capable of phosphorylating both Spo0F and Spo0A in vitro but was more active on Spo0F. However, deletion of the *kinA* gene resulted in a strain with only partial deficiency in sporulation, suggesting that multiple kinases played a role in the initiation of sporulation (56). This proposal was found to be correct when we cloned and sequenced the other major kinase for sporulation, KinB (86). Véronique Dartois identified several genes specifically needed for KinB function (10).

It was at this time in 1989 that I was disillusioned with genetics as a means to solve the initiation problem and turned the lab toward the biochemistry of phosphorylation, first by purifying the protein products of the *spo0* and kinase genes. This turned out to be a prescient decision. David Burbulys and Kathleen Trach purified and studied the phosphorylation biochemistry of Spo0 proteins and discovered the pathway to the ultimate phosphorylation of the Spo0A transcription factor. This pathway for which we coined the seminal name "phosphorelay" reflected the relay-like transfer of a phosphoryl group from ATP to kinase to Spo0F to Spo0B to Spo0A (6). The phosphoryl group moved from ATP to a histidine on the kinase to an aspartate on Spo0F and back to a histidine on Spo0B, finally residing on an aspartate in Spo0A (**Figure 1**). Phosphorylated Spo0A was the transcription factor driving the initiation of sporulation!

Although this longtime mystery of what some of these proteins were doing was finally understood, I wondered why nature dumped Spo0F and Spo0B in the middle of a phosphorylation

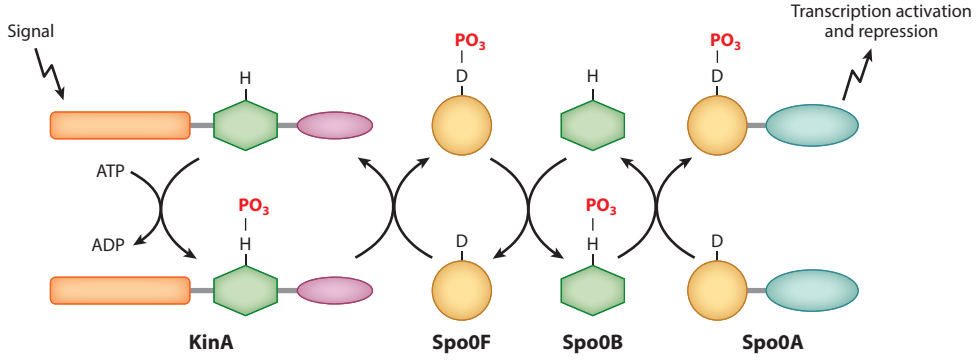

Figure 1

The phosphorelay pathway activating the response regulator and transcription factor Spo0A. The pathway is initiated by signal activation of KinA [or any of the other sporulation kinases (KinA–E)]. Phosphoryl transfer in this pathway occurs on either histidine (H) or aspartate (D) residues. Structural domains depicted in the same color are common to response regulators (*yellow*) or HisKA domains (*green*). The dimeric structures of KinA and Spo0B are not shown.

pathway that could be accomplished by kinase alone. The answer, I believed, lay in providing targets for the multiple cell cycle, biochemical, and environmental signals that needed to have a say in controlling the switch between growth and sporulation and did so by regulating the level of Spo0A phosphorylation. Our rationale at the time was that two-component systems such as those regulating chemotaxis and transcription of other genes responsive to simple environmental signals were not essential for life, whereas sporulation involved a massive morphological energy-requiring change in the cell and was a nontrivial event. Each component protein of the phosphorelay may well have served as an entry point for regulation of this crucial phosphorylation, thus providing a system of checks and balances (6). This theory turned out to be prophetic.

However, it should be noted that the spore-forming clostridia appear to not employ a phosphorelay for sporulation. Direct phosphorylation of Spo0A by histidine kinases and dephosphorylation by kinase-like proteins appear to be common features of the anaerobic clostridia. We speculated that this may represent the ancestral state before the great oxygen event some 2.4 billion years ago, after which additional phosphorelay proteins were recruited in the evolutionary lineage that led to the aerobic bacilli (75).

REGULATORS OF THE PHOSPHORELAY: HOW EVERYTHING CONTRIBUTES TO THE DECISION BETWEEN GROWTH AND SPORULATION

Little did I realize at the time the simple checks and balances I hypothesized were mediated by an exceedingly complex maze of regulators of regulators of regulators. We soon discovered that multiple regulators fed both positive and negative information to the phosphorelay, and phosphorylation-activated Spo0A was a negative regulator of growth as well as the inducer of transcription for sporulation (64).

Phosphatases of Phosphorylated Spo0A

Not surprisingly, some antisporulation growth stimulatory regulators turned out to be phosphatases. The first phosphatase specific to the phosphorelay was the product of the *spo0E* gene, which had been cloned and sequenced earlier by Marta (59). Moreover, it was postulated to be a negative regulator of the phosphorelay because it decreased sporulation when overproduced and deletion of this gene upregulated sporulation (61). Kari Ohlsen purified the protein encoded by the *spo0E* gene and showed that it was a dedicated protein phosphatase specific for the Spo0A~P transcription activator, thus providing evidence that our theory of each component of the phosphorelay being a target for signal information might be correct (**Figure 2**) (48).

Marta recognized the products of the *ynzD* and *yisI* genes were homologous to Spo0E and found that Spo0A~P was also their target (53). These new phosphatase genes were differentially regulated but generally induced by growth conditions antithetical to sporulation; thus, their regulation was consistent with negative regulatory roles in the phosphorelay controlled by independent growth-dependent signals as predicted.

Phosphatases of Phosphorylated Spo0F

A second type of phosphatase was discovered when Marta cloned and sequenced the *spo0L* gene. The sequence identified it as identical to the *gsiAA* gene of Mueller and Sonenshein (42). They had shown that *gsiAA* was regulated by the ComA/ComP two-component system that served to initiate the competent state as an alternative to sporulation at the end of growth. Moreover, they predicted that GsiAA was a negative regulator of sporulation (43). Ultimately, Perego et al. (57)

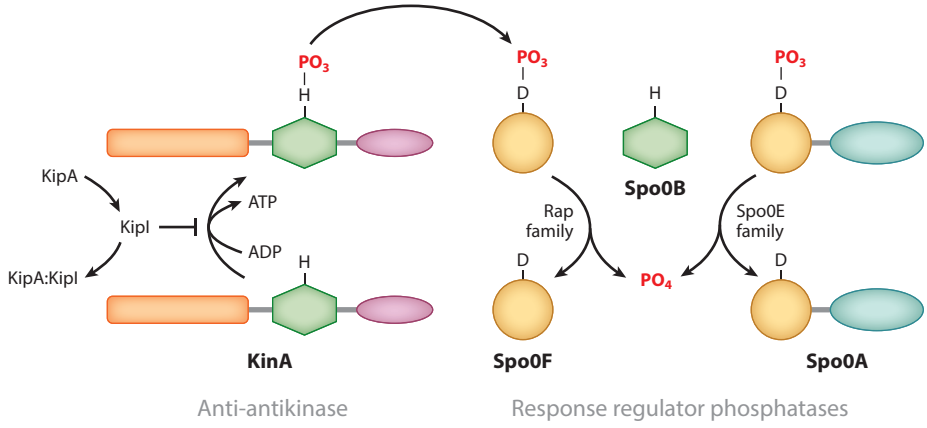

Figure 2

Negative regulation of the phosphorelay. Antisporulation mechanisms exist to counteract the activation of kinases and subsequent phosphoryl transfer. KipI is an antikinase inhibiting the ATP-dependent autophosphorylation of KinA. KipI inhibitory activity is neutralized by complex formation with KipA. Two families of aspartyl phosphate phosphatases specific for either phosphorylated Spo0F or Spo0A were discovered. Six known members of the Rap family, each independently regulated and some controlled by specific secreted peptides, act on Spo0F∼P. Three members of the Spo0E family act on Spo0A∼P upon activation by conditions favoring active growth rather than sporulation.

showed that Spo0L was a phosphatase of Spo0F∼P. The Spo0L protein and its homolog Spo0P were renamed Rap phosphatases. Genome sequencing revealed genes for 11 such proteins in *B. subtilis*, 6 of which acted as phosphatases of Spo0F∼P. These studies implicated phosphatases in a tripartite battle between growth, sporulation, and competence (**Figure 2**) (55, 66, 67).

The regulation of Rap phosphatases was about to get complex! The *rapA* gene was located in an operon adjacent to *phrA*, a gene coding for a small 44–amino acid protein with a secretion signal sequence, suggesting it was secreted by the bacterium. Why? Genetic evidence indicated that this small protein was a regulator of RapA phosphatase activity and may have served as a means for cell-cell communication (62). The nature of the secreted peptide that was the ultimate regulator remained unknown until Alan Grossman's laboratory (73) purified a competence-inducing peptide from culture supernatants as a 5–amino acid peptide corresponding to the C terminus of the PhrC protein. Marta demonstrated, using synthetic peptides, that the carboxyl 5 amino acids were sufficient for inhibition of the phosphatases (52) and single–amino acid changes among these 5 were responsible for differential specificity between Rap phosphatases. Subsequently Shu Ishikawa, Leighton Core, and Marta (33), using purified RapA phosphatase, were able to show that the peptide was bound to the tetratrico repeat domains in RapA and functioned by preventing or dissociating the complex between Spo0F∼P and RapA. This export-import cycle of regulatory peptides explains the sporulation deficiency in peptide transport mutants (58, 62) and mediates specificity control of eight different Rap phosphatases (54). The cycle may be a mechanism to couple sporulation and competence to population density (57).

Antikinase and Anti-Antikinase Regulators

The discovery of antikinase and anti-antikinase genes coding for new negative regulator proteins came when we realized that mutations in genes for negative regulation of sporulation would not necessarily be recognized in a search for sporulation-deficient mutants. We generated a library of *B. subtilis* DNA in a multicopy plasmid under the rationale that overproduction of a negative

regulator would manifest as a sporulation-deficient phenotype. This method led to the discovery of a pair of genes, *kipI* and *kipA*, encoding regulatory proteins (94). KipI protein was found to be an antikinase inhibitor of the ATP-dependent phosphorylation of the sporulation kinase KinA but did not affect its phosphotransfer reactions. KipA functioned as an anti-antikinase by inhibiting KipI from associating with KinA (**Figure 2**). The crystal structure of TTHA0988, a *Thermus thermophilus* protein of unknown function that is homologous to a KipI-KipA fusion, confirmed these conclusions (34). Many homologs of KipI-KipA have now been discovered in other bacteria, sometimes found as fused proteins, but the mechanism regulating the association-dissociation of KipI-kinase and KipI-KipA complexes and their homologs remains obscure (34).

TRANSITION STATE GLOBAL REGULATORS

Global regulators that affect the transcription of large numbers of genes expressed during the transition between growth and sporulation are controlled by Spo0A.

AbrB

The enigmatic regulatory protein AbrB was discovered by John Trowsdale when he was studying second-site phenotypic revertants of *spo0A* mutants (87, 88). Mutations in the *abrB* gene suppressed the many phenotypes of *spo0A* mutations. This property led us to label the AbrB protein a transition state regulator—a designation I stole from chemistry referring to the metastable intermediate in a chemical reaction, thus proving all those years of chemistry courses paid off.

Cloning and sequencing of the *abrB* gene by Marta revealed a small protein (63), under repression control by Spo0A (79). Mark Strauch, George Spiegelman, and others showed AbrB was capable of binding to DNA (78) and was able to act as a repressor or an activator, depending on the promoter to which it was bound. AbrB was ultimately found to control hundreds of genes either directly or indirectly, by regulating the transcription of other regulators that controlled further regulators. Therefore, many of the multitude of phenotypes exhibited by *spo0A* mutants were consequences of the lack of Spo0A repression on *abrB* transcription leading to overproduction of AbrB (77, 79). Subsequent studies by Strauch, after he left the laboratory, and John Cavanagh revealed the structure and further functions of this regulatory protein (49, 93).

Hpr

In the early studies of protease production we isolated and mapped a series of *hpr* mutations in which protease expression was much higher than normal (24). Later we began a study of the mechanism of this hyperproduction and showed that the effect of *hpr* mutation was located next to the promotor of the subtilisin gene and that *hpr* mutations as well as *sacU* mutations increased the mRNA level in the cell for this locus. This suggested that Hpr and SacU proteins were repressors and part of a global response to nutritional conditions (23). Marta cloned and sequenced the *hpr* gene, confirmed its product was a repressor, and found that transcription of *hpr* was under control of Spo0A (60). Pauli Kallio purified the Hpr protein and located its repression binding sites on the promoters of subtilisin and neutral protease genes (35). Ultimately these mutants and others established the basis for the formation of the enzyme production company Genencor by former postdoctoral fellow Dennis Henner when at Genentech, with subsequent strain development by another former fellow, Eugenio Ferrari.

Whereas these regulators were found and characterized in my laboratory, other investigators discovered additional regulators affecting sporulation. The most important discoveries were the developmentally regulated RNA polymerase sigma factors and their roles in progression of

sporulation, by the Losick laboratory (e.g., 5), and specific to gene expression in the forespore, by Setlow and colleagues (80). The conclusion was that sporulation, once viewed as simple, was complicated beyond belief.

AN ESSENTIAL TWO-COMPONENT SYSTEM CONNECTING CELL WALL GROWTH WITH CELL DIVISION

A major unanswered question in sporulation was how cell division is shut down by the onset of sporulation.

We wondered if any of the two-component systems in *B. subtilis* might be essential for viability, which would allow us to investigate the interaction between division and sporulation. Celine Fabret found a single system essential for growth whose transcription was repressed upon the initiation of sporulation (11). The genes for the kinase, YycG, and the response regulator, YycF, were in an operon with proteins of unknown function, YycI and YycH, later revealed to be activity regulators of YycG kinase, not transcription regulators (83). A temperature-sensitive YycF mutant stopped growth within 30 min, and microscopy revealed that cells were devoid of internal contents, suggesting leakage through the cell wall and membrane barriers. This indicated that whatever its role, it might be similar in gram-positive pathogens and a target for anti-infective drugs. This supposition was proven to be true (44, 45) and led us to spend time working with Johnson and Johnson Corporation trying to produce such antibiotics (4).

Our crystal structure of YycH (84) and YycI (69) revealed that both proteins were structurally similar despite having very little amino acid sequence homology. Research by Hendrik Szurmant and others in the laboratory showed that YycH and YycI formed a complex with the sensor kinase YycG that regulated its activity (82). YycH, YycI, and YycG are all proteins with extracellular domains and transmembrane helices. Hendrik and Charles Brooks applied a replica exchange molecular dynamics computational approach that generated in silico structural models of the transmembrane helix complex. The results predicted that signal recognition by any of the extracellular domains of the sensor histidine kinase YycG or the associated proteins YycH and YycI is transmitted across the cellular membrane by subtle alterations in the positions of the helices within the transmembrane complex of the three proteins, and mutational analysis substantiated the prediction. Moreover, truncation studies of YycH and YycI demonstrated that the individual transmembrane helices of these proteins, lacking the extracellular domains, were sufficient to control YycG activity (81). What the extracellular domains of YycH and YycI are doing is still a mystery.

Bigger surprises were yet to come. Tetsuya Fukushima and Hendrik showed that YycG kinase activity on YycF was dependent on YycG localization to the division septum, indicating that the YycG sensor kinase perceived information at the division septum to coordinate growth and division with cell wall restructuring (20). How did it move there? What domain of YycG kinase was interacting with the septum? Tetsuya was able to show that the cytoplasmic PAS domain of this multiple-domain transmembrane kinase was a determining factor translocating the kinase to the division septum (19).

The YycG-YycF two-component system is being studied in many organisms in many labs. It is an important component of what is sure to be a fascinating regulatory network controlling cell division to be revealed in the coming years.

UNDERSTANDING MOLECULAR RECOGNITION BY PROTEIN STRUCTURE AND STATISTICAL PHYSICS

The physical structure of phosphorelay signal transduction proteins reveals the mechanisms by which they function.

Research on understanding the basis for multiple phenotypes where regulators are regulating other regulators and they, in turn, regulate more regulators that affect the phosphorelay can drive one mad. I avoided this fate by beginning studies on protein structure and returning to biochemistry of regulator proteins, which has its own, different, frustrations, but I felt comfortable there.

Our crystal structure of the Y13S, phosphatase-resistant, Spo0F mutant (40) showed how surface residue changes around the active site aspartate could yield a protein resistant to the Rap phosphatases while still active with the phosphorelay kinases. Comparing the structure to that of the chemotaxis protein CheY allowed speculation as to the mechanism behind differential stability of the acyl-phosphate in the two proteins, a concept that was quickly proven in studies by Yih-Ling Tzeng and me (90) and by Jim Zapf et al. (99). Our nuclear magnetic resonance (NMR) structure of Spo0F with John Cavanagh defined the flexible regions found in conformers that we proposed to be the regions responsible for interaction with other proteins of the phosphorelay (12). Moreover, we captured the structure of Spo0A bound to its DNA target, in a crystal, allowing determination of residues important for binding (101).

The molecular recognition of Spo0F with KinA and Spo0B was investigated by Yih-Ling using a comprehensive strategy of alanine-scanning mutagenesis. Of 124 residues, 79 in the region of surface helices and loops were individually changed to alanine using site-directed mutagenesis. The mutants with notable in vivo sporulation phenotypes were further examined in vitro to identify the corresponding effect in each protein-protein interaction. This study revealed that most, if not all, protein-protein interactions involve the residues in the vicinity of the active site and identified the surface-exposed residues critical for the interactions with KinA or Spo0B (90). This beautiful study was very important, as it drove our thinking about how protein-protein interaction specificity is generated by evolution.

One question in two-component and phosphorelay signal transduction kept nagging me. How did a protein recognize its partner to the exclusion of 30 or more other structurally identical homologs in the cell? How did specificity arise?

While all of our studies were directed toward this question, we did not have a physical structure of the interaction between signaling proteins. The crystal structure of the Spo0B phosphotransferase of the phosphorelay was the first step in solving this problem. It was found in a crystal structure that Spo0B existed as a dimer in solution and that it dimerized primarily through helical hairpin domains to form a novel four-helix bundle; the His-30 residue in this bundle was identified as the catalytic histidine (91, 92). The overall importance of this structure rests on the fact that it was the first structure to be determined of the phosphotransferase domains of essentially all histidine kinases in two-component systems.

The major breakthrough in molecular recognition occurred when Madhusudan and Jim Zapf accomplished the impossible by obtaining a crystal of Spo0B complexed to Spo0F. The analysis of this cocrystal gave for the first time a physical structure of a response regulator bound to the active site of its partner phosphotransfer domain and defined the amino acid residues important for interaction (100).

WHAT THE Spo0F-Spo0B INTERACTION SURFACE REVEALED ABOUT MOLECULAR RECOGNITION

The surface by which two-component proteins interact not only serves as the means for recognition by compatible pairs of signaling proteins but also aligns the catalytic site for phosphoryl transfer between them. Therefore, it should not be surprising that the active-site residues, including those involved in catalysis and those residues for metal binding and stability that are closely associated with catalytic residues, are resistant to evolutionary change because the environment of

the active site is the primary determinant of directionality and equilibrium in phosphoryl transfer. Yih-Ling showed that relatively minor side chain changes in these residues have profound deleterious effects on both of these properties (90). Since the evolutionarily stable residues are responsible for catalysis, recognition must be the province of the other residues surrounding the active site that are part of the surface of interaction.

In order to identify residues involved in specificity recognition, it made sense to me to examine in detail a closely related family of two-component systems that were recently evolved by gene duplication and whose individual proteins needed to have acquired unique recognition properties to distinguish themselves. Furthermore, it seemed intuitive to examine the family within a single bacterial species since that is where recognition discrimination is most important. Thus I compared the related response regulators from 13 OmpR family members in *B. subtilis*. The residues making up the potential surface of interaction with the sensor kinase were superimposed on the Spo0F interaction surface revealed by the crystal structure of the Spo0F-Spo0B complex and by alanine-scanning mutagenesis studies (31).

This comparison revealed that the conserved catalytic residues had little, if any, determinative properties on recognition. Highly variable residues in the interaction surfaces were found that established discrimination between protein partners. These recognition residues either allowed two proteins to fit together or prevented them from fitting together by introducing charge and size constraints at several points within the surface. The greater the incompatibilities in recognition residues between the wrong partners of two-component systems, the less chance that signals would go astray (31).

I should point out that recognition within families of evolved two-component systems is fragile. In vitro studies of cross recognition or in vivo overproduction of kinases, by others, do not respect the environment or cellular levels in which these systems work and ultimately fill the literature with questionable conclusions.

STATISTICAL PHYSICS APPLIED TO IDENTIFYING PROTEIN INTERACTION SURFACES

Our studies revealed the important fact that the evolution of surface residues determining interaction between two proteins evolved at a rate different from noninteractive residues. This enabled statistical physicists to determine how any two proteins interact.

Evolution is our friend; we can determine how and where two proteins interact based on the rate of evolution of amino acids at specific positions in both proteins. Since every surface residue could be any one of 20 amino acids, what one hopes to find are residue positions in one protein of a pair that evolve with a residue position of the partner protein. Remember, only certain amino acid pairs are compatible with maintaining the correct structure of the complex, whereas other residues are free to evolve into any of the other amino acids.

This study was done at a time when the genomes of very few bacteria had been completely sequenced. The two-component systems were highly amplified in each organism, thereby increasing the size of the database by a factor of 30 or more, which made it amenable to statistical analyses. Being statistically challenged, I turned to physicist Terry Hwa (University of California, San Diego) for help. Bob White, a postdoctoral fellow, began a comprehensive analysis of the amino acid residues at every position of two-component systems using a coupled approach employing a mutual information measure followed by a log-likelihood score to potentially interacting protein pairs (96). It was predicted that even single-copy protein pairs would be amenable to this method if enough genomes were sequenced. Subsequently, covariance-based methods were found to have an important shortcoming in that they could not distinguish between directly and indirectly correlated residues.

Martin Weigt and Terry Hwa developed a method, Direct Coupling Analysis (or DCA), that combined covariance analysis with global inference analysis and, with Hendrik Szurmant, applied it to a set of >2,500 representatives of the bacterial two-component signal transduction system (39, 95). This combination of covariance with global inference successfully and robustly identified residue pairs that were proximal in space both for hetero-interactions between sensor kinase (SK) and response regulator (RR) proteins and for homo-interactions between RR and SK dimer proteins. The success of this approach illustrated the effectiveness of the global inference approach in identifying direct interaction based on sequence information alone.

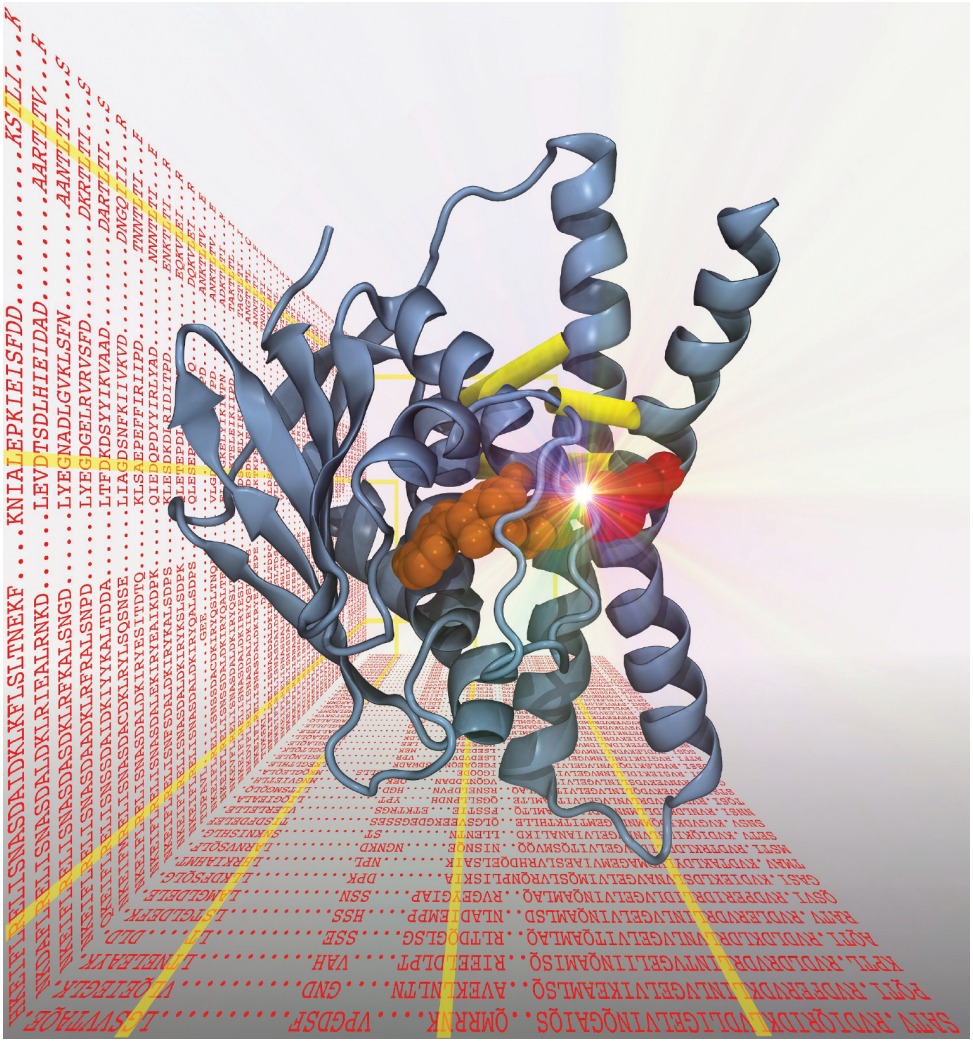

Figure 3

Generation of protein interaction structures from databases of protein homologs by statistical analyses. Illustrated here are direct coupling analyses with molecular dynamics simulations of HisKA histidine kinase catalytic domains (*abscissa*) with ATP-binding HATPase domains (*ordinate*). The program generates contact residues (*yellow*) between the two domains, forming the three-dimensional structure of the active-state kinase in autophosphorylation (9, 70, 71). Figure courtesy of Alex Schug.

Subsequently, Schug and colleagues (70, 71) developed a method capable of generating structural models of transient protein complexes by using existing structures of the individual proteins. The method combined bioinformatically derived contact residue information with molecular dynamics simulations (**Figure 3**). They found crystal resolution accuracy with existing crystallographic data when reconstituting the known system Spo0B/Spo0F. Later studies by Angel Dago, Hendrik Szurmant, and the physicists showed they were able to assemble a structural model using genomically derived contacts and mutagenesis techniques to repair a severely defective hybrid protein to full in vivo and in vitro function (9). Thus structures of unstable transient complexes of interacting proteins and of protein domains now were accessible by applying this combination of cross-validating technologies (9).

This program continues with the promise of deducing the structural interactions of many of the cell's protein combinations. It was clear that this program I began and promulgated had moved beyond my mathematical capabilities, leaving me in the dust.

EPILOGUE

Upon reflection I realize now my greatest interest was in proteins and the mechanisms by which they recognize each other and transmit signals to transcription controllers thereby converting a signal to gene activation or repression. *B. subtilis* is the great model of "simple" development where the cell shuts down the networks necessary for division and fires up new signaling networks to form a spore. Bacteria have provided the tools for and a window to seminal mammalian discoveries. Perhaps developmental mechanisms in mammals may retain features of their progenitor microbial differentiation systems.

DISCLOSURE STATEMENT

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