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A Career on Both Sides of the Atlantic: Memoirs of a Molecular Plant Pathologist

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Abstract

This article recounts the experiences that shaped my career as a molecular plant pathologist. It focuses primarily on technical and conceptual developments in molecular phytobacteriology, shares some personal highlights and untold stories that impacted my professional development, and describes the early years of agricultural biotechnology. Writing this article required reflection on events occurring over several decades that were punctuated by a mid-career relocation across the Atlantic. I hope it will still be useful, informative, and enjoyable to read. An extended version of the abstract is provided in the **Supplemental Materials**, available online.

Supplemental Material

INTRODUCTION

I am especially honored to be invited to write an autobiographical review for the *Annual Review of Phytopathology*. As my assignment allowed for considerable latitude, my story deals with some of my experiences, the circumstances that shaped my career as an academic plant pathologist, and selected aspects of my work in the historical/technological context in which they occurred. These were inextricably linked to the early developments in the recombinant DNA field and their exploitation for the advancement of molecular phytobacteriology as a disciplinary field. As my own work dealt mostly with phytopathogenic pseudomonads, references to other phytobacterial genetic models are made only occasionally, mainly with regard to some genetic analysis tools that served the early years of molecular phytobacteriology. This article does not address extensively disease-relevant developments in plant genetics and genomics.

MY ROOTS

I was born to an agricultural family in a small village in southern continental Greece in the midst of World War II (WWII). I was fortunate that my family survived WWII, the occupation of the country by the Axis powers, and the civil war that followed unscathed. I have only vague recollections of the war itself, but vivid memories of the hardships people endured during those periods in the Greek countryside. It is ironic that seven decades later, economic recession was followed by severe austerity that matches the post-WWII hardships. Most of the village inhabitants were small-size farmers, growing a variety of crop plants in two regions of different elevations: one suitable for olives, winter vegetables, and citrus crops and the other suitable for fruit and nut trees, grapes, summer vegetables, and field crops. Thus, I had the opportunity to become familiar with the ins and outs and hardships of small-farm, low-tech agriculture as well as annual and multiannual crops and their growing cycles, diseases, pests, and vulnerability to unseasonal weather conditions. I am thankful my parents did not place high demands on my siblings and me to work on the farms except during the summer and school holidays. In primary school and high school, I usually got top grades in most classes, but I was fixated on nonscience subjects, especially modern and ancient Greek and Latin (however, no foreign language teachers were appointed in countryside schools in those years). Thus, I had relatively little exposure to biology, and what I had was mostly descriptive, but I did learn how olive trees could be propagated through cuttings and how scions could be grafted onto rootstocks. In the last two years of high school, I discovered my attraction to the physical sciences, a subject that was not really covered in the curricula of earlier years. After an unsuccessful bid to enter the Athens Polytechnic (engineering professions offered better employment opportunities), I gained admission to the Agricultural University of Athens (AUA), thanks to the top scores I received in the composition/language essay exam.

HOW I BECAME A PLANT PATHOLOGIST

Undergraduate Years

AUA ran a five-year undergraduate program in agricultural sciences that covered plant agriculture, animal husbandry, food science, agricultural chemistry and engineering, soil science, meteorology, and economics, with opportunities for specialization in several of these areas during the fifth year of study. My exposure to and indoctrination in plant pathology occurred in several distinct stages. During my undergraduate years, I was strongly attracted to courses like plant physiology (which included biochemistry), phytopathology, and (then classical) genetics. The plant physiology professor was not the most eloquent teacher, but he was highly disciplinary, very demanding, and

AN INSPIRING EXCERPT FROM A CLASSICAL GREEK PHILOSOPHER

"Μή κατόκνει μακράν οδόν πορεύεσθαι πρός τούς διδάσκειν τι χρήσιμον επαγγελλομένους. αισχρόν γάρ τούς μέν εμ πόρους τήλικαύτα πελάγή δια περάν ένεκα τού πλείω ποιήσαι τήν υπάρχουσαν ουσίαν, τούς δέ νεωτέρους μήδέ τάς κατά γήν πορείας υπομένειν επί τώ βελτίω καταστήσαι τήν αυτών διάνοιαν."

"Do not hesitate to travel a long road to those who profess to offer some useful instruction; for it were a shame when merchants cross vast seas in order to increase their store of wealth, that the young should not endure even journeys by land to improve their intellect."

(Excerpt from Isocrates, to Demonicus, speech 1, letter 19. In George Norlin, Ed. 1980. Isocrates with an English Translation. London, William Heinemann Ltd.)

always worked in his lab (although he rarely published; those were not "publish or perish" days). I was impressed by such things as his spooling of plant DNA in a test tube after it precipitated upon addition of alcohol in lab sessions. The genetics professor strongly appealed to me partly because he was the youngest faculty member (under 30 years old) among the mostly senior faculty and maintained an active lab. In the plant pathology course, I saw a good mix of familiar themes and challenges (my farm background played a role in this) and a basic science focus, mostly the use of the microscope and pure (fungal) culture techniques that enabled students to come in close contact with the microbial world. Most important was the great devotion of the plant pathology Lecturers and Research Assistants who supervised the trainees specializing in phytopathology. One of them recommended me for a short-term internship at the National Center for Scientific Research "Demokritos" (NCSR) in Athens. The NCSR Genetics lab was run by Dr. Spyros Georgopoulos, who specialized in fungicide resistance mechanisms in phytopathogenic fungi. My assignment was to investigate the relative mutability of *cnb* loci involved in pentachloronitrobenzene (PCNB) and biphenyl resistance in *Fusarium solani* f. sp. *cucurbitae* race 1. Of some 100 mutants I isolated, one turned out to be a new *cnb* locus, which is how I got my first publication (36). This assignment enabled me to get experience in "hands on" petri dish biology, single-spore and hyphal tip cultures, and genetic analysis in a standard sexual system of a plant-pathogenic fungus. Crucial to my subsequent engagement in plant pathology was the fact that Dr. Georgopoulos was a recent PhD graduate from the Department of Plant Pathology at UC Berkeley (UCB). He guided me to apply for graduate study in the same department (I believe that he actually uttered the often-quoted proverbial phrase "Go west, young man, go west") and supported my application with a strong recommendation letter. This was my sole application for graduate training abroad, and I was lucky to be offered financial support in the form of a research assistantship. I am immensely thankful to my mentor and to the department. After I submitted my undergraduate thesis and took the institutional oath off schedule, I requested a deferment from army duty (thanks to new legislation that had just been enacted by the newly elected Greek government) and started attending classes at Berkeley in the spring of 1966. I felt as if the advice of the ancient Greek philosopher Isocrates marked my graduate studies and my career (see sidebar titled An Inspiring Excerpt from a Classical Greek Philosopher).

Graduate Years and My Switch to Phytobacteriology

The UCB plant pathology department hosted a cosmopolitan group of graduate students (including two other Greek students). Socially, the campus was going through very intense and interesting times: The free speech movement was at its height, the Vietnam War was escalating, racial

equality and gender issues were demanding their share of attention, growing a beard came into fashion among college students (but for some conservative professions, it carried the risk of not getting a strong recommendation letter) and noontime rallies and student sit-ins were the order of the day. In this context, the integration of graduate students and faculty into the department's social life was comforting and reassuring. My first graduate research assignments at UCB had little to do with test tubes, except to make and test various buffers and additives to improve mechanical transmission of viruses to indicator plants. Because the 60s were exciting and romantic days for plant virology, I felt more attracted to basic research on viruses and virus diseases, and eventually joined the laboratory of Dr. Herbert Gold, a plant physiologist working on *Beet curly top virus*. The viral nucleic acid had not yet been characterized, and my supervisor was not willing to address this issue. Instead, my doctoral research focused on physiological effects of the virus on photosynthesis, carbohydrate translocation, and macroelement uptake/translocation in tomato plants; it eventually led to the publication of five papers in refereed journals, a very productive record by the department's standards at the time.

Upon completion of my graduate studies, I faced the hard realities of a post-PhD career option for a non-US citizen facing an uncertain future in both the United States and his home country. I had not developed a particular liking for private sector jobs (mainly agrochemical or horticultural in those days), and the biotech startup era had not yet arrived. I applied for postdoctoral work in plant virology laboratories and came close to being offered one or two positions (not having a green card may have played a role in not getting a final offer). At this juncture, I realized that university positions in plant virology were rapidly becoming saturated; thus, a few years of postdoctoral work in plant virology seemed not to be the best option. I took notice of a new academic program (PhD/MD) at the University of Miami's Medical School. After vacillating for several months, I asked my major professor whether he would provide a recommendation letter to support my application and was relieved at his very positive response. Had I pursued this program, I would probably be an MD today.

My switch from plant virology orientation to phytobacteriology was serendipitous. Milton Schroth, the department's resident bacteriologist, offered me work in his lab, initially for six months, while I was still searching for postdoctoral positions. We held regular lab and one-on-one meetings, one of which started like this: "Nick, I have a favor to ask of you: Some students/colleagues call me Milt, others Dr. Schroth, and others are not sure how to address me. I encourage you to use the first one;" I took this as a signal that our former student/professor formalities belonged to the past. After tossing around different ideas for short-term research projects, we agreed to investigate the role of flagellar motility in bacterial infection. We isolated several nonmotile mutants of *Pseudomonas syringae* pv. *phaseolicola* using chemical mutagenesis and their motile revertants by employing methods used for the study of bacterial chemotaxis, and compared their rates of invasion of bean leaves that had been infiltrated with water or various attractants/repellents. The mutant-revertant approach intended to sidestep the common problem of chemical mutagenesis: unwanted mutations. Not surprisingly, nonmotile mutants of *P. syringae* pv. *phaseolicola* invaded water-soaked leaves at much lower rates (85). This was my first "baptism by fire" in routine bacteriological techniques and classical bacterial genetics. On the side, I ended up collaborating with Dr. Schroth's PhD students Walter Guimaraes and John Cho. We worked with *Erwinia* spp. and decided (clandestinely at first) to explore options for developing gene transfer systems for phytopathogenic pseudomonads and erwinias. Once our first results on R-plasmid transfer to phytopathogenic bacteria came in (published in 1975) (16, 83), Milt swiftly secured additional funding and extended my tenure in his lab. As the molecular phytobacteriology era gained speed, I was fortunate to be appointed as a Researcher and Lecturer, and later to a tenure-track academic position at UCB. Occasionally, some colleagues called me a "Berkeley auxotroph"

because I was hired by the same department where I had pursued my doctoral and postdoctoral studies.

THE EMERGENCE OF MOLECULAR PLANT PATHOLOGY

During the period from the mid-1960s to approximately 1980, research on mechanisms of plant disease susceptibility and resistance and pathogen virulence remained focused primarily on preformed and postinfectional inhibitors of infection (phytoalexins and their elicitors and suppressors), cell wall–/cuticle-degrading enzymes, and host-specific (fungal) and nonspecific (bacterial) toxins as well as the role of these factors in the host-pathogen interaction (1, 26, 86, 122). Conventional genetics in fungal-plant pathosystems, in parallel with disease resistance breeding, provided the basis for the concepts of vertical and horizontal resistance. Host macromolecules, such as pathogenesis-related proteins, and their possible role in plant defense were first brought to the forefront by the research on plant viruses in 1970 (37, 119). Classical genetics continued to address long-standing questions about the Mendelian genetics basis of pathogen virulence/host specificity of model phytopathogenic fungi producing host-specific phytotoxins and having standard sexual systems. These studies provided a large volume of data on the biochemical basis of virulence and the biochemistry and physiology of plant infections but, with few exceptions, did not sufficiently explain virulence, pathogenicity, or host-pathogen specificity mechanisms at the molecular genetics level. It was generally recognized that in host-pathogen systems where host-specific, generally necrosis-inducing toxins were involved, mutual recognition between host and pathogen resulted in compatibility. In regards to biotrophic/hemibiotrophic gene-for-gene systems, there were strong disagreements as to whether the specific recognition between host and pathogen molecules determined incompatibility or compatibility. It was generally presumed that recognition specificity rested in proteins, nucleic acids, or complex polysaccharides, rather than small molecules, because these could store enough structural/functional information to explain gene-for-gene interactions in molecular terms (23, 27, 116, 117). I recall long discussions with A.H. Ellingboe during occasional encounters, defending the recognition for the incompatibility view by reference to an R-gene allelic series in cereal hosts controlling resistance to different races of rust fungi and the temperature sensitivity of some resistance interactions involving single host and pathogen genes. However, disagreements regarding recognition specificity lasted well into the following decades, and broad-spectrum host resistance remained genetically undefined.

Shifts toward molecular genetics came primarily from plant virus and viroid research. By their very nature, viruses were viewed and studied as macromolecules (Wendell Stanley wondered whether a virus should be considered an organule or a molecism) and the important contribution of plant virus studies in the development of molecular biology at large was widely recognized. Full molecular characterization of plant viral and viroid genomes followed the development of nucleic acid chemistry and sequencing methods. An early paradigm shift in molecular plant virology incorporating genetics (mutation analysis) was an attempt to determine the location of the local lesion and coat protein genes along the *Tobacco mosaic virus* RNA by chemical mutagenesis methods (59, 60). Further studies on genes responsible for encoding viral proteins and particular disease symptoms became possible with the multicomponent plant viruses, virus satellites, and co-virus systems (118). The study of viral genome organization, subgenomic mRNAs, and viral (poly)proteins occupied researchers in subsequent years (133). The demonstration that mitochondrial DNA (mtDNA) may be the disease susceptibility trait in male-sterile maize signaled the beginnings of DNA-level analysis of fungal-plant interactions on the plant side (90).

Molecular genetics and recombinant DNA methods in bacteria and yeast made their entry in the early to late 1970s, similar studies in phytopathogenic fungi followed a decade later, and the

first cloning of an R gene came in the early 1990s (53). A main driver in these areas was the state of development of recombinant DNA technology and related tools for nucleic acid analysis. This brief account does not address the developments in molecular plant virology, viroid research, fungal pathogens/diseases, or plant disease resistance genes or in *Agrobacterium*; the reader is referred to recent reviews (15, 44, 58, 65, 77, 78, 101, 132). It is worth pointing out these three areas cross-fed into each other vis-à-vis concepts and mechanisms of plant-pathogen interactions. In addition, progress in these areas enriched the modern molecular toolbox for the study of plant-microbe interactions in important ways. Examples of this enrichment are the development of viral transient expression systems that emerged from molecular plant virology, the Ti-based stable expression of foreign genes in plants, the transcription activator-like effectors (TALEs) technology for genome engineering (4), and the recent use of type III secretion-based system (T3SS) as a tool to deliver fungal effectors using bacterial pathogens to plants (113).

BACTERIAL MODELS: TACKLING TECHNICAL HURDLES—THE INFANT STAGE

Plasmids and Chromosome Mapping

In the 1970s, several phytobacteriology laboratories engaged in analyzing the suitability of various bacterial plasmids and transposons for genetic studies of phytopathogenic bacteria and other bacteria of agricultural importance. To those "skilled in the art of bacterial genetics," the temperature-sensitive conjugative plasmids or phage derivatives used in model bacteria (e.g., Hfr strains desired for gross- or fine-structure mapping or transposon delivery) were not deemed broadly usable for such purposes in many plant pathogens because these did not grow at the nonpermissive temperature (typically 37°C). Strain/species/genus variability in terms of conjugational proficiency and transformability were commonly experienced. The majority of efforts during this period dealt with descriptions of indigenous plasmids and plasmid-mediated chromosome mapping. Research on bacteriophages was aimed primarily at their possible use in strain typing, with occasional reports on transductional mapping. There were earlier reports on transformation in *Xanthomonas*, but there was no follow through on these reports. Their statuses and those of other pre-1985 reports have been extensively reviewed (14, 63, 84).

Most genetic techniques were originally developed for gram-negative model bacteria, such as *Escherichia coli* and *Salmonella typhimurium*, and their use with gram-negative bacterial phytopathogens was restricted to members of the enterobacterial group. Many groups attempted to broaden the range of their application with some success. However, transposon mutagenesis and cloning technologies were fervently being pursued internationally by the *Agrobacteriun* and *Rhizobium* communities. Some of this progress eventually trickled into nonenteric gram-negative bacterial phytopathogens. Several groups, including mine, spent multiple years experimenting with transfers of different *E. coli* and *Pseudomonas aeruginosa* plasmids to and from phytopathogenic *Pseudomonas*, *Erwinia*, and *Xanthomonas.* In our initial studies, we were encouraged and guided by the early reports that broad host range antibiotic resistance plasmids originating in *P. aeruginosa* (later classified in the P1 incompatibility group) could be transferred by conjugation from *E. coli* to bacterial genera outside the Enterobacteriaceae, such that plasmids could mobilize chromosomal genes in *P. aeruginosa* and that the enterobacterial F′ derivatives and R factors could be transferred to phytopathogenic *Erwinia* spp. (14, 46, 63, 84). Following the first report on F′-mediated chromosome mapping in *Erwinia*, reports on the conjugative transfer of IncP-1 group R plasmids to/from diverse bacteria, including plant pathogens and symbionts, and their use in chromosomal gene mapping came in rapid succession. By the decade's end, rudimentary chromosome linkage maps of a few phytopathogenic bacteria were published (14, 31, 63).

Broad Host Range Cloning and Transposon Delivery Vectors

Following the excitement of our initial experience with IncP-1 plasmids, I sought to enlist the interest of mainstream molecular plasmidologists to consider modifying these replicons to serve the needs for cloning and molecular mutagenesis in phytopathogenic *Pseudomonas*. Donald Helinski and his group were among the pioneer plasmidologists whose work with small high-copy number *E. coli* plasmids served as a basis for the first-generation DNA cloning vectors. During a tour arranged in the context of the First Intersectional Congress of the International Association of Microbiological Societies (IAMS) in Tokyo in 1974, I had casual discussions with another attendee and indicated that I would like to meet Don Helinski. The person I was talking with responded, "I am Helinski." He expressed interest in my work on P-1 plasmid transfer to phytopathogenic *Pseudomonas* and *Erwinia*, which I had presented at the conference. Upon my return to California, I sent him *E. coli* strains harboring the plasmids, and a year later Don's group published their first of several papers on the molecular vehicle properties of the broad host range plasmid RK2, whose various reduced-size and cosmid derivatives were transformed into some of the most frequently used cloning vectors for nonenteric bacteria in the following years. Additional broad host range cloning vectors suitable for use in phytopathogens were also developed based on P, Q, Sa, and W incompatibility group and other plasmids (2, 3, 22, 109).

Along with the search for chromosome-mobilizing plasmids and cloning vectors, a need for tools suitable for molecular mutagenesis through transposon delivery to phytopathogens was widely perceived. Aside from the spillovers from the ongoing research on *Rhizobium*, efforts were made to develop such tools within and outside the molecular phytobacteriology community. While attending an international meeting in Europe, I took notice of a presentation by a Russian attendee reporting on the properties of a novel hybrid plasmid, pAS8 (a derivative of the RP4-ColE1 hybrid), displaying ColE1-dependent replication/maintenance due to insertion of the transposon Tn7 in a gene necessary for RP4 replication. This immediately raised the prospect of the potential utility of the plasmid as a general purpose suicidal transposon delivery vehicle for bacteria that did not support stable replication/maintenance of ColE1 but were within the conjugational host range of RP4. In casual talk, some western colleagues thought that Eastern Bloc researchers would probably not provide the plasmid to a Westerner (these were Cold War times). However, I was thrilled that the authors honored my subsequent request for the pAS8-Rep::Tn7 plasmid. Suicide plasmid technologies for phytopathogenic bacteria were also developed by taking advantage of the suicidal properties of bacteriophage *Mu*-containing derivatives of incP-1 plasmids. Useful as they were, certain transposons and suicide delivery vehicles displayed some undesirable properties in different bacteria. For example, transposon Tn7 tended to insert in certain "insertion hot spots." Some plasmids had additional antibiotic resistance genes coding for the same antibiotic resistance as the transposons that were called to deliver to recipient bacteria, and such genes had to be inactivated in advance; in others, such as Mu-containing derivatives, the suicidal properties were not universally expressed and simultaneous transposition or re-transposition of Mu sequences complicated genetic interpretation, as did transposition of Tn*5* and its terminal inverted repeat arms (IS50-L and -R) in some bacterial hosts. A detailed review published in the mid-1980s (75) covers the early stages and potential uses of transposon mutagenesis for studying virulence genes in plant pathogens.

It is important to mention here that cloning vectors, transposons, and suicide plasmids were continually being improved and refined for general and specific purposes. With regard to cloning vectors, derivatives with reduced size, alternative selection markers, multiple cloning sites, and cohesive end sites (cos), and those that were mobilizable by broad host range plasmids, more easily transformable, or endowed with other useful features continued to enrich the molecular toolbox

well into the 1980s and beyond. With regard to transposons, early examples of tools that were enlisted for the analysis of bacterial pathogenicity were the incorporation of various reporter genes, enabling visual identification of mutants, gene expression analysis in planta, negative selection for transposon excision/marker exchange mutagenesis, and transcriptional and translational fusions to reporter genes suitable for gene expression analysis and gene product localization studies. More versatile tools for generating series of mutants in different genes, each disrupted by a different antibiotic marker, to obtain all possible double or multiple mutant combinations were developed only recently (e.g., 80). It is also instructive for present-day students to highlight the fact that, quite often, the lack of suitability of genetic elements for particular purposes was exploited in positive ways. To mention just a few, suicide vector development was essentially based on the inability of plasmids to replicate in a particular host; plasmid incompatibility was exploited in plasmid eviction; the polarity of transposon insertions provided first indications that genes relevant to a particular phenotype were located downstream from the insertion points; and the chromosomal hot spots of certain transposons, such as Tn7, enabled researchers to produce collections of isogenic pathogen strains tagged with different fluorescent proteins for confocal imaging. The technical and sociological context that shaped the molecular biology of phytopathogenic bacteria is elaborated further in **Supplemental Materials Sections 6.1** and **6.2.**

Supplemental Material

WHEN IT RAINS, IT POURS (GENES AND GENE CLUSTERS EVERYWHERE)

Having assembled many tools of molecular genetics, phytobacteriologists were well positioned to address old and new questions relevant to bacterial pathogenesis from new perspectives. Starting in the early to mid-1980s, a flurry of gene discoveries came in rapid succession. As in other areas of biology, these discoveries were technology driven: Through the combined use of transposon or chemical mutagenesis, cosmid cloning, complementation, and other techniques, genes and gene clusters began to spring up everywhere. Among them was the cloning of the first bacterial avirulence gene (103). In this review, I discuss mainly genes with which my laboratory had some early or continued involvement. Among them were ice nucleation genes from epiphytic ice nucleating *P. syringae* and *Erwinia herbicola* (79), genes involved in the production of and immunity to a phytotoxin (*tox-argK*) (87), the hypersensitive response and pathogenicity (*hrp*; phonetic "harp") (69) from *P. syringae* pv. *phaseolicola*, and the production of fluorescent pigment-siderophore from *P. syringae* pv. *syringae* (72). Preliminary reports of these efforts were published either in abstract form or in conference papers as early as 1982. By employing similar methods as a starting point, and using progressively more advanced DNA sequencing methods, important gene discoveries in phytopathogenic bacteria continued throughout the decade of the 1980s and well into the 1990s and beyond, and were subsequently propelled by the advent of whole-genome sequencing in the genomics era. Because of space limitations, my involvement in ice nucleation and phaseolotoxin research was moved to **Supplemental Materials Sections 7.1** and **7.2**.

The Enigmatic Avirulence Genes

An early case of gene discovery in the field of molecular plant bacteriology was the cloning of bacterial avirulence genes during the 1980s and beyond. These discoveries made a major splash in the field of plant pathology: On one hand, they helped to extend the gene-for-gene concept to race-specific interactions in pathogens in which classical genetic analysis had not been possible; on the other hand, they seemed to conform to the basic tenets of the gene-for-gene hypothesis. However, the fact that these genes were genetically dominant for incompatibility (i.e., did not

seem to promote infection) left unanswered the enigma in Ellingboe's conceptualization; namely, if gene-for-gene specified incompatibility is superimposed on a basic ability to parasitize, what makes a bacterium pathogenic to plants in the first place? Whereas the identification of phytobacterial *hrp* genes (see below) seemed to provide a possible way out of this dilemma, their actual function in pathogenesis also remained elusive for many years. Over the next 25-year period, the requirement of a functional *hrp* system for the phenotypic expression of *avr*-coded avirulence function and the mode of action of Avr effectors in bacterial pathogenesis were established: Avr effectors [currently often referred to as type III effectors (T3SEs)] travel the Hrp/Hrc pathway to the plant cell interior. In susceptible plants, T3SEs suppress basal host defenses, thus promoting bacterial virulence on susceptible hosts. On resistant hosts, they are recognized by R genes or other receptors either directly or when they modify host targets connected to nodes of the immune signaling pathways. Beyond anybody's guess was the finding that a particular class of bacterial Avr proteins was in fact made up of transcription factors modulating the expression of plant R genes. These developments are covered well by Boch et al. (4) and Mansfield (74) and only occasionally mentioned in this review. At this juncture, the enigmas posed by the different interpretations of the gene-for-gene hypothesis seem to approach a long-awaited resolution. Indeed, various conceptual models to describe the plant immune system have been presented that take us far beyond most imaginative hypotheses based on classical genetics (6, 17, 25, 56, 99, 115, 126).

The Discovery of *hrp* Clusters

Historical accounts of the discovery and characterization of *hrp* genes in phytopathogenic bacteria are provided in past and more recent reviews, including one that I coauthored in 2010 (108). Here, I have chosen, arbitrarily, to revisit only some areas of my involvement in this journey. The conceptual framework for our search for genes controlling the bacterial hypersensitive response was provided by the seminal work of Klement and associates (61), which were presented during a seminar at Berkeley several years before I started working with bacterial pathogens. He showed that phytopathogenic bacteria, unlike their saprophytic relatives, elicited a rapid confluent necrosis [hypersensitive response (HR)] when injected in leaf intercellular spaces of resistant plants (nonhosts, heterologous hosts) at inoculum concentrations above a certain threshold. In essence, Klement's work made macroscopically visible a necrotic reaction of plant cells that are in immediate contact with the pathogen, as described decades earlier for phytopathogenic fungi (1), and brought to the foreground a hitherto unexplored phenotype of bacteria-plant interactions. Initially, the biological significance of the bacterial HR was not universally accepted by phytobacteriologists, as it was considered a likely artifact caused by the high inoculum levels needed for confluent necrosis. These doubts were partly dispelled by the demonstration of Turner & Novacky (112) that HR occurred at the single-cell level in tissues inoculated with dilute bacterial suspensions and partly by the eventual adoption of the tobacco HR assay as a standard test for pathogenicity of phytopathogenic *Pseudomonas* spp. Nevertheless, early attempts to isolate HR-inducing substances from bacterial cultures were not successful. It is known today that the majority of bacterial HR elicitors are effector proteins that are injected into the plant cell interior via the *hrp/hrc*-coded T3SS apparatus (T3SEs), are generally produced under conditions simulating the in planta environment, and, with the exception of harpins and harpin-like proteins, do not trigger the HR when administered to plant cells externally in purified form.

Armed with the necessary tools for molecular mutagenesis, my graduate student Peter Lindgren sought to obtain mutants of *P. syringae* pv. *phaseolicola* with altered host and nonhost plant responses. Because HR assays on tobacco leaves could be scored more rapidly and on fewer plants than disease symptoms on bean, he screened more than 3,000 presumptive Tn*5* insertion mutants for

their HR phenotype; those found to be HR$^-$, he subsequently screened for pathogenicity on a susceptible bean cultivar. To our surprise, the mutants that did not elicit the tobacco HR had altered host responses on bean as well: Some were completely nonpathogenic, whereas others had reduced virulence to varying degrees (incited fewer or smaller water-soaked lesions on bean leaves). We decided to designate these mutants *hrp*. The next reward came when the location of these mutations was mapped by complementation analysis and physical mapping of cosmid clones and the Tn*5* insertion region: All but one of the mutations mapped in a single contiguous region, approximately 25 kb on the bacterial chromosome, named the *hrp* cluster. Southern blotting and marker exchange analysis revealed structural and functional conservation in other pathovars of *P. syringae* (69, 70). The solitary mutation had been identified by other groups in *P. syringae* strains (reviewed in 125).

By the end of the 1990s, similar mutants had been described and characterized in representatives of all major taxonomic divisions of gram-negative bacterial plant pathogens: several pathovars of *P. syringae*, *Xanthomonas campestris*, *Erwinia amylovora*, *Ralstonia* (formerly *Pseudomonas*) *solanacearum*, and *Erwinia stewartii*. These reports are not cited here for reasons of economy but are well detailed in various reviews (7, 8, 21, 67, 108, 125). In all cases that were studied in sufficient detail, *hrp*-type mutations were clustered in largely contiguous chromosomal or megaplasmid regions, which were later shown to have the molecular footprints of "pathogenicity islands," i.e., evolutionarily mobile genetic elements. Important from a model system point of view was the isolation of a cosmid clone, pHIR11, carrying the *hrp* cluster from *P. syringae* pv. *syringae* 61 (48), which enabled nonpathogenic *Pseudomonas fluorescens* and laboratory strains of *E. coli* to elicit HR on tobacco thanks to the presence of a *hrp*-linked effector gene (*hopA1*, originally called *hrmA*). This cosmid and its effector-free derivatives proved instrumental in later studies on functional assays of T3SS effectors.

The next task was to define the *hrp* cluster of *P. syringae* pv. *phaseolicola* in molecular genetics terms. Lindgren et al. (68) and Rahme et al. (91) saturated the cluster with randomly distributed insertions of a newly designed transposon, Th3-Spice, which carried a terminally positioned promoterless ice nucleation gene as a reporter that allowed transcriptional (but not translational) fusions and provided a sensitive tool for the study of bacterial gene expression in planta at low inoculum levels where other reporters were deemed not ideal. Rahme et al. (91) produced the first genetic and transcriptional organization of the cluster in a phytopathogenic bacterium, defined seven complementation groups and the physical size of the cluster more precisely, and determined the transcriptional orientation of putative operons. Similar organization was also found in *P. syringae* pv. *syringae* C61 by Hutcheson's group (130), who found eight apparent transcriptional units within the *hrp/hrm* cluster, several of which were multicistronic.

EARLY CLUES ABOUT HRP GENE/PROTEIN FUNCTIONS

Clues about the possible or actual functions of *hrp* genes came in overlapping waves from many quarters over the next several years. The sections below deal with some of the early clues to which my group had some contribution or that paved the way for investigations that continue to this day. Key contributions of other groups are included somewhat arbitrarily to provide perspective.

Identification of *hrpR/S/L* Regulatory Trio: Only the Beginning

Early on, my hopes of obtaining clues about the possible function of *hrp* genes rested squarely on sequencing rather than blindly undertaking biochemical analysis. Around 1984–85, when the Hrp cluster of *P. syringae* pv. *phaseolicola* was physically and genetically characterized to some extent

but not yet functionally characterized in any respect. I submitted a grant application to a US federal agency in which I proposed, among other things, to sequence the entire cluster to enable more detailed functional characterization. Unfortunately, the application was turned down, and a comment made by one of the reviewers is illustrative of the thinking of times: "Suppose you knew the sequence today, what would you do with it?" Not having commercial sequencing as an option, or funds for the limited institutional sequencing services at UCB at the time, I took advantage of a sequencing opportunity at the Institute of Molecular Biology and Biotechnology (IMBB) during my sabbatical leave (see below) by enlisting the involvement of Charlotte Grimm, a postdoc, to begin sequencing the cluster. Given the size of the cluster and the lack of complementation and transcriptional analysis (see above), one question was where to begin. Somewhat arbitrarily, we decided to start from the "right end," where the limited complementation analysis by Lindgren et al. (69) had suggested that one average-sized gene would likely be found. It turned out that it was a lucky strike: The region harbored the *hrpS* gene, whose predicted product had significant similarity to a highly conserved central (activator) domain of several response regulators described for two-component bacterial regulators, among them NifA, NtrC, and DctD, which were being intensively studied at the time in nitrogen-fixing bacteria (41). Sequencing further upstream of *hrpS* revealed the presence of part of another likely regulatory gene with similarity to *HrpS*, which appeared to be truncated because of a scrambling of the cosmid insert during cloning. In a later study (40), sequencing and expression analysis of the approximately 1-kb region upstream of *hrpS* fully defined this gene, named *hrpR*. Our results were in line with those of Xiao et al. (128), who further proposed that HrpR/HprS participated, alone or as a heterodimer, in a regulatory cascade in which HrpR and HrpS activate expression of another gene, *hrpL*, located at the "left end" of the *hrp* cluster (91), whose expression requires an enhancer (the alternative sigma factor σ^{54}) (40, 50, 100, 128), which, in turn, recognizes a single promoter sequence called *hrp* Box upstream of *hrp/hrc* operons and *avr* genes in *P. syringae* pathovars (54, 96, 121, 129). These and other findings (51) enabled the first functional link to be established between *avr* and *hrp* genes in *P. syringae* strains by demonstrating that expression of *avr* genes was dependent on *hrpRS*. The Hrp Box later served as one criterion for the bioinformatic identification of T3SS-secreted proteins (putative effectors) in *P. syringae* genomes, although it is also found in variable suites of noneffector genes that are coregulated with T3SS secretion across the *P. syringae* phylogeny (76, 121). One puzzling aspect of HrpS and HrpR structure was the absence of an extended sensor domain N terminal to the activator domain, as found in most two-component system regulators. In many ways, these initial discoveries were the tip of the iceberg: A long list of subsequent studies of various *hrp* regulons continue to reveal intricacies of what turned out to be very complex regulatory networks, involving genes from both within and outside the *hrp/hrc* clusters acting both upstream and downstream of HrpRS$\sigma^{54}\sigma^{L}$ in some *hrp/hrc-I* systems (T3SS of *P. syringae* and *E. amylovora*, see below). Interestingly, the complexity of regulation of these systems has recently attracted the interest of synthetic biologists for the design of modular and tunable genetic amplifiers in cascade gene networks for scaling advanced intra- and extracellular control functions (57, 123).

Among the proteins that partake in T3SS regulation in some *P. syringae* pathovars under different environmental conditions are the GacA/S two-component system, the Lon protease, the RhpS/R two-component system (RhpS, a kinase, and RhpR, a phosphatase, global regulators that coordinate the T3SSs and many other cellular activities), and several nonconserved (Hrp) proteins coded within the cluster, among them HrpV, HrpT, the chaperone-like protein HrpG, the T3SS-secreted/translocated protein HrpJ, and the pilus protein HrpA (20, 24, 73, 81, 82, 124). The regulatory complexity of these systems appears to be even more enigmatic: a recent study proposes a "bistability" model for *hrp/hrc-I* systems involving the action of the double-negative regulatory feedback loop involving HrpV and HrpG and a positive loop involving the

main component of the pilus, HrpA (95). It appears that protein secretion through the T3SS is a highly regulated process, involving multiprotein interaction networks coupling expression regulation to secretion, making the T3SS one of the most complex bacterial protein secretion systems known (88). In contrast to the structural complexity of T3SSs, most *hrp/hrc* operons seem to be induced almost simultaneously without any pattern that would help integrate the ordered assembly of the export apparatus (74, 110). The detailed characterization of several conserved components of T3SSs, including a few protein crystal structures, has shed some light on the structure, assembly, and role of type III chaperones in bacterial plant-pathogen T3SSs (18, 19, 29, 30, 71, 106, 108). However, the schedule of effector export remains largely an open subject (11, 13).

Plant Inducibility

With regard to expression, a preliminary analysis by Lindgren et al. (68) indicated that transcription of certain regions of the *hrp/hrc* cluster of *P. syringae* pv. *phaseolicola* was strongly induced in planta within 2 h post inoculation, and induction was nearly complete by the time the Hrp^+ bacteria began to multiply in the leaf tissue. In follow-up studies, Rahme et al. (92) extended these findings in important respects: (*a*) A plant signal(s) was required for the induction of all seven *hrp* operons during both compatible and incompatible interactions; (*b*) expression of the key regulatory genes (see below) was regulated differently in planta versus in vitro; and (*c*) expression of individual *hrp* loci was differentially affected by pH, medium osmotic strength, and certain carbon sources. Similar results were published the same year for *P. syringae* pv. *syringae* 61 (130). These findings suggested complex signaling mechanisms taking place during plant-pathogen interactions, aspects that have not been fully elucidated to date. The nature of plant/environmental cues regulating T3SS gene expression in *P. syringae* pv. *phaseolicola* is also puzzling, as are the sensory proteins perceiving the signals, the links between signal sensors and the possible cross talk between T3SSs and other regulated processes in phytopathogenic bacteria (43). Recently, structurally diverse secondary metabolites have been identified as modulators of T3SS expression and assembly in various phytopathogenic bacteria (62, 114, 120, 131).

Sequence Similarities to Mammalian Type III Secretion Systems and Flagellum Proteins

Other clues about the nature and role of *hrp* genes in plant-bacterial interactions came in steps, beginning in the early 1990s. First, sequence comparisons established the occurrence of *hrp* genes in all gram-negative phytopathogen lineages beyond what Southern blot hybridizations were able to show initially. Second, several groups reported significant sequence similarities between several Hrp proteins and structural proteins of bacterial flagella as well as virulence gene clusters found in gram-negative mammalian pathogens, thus establishing a link between virulence-related genes across pathogens of the plant and animal kingdoms (7, 12, 19, 45, 49, 108). Third, some *Yersinia* virulence proteins that were secreted in culture [*Yersinia* outer proteins (Yops)] did not possess a canonical signal peptide necessary for secretion via the main bacterial protein secretion system (Sec) known at that time; however, they could be detected intracellularly in cultured mammalian cells after contact with the pathogen. It was soon established that certain avirulence proteins are translocated to the plant cell interior by use of Avr-Cya (calmodulin-activated adenyl cyclase) fusions (98), that the biogenesis of extracellular appendages (Hrp pili) was dependent on *hrp* genes, that *hrp* genes were also required for secretion in cultures of certain phytobacterial effector

proteins (harpins) of these bacteria that were able to elicit HR-like necrosis when applied externally to plants, and that the Hrp pilus served as a conduit for delivery of harpins and Avr proteins. On the basis of sequence similarities detectable by BLAST algorithms, nine of the proteins encoded by *hrp/hrc* clusters share significant sequence similarities among phytopathogen T3SSs (these were designated Hrc for Hrp conserved), whereas eight of them share significant sequence similarity with the components of the flagellar assembly of gram-negative bacteria [flagellar T3SS (F-T3SS)]. Accordingly, the *hrp/hrc* clusters are referred to as nonflagellar (NF) T3SSs. In summary, direct and indirect experimental evidence established that effector proteins may be translocated to the plant cells via the Hrp/Hrc apparatus, analogous to the T3SS mechanism in animal pathogens (19, 32, 49, 108, 111). Historically, *hrp* genes and their corresponding proteins were given different names in different plant pathogens and other bacteria that possess T3SSs. This situation led to confusion, and efforts to standardize the nomenclature were eventually made (5). For example, the nine highly conserved type III secretion genes have been named *hrc* (for *hrp* gene conserved) in plant-pathogenic and other bacteria that possess T3SSs (5). Gene Ontology, along with numerous biochemical studies, established the existence of at least six major protein secretion systems in gram-negative bacterial species. The pathways have been numbered Type I through VI (88).

THE AGE OF GENOME SEQUENCING

By the turn of the millennium, the completion of the human and *Arabidopsis* genome sequencing projects had drawn much public and scientific attention. Genome-sequencing projects of phytopathogenic bacteria began to show up in top scientific journals early in the 2000s and claimed their share of attention among phytobacteriologists, beginning with *Xylella fastidiosa* in 2000 (102) and followed by *Agrobacterium tumefaciens* in 2001 (38, 127), *R. solanacearum* in 2002 (97), and *P. syringae* pv. *tomato* DC3000 in 2003 (10). In the ensuing years, partial or complete phytobacterial genome sequences showed up by the dozens. Genomic mining became a fashionable practice among prospectors, enabling searches for everything that comes to mind: T3SSs and effectors, pathogenicity islands, phylogenetic and evolutionary studies, evaluation of plant-associated microbial communities and pathogen typing, and rapid identification of bacterial pathogens are becoming mainstream. The genomic technologies enabled, among other important discoveries, the identification of multiple T3SSs in some phytobacterial pathogens, detection of unwittingly large effector repertoires and other virulence-related genes, and comparative analysis of multiple genomes and metagenomes of complex microbial environments. These discoveries are recorded in many review articles (too many to be cited here) as well as in books (42, 52). The rapid pace at which new sequencing technologies emerged and the associated computational analysis created a growing disparity between the rate of data generation and biologically meaningful analysis. Today, fully annotated and well-curated genome databases are present-day equivalents of bacterial culture collections of the past and bioinformaticians/statisticians are still in short supply. In short, the era of genomically enabled microbiology had suddenly dawned upon the new generation of plant pathologists who were ready to take charge and capitalize on these challenges (105).

MY RELOCATION BACK TO THE OLD WOLRD

In the mid-1980s, I received an unsolicited phone call from Dr. Fotis Kafatos, then a professor at Harvard, who had maintained close ties with Greek universities and founded the IMBB and the Department of Biology at the University of Crete (UoC). I had met him only once at a social gathering following a seminar held at the University of California's Medical School in San

Francisco. He inquired whether I had any plans to repatriate to Greece and tried to entice me to consider a package of proposals: a faculty position in UoC's newly established biology department, a researcher's position at the IMBB, and a place on the scientific advisory board of a government-sponsored start-up biotech company. Although the package was enticing, I had no reason to make such a move at that juncture, as my research activity was gaining speed and my laboratory was well funded through federal grants. However, contributing to the new academic and research landscape in Greece, which had joined the European Union (EU), appealed to me as a challenge, and I opted to spend a sabbatical leave at the above institutions as a practical way to assist Kafatos in his effort. After spending a sabbatical leave in 1985–86 at the IMBB/UoC, I returned to Berkeley but maintained ties with the Greek institutions by continuing to teach in UoC's graduate program and hosting two former postdocs and two graduate students at Berkeley in the following years. That is when I met my second spouse, who followed me to the United States a year later. My lack of interest in repatriating changed in the early 1990s because of a series of events that followed the fall of the Berlin Wall: severe budget cuts in the University of California system, revolving temporary salary cuts for faculty, successive reorganization of many life science departments, including plant pathology, and implementation of sequential voluntary early retirement programs (VERIPs) for qualifying faculty. My final decision to relocate was difficult and took into consideration family reasons and the changing landscape of research funding in the United States and EU.

STRUCTURAL BIOLOGY AND FUNCTIONAL ARCHITECTURE OF TYPE III SECRETION SYSTEMS

Although in regards to the structural biology of T3SSs, studies with mammalian pathogens generally lead the field, important progress also came from studies with plant pathogens. Early examples are the visualization of the Hrp pilus appendage of *P. syringae* penetrating the plant cell, the identification of HrpA, a small hydrophilic protein of 11 kDa, as its main structural protein able to reassemble into pili in vitro, the immunocytochemical visualization of HrpA elongating from the pilus tip rather than from the base, and the discovery that HrpZ and Avr effectors are secreted only from the pilus tip, which led to the proposal that the pilus acts as a moving conveyor of effector proteins from bacteria to the plant cell interior across the plant cell wall (9, 47, 55, 66, 93, 94).

Upon relocation to UoC, my research direction adjusted to the pressures and opportunities in the national and EU funding priorities and to the need to build new allegiances with European and Greek colleagues. Research into structural aspects of T3SS proteins became a new adventure. However, I continued working on functional *hrp*- and effector-related aspects and was also drawn concurrently into several areas unrelated to T3SSs, where I had limited or no previous experience, and, often, I felt that I spread myself too thin. Owing to space limitations, I do not elaborate further on the latter two aspects and refer the readers to the **Supplemental Materials Section 8.1**. My involvement in the study of the structural aspects of T3SSs came largely as a diversion from my previous research. It was becoming increasingly clear that the T3SS is a very complex nanomachine (12, 18, 19, 28), and its full understanding would require the use of advanced structural analysis methods along with bioinformatics tools, with which I had no previous experience. Venturing into this new terrain entailed the sharing of graduate students, gene/clone resources, and my phytopathology perspective in multidisciplinary collaborations with UoC and IMBB groups led by Michael Kokkinidis and Anastasios Economou. This venture employed an array of structural biology and bioinformatics techniques and brought several rewards. Owing to space limitations, some of them are epigrammatically stated here (others were moved to the **Supplemental Materials**): the ability of the $HrpZ_{Psph}$ harpin to elicit cell death in planta when extracellularly targeted (107) and to form an ion-conducting pore in lipid bilayers in vitro (64), the

Supplemental Material

oligomeric structure of the HrcN ATPase (89), the determination of the tertiary and quaternary structure of the C-terminal domain of the HrcQb protein (29, 30), the coiled-coil interaction mode of T3SS proteins (33–35), and the demonstration of several binary and some ternary interactions among proteins of the *P. syringae* pv. *phaseolicola* and *E. amylovora* T3SS (it seems that, when it comes to its regulation, it takes many more than two to tango), which provided leads into ongoing advanced proteomics studies at the UoC/IMBB after my retirement.

CONCLUDING REMARKS

In his article recounting the historical developments in molecular biology, Gunther Stent (104) remarked that autobiographical essays about a scientific discipline may be symptomatic of an approaching decline. In light of the many reviews on the subject in the recent past [among them are several articles in a special volume of the *Molecular Plant Pathology* journal published in 2011 (see Reference 52)], such a fate may be in store for the field of molecular phytobacteriology. However, I would suggest that in the post-genomics era, the field may reconceptualize from "molecular" to "systems" biology and change direction, gravitating into new areas. The challenge will be to understand the huge amounts of readily accessible genomic, proteomic, transcriptomic, and metabolomic data and transition from bacterial genomics studies to application to real world problems in disease management (74, 105). After some 50 years, the story of molecular phytobacteriology is not yet finished.

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