# *PSEUDOMONAS SYRINGAE* TYPE III SECRETION SYSTEM AND EFFECTORS

by

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# *PSEUDOMONAS SYRINGAE* TYPE III SECRETION SYSTEM AND EFFECTORS

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*Pseudomonas syringae* pv. tomato DC3000 is a gram negative bacterial pathogen that causes bacterial speck disease on *Arabidopsis* and tomato. The pathogenicity of *P. syringae* relies on effector proteins that are injected into plant cells by the type III secretion system (TTSS). *hrpJ* is one of the genes in the *hrp*/*hrc* gene cluster, which encodes the TTSS apparatus. I demonstrated that HrpJ was secreted and injected into plant cells via the *P. syringae* TTSS. A DC3000 *hrpJ* mutant, UNL140, was greatly reduced in its ability to cause disease. UNL140 was defective in the injection of AvrB1, AvrRpt2, AvrPto1, HopB1, and AvrPtoB effectors into plant cells. Moreover, UNL140 secreted AvrB1, AvrPto1, HrpA1, but not HrpZ1. This defect in the secretion of HrpZ1 and possibly other putative translocators in the *hrpJ* mutant probably results in disabled injection of effector proteins into plant cells. Genome investigation in DC3000 identified three effectors, including HopU1, that share similarity with mono-ADP-ribosyltransferases (ADP-RTs). HopU1 inhibited outputs of plant innate immunity including the hypersensitive responses and callose deposition dependent on the ADP-RT active site. We identified three chloroplast and two glycine rich RNA binding proteins in *Arabidopsis* extracts that are ADP-ribosylated by HopU1. T-DNA knockout mutants of one of these glycine rich RNA binding proteins, AtGRP7, exhibited enhanced susceptibility to DC3000 infection indicating that this protein has a role in the plant innate immunity. When co-expressed in plants, HopU1 was capable of ADP-ribosylating AtGRP7 indicating that HopU1 could modify AtGRP7 *in planta*. Two arginine residues

within AtGRP7's RNA-binding domain were required for it to be ADP-ribosylated by HopU1-His, suggesting that ADP-ribosylation interferes with AtGRP7's RNA binding ability. Our results suggest a novel strategy employed by a bacterial pathogen where ADP-ribosylation of plant RNA-binding proteins results in posttranscriptional inhibition of host innate immunity. Using a bioinformatic approach, we identified seven putative ARTs from plant bacterial pathogens. At least one of them, XAC3230 from *Xanthomonas axonopodis* pv. citri strain 306 has the characteristics of being a type III effector.

## Acknowledgements

The Ph.D. is one of the most advanced degrees in current education system. For me, Ph.D. study was a really wonderful and unforgettable experience in my life. Although it took a little longer than I first thought, the reward was also much more than I expected. Before I started my Ph.D. study, I told myself that all I can do is my best, but now I believe I really can not accomplish these achievements without other people's help.

The first person I want to acknowledge is my major advisor Dr. Jim Alfano. I feel lucky to have him as my boss and friend and I am grateful for his help. I remembered that when I first started my Ph.D., he said to me "A job well begun is a job half done". This kind of quote, which Dr. Tom Clemente refers as California Mumbo Jumbo, really helped me getting started. For my first seminar in the Department of Plant Pathology, Jim gave a lot of valuable advices that improved my skills. It was Jim who recognized that three effector proteins including HopU1 from *Pseudomonas syringae* pv. tomato DC3000 appeared to share similarity to mono-ADP-ribosyltransferases. Even after a postdoc and a graduate student worked on this project for almost two years without much progress, Jim did not give it up and he was still very enthusiastic on this project. Right before they left our lab, Jim gave this great project to me. Luckily I was able to show several months later that HopU1 indeed had mono-ADP-ribosyltransferase activity on plant proteins. One thing I appreciate a lot about Jim is that we always communicated well on projects. He really spent time with me guiding my projects in the right direction. I had a hard time finding the substrates of HopU1 using two-dimensional gel electrophoresis. After I had been working on that for one and a half years without success, I was very frustrated and wanted to give up on it. Thanks to Jim's encouragement, I continued the project. I was in a Journal club when we were

discussing a paper on the identification of protein by ion exchange chromatography, we got the right idea to fractionate the plant protein samples prior to electrophoresis and then we finally identified the substrates of HopU1. Jim tried to help me to do things right in every step. In the process of applying for a postdoctoral position, Jim helped me a lot in the writing of cover letters. In the summer of 2005, we rented a van to get to Austin, Texas, for the 2005 annual meeting of American Phytopathological Society. It took us two days to get there. At the end of the first day, we camped at a park in Oklahoma. At that park, Jim, Ming and I successfully swam across a pretty wide lake. This is something I would not have done by myself, but we together made a difference. I am also very impressed by the way he dealt with the comments from the reviewers on our Nature paper. He handled it in a very professional way, and our paper was finally published as an article in the journal Nature.

Besides Jim, I would also like to thank other faculty whom helped me with my projects. Dr. Tom Elthon helped me with two-dimensional gel electrophoresis for about two years. We first tried to use tube gels, and then we changed to use strips, which greatly improved the separation of the proteins. We switched from Bio-Rad preparative isoelectric focusing Rotofor cell to ion exchange chromatography to fractionate the proteins. Dr. Tom Elthon and his protein core facility really provided a lot of convenience for my project on HopU1. I would like to thank Dr. Tom Clemente who helped me with the *Arabidopsis* plant transformation by generously providing solutions and protocols. He is also a very good neighbor to our lab. I can always get help from his lab for what I needed for my experiments. Dr. Tom Clemente's interesting comments on different topics are hard for me to forget. He is a very funny person who loves to tell people that Chinese in his lab call him "Zhou Bapi", a landlord who pretended to be a rooster that crows at midnight in order to make his peasants to get up earlier and work harder. Tom

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I really can not finish my Ph.D. without the support from my wife Kaimei Xu. She took most of the responsibilities of taking care of family. We have three lovely boys, Mingke, Luke and Matthew. There is a lot of work to do at home. She could have gone for a Ph.D. as well, but instead she sacrificed herself for my Ph.D. I remember the day I confirmed the substrates of HopU1, my wife and my two kids brought me supper, and we together enjoyed seeing a beautiful rainbow in the sky right outside of the Beadle Center. Rainbows are very common in Hawaii, but in Nebraska they are much more rare. It was just wonderful. During my Ph.D., my parents had a chance staying with us

for one year. My parents' visit really made me very happy. We have not seen each other for more than 5 years. They helped in a great deal with our house work. They brought me up and made me a confident and ambitious person. After my parents left, Kaimei's parents also stayed with us for 1 year. Kaimei had not seen her parents for about 6 years. They also helped us a lot by taking care of the kids and cooking food. We rented a plot of garden for my parents and my in-laws. They all grew vegetables so we enjoyed fresh and organic vegetables. My dad and father-in-law both went fishing with me a lot. We caught a lot of rainbow trout fish. I often thought that catching fish out of the lake is just like identifying HopU1 targets among the about 25,000 proteins present in *Arabidopsis*. If I went fishing on a weekend, then the next week I was in a very good mood to work hard to get more research done. In July 2006, Kaimei's brother, Kaiming, came to visit us. All my family, my in-laws and my brother-in-law went to Yellowstone National Park. It took us two days to get there. One thing I thought on the top of my head was if I keep driving up, sooner or later, I will get close to the top of the hill and enjoy the beautiful views.

I also want to give special thanks to Dr. Anne Alvarez. In 2001, I was almost done with the requirement for a M.S. degree at University of Hawaii-Manoa. Dr. Anne Alvarez came to me and asked me what I want to do next. To be honest, I really did not know what to do. She suggested that I take some time to think about it very carefully. She asked among all the jobs in the world excluding those ones that are unrealistic, which one would be my best interest and which one I would enjoy most. It was a tough decision for me. I thought about all kinds of possibilities. I really spent around 2 months thinking about my future and decided to begin to study as a Ph.D. student after I had two master's degrees in Plant Pathology. I joined Dr. John Hu's journal club on gene silencing and systemic acquired resistance. We discussed many interesting papers and

my interest in the research was greatly stimulated. I spent one month writing the statement of interest before I applied 5 universities. With the connection that Dr. Martin Dickman's had with UNL I decided to apply to it and I was accepted as a Ph.D. student in Dr. Jim Alfano's lab. I guess the rest is history, but I'm very glad that I decided to come to Nebraska.

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# CHAPTER I

## BACKGROUND

We use *Pseudomonas syringae* pv. tomato DC3000 as a model organism to study the type III secretion system (TTSS) of plant pathogenic bacteria. This gram-negative bacterial pathogen causes bacterial speck disease on the widely used model plant *Arabidopsis* and on the economically important tomato. Because the genome of the pathogen DC3000 and host plant *Arabidopsis* are both completely sequenced and this pathogen causes disease on an economically important tomato, DC3000 is an excellent model pathogen to study molecular interactions between microbial pathogens and host plants.



## *P. syringae*

### General description and significance

*P. syingae* are gram-negative, rod-shaped bacteria with multiple polar flagella and aerobic metabolism. The function of flagella is to provide motility so the bacterium can swim towards or away from environmental stimuli. *P. syringae* can be characterized by its inability to properly utilize arginine due to the lack of arginine dihydrolase and a negative oxidase reaction resulting from the lack of a specific cytochrome C oxidase in the respiratory electron transport chain. Most, but not all *P. syringae* strains are plant pathogens. Plant pathogenic *P. syringae* strains cause diseases on a wide range of host plants. Non-pathogenic *P. syringae* strains have been used to inoculate plants for potential antifungal treatment. Because *P. syringae* is also associated with ice nucleation, *P. syringae* has also been used to produce artificial snow in ski resorts (71, 72).

### Taxonomy and nomenclature

. *P. syringae* is a member of the family Pseudomonadaceae under the order of Pseudomonadales. As is well known, Pseudomonadales are classified in the gamma class of Proteobacteria in the bacteria kingdom. *P. syringae* was initially isolated in 1902 by van Hall from a diseased lilac (*Syringa vulgaris*), therefore leading to the species designation of *syringae* (71, 128)

### *P. syringae* and ice nucleation

Ice nucleation induced by *P. syringae* was first described in 1974 when bacterial suspensions of *P. syringae* isolated from decaying alder leaves (*Alnus tenuifolia*) were found to freeze at a warmer temperature than the suspensions of most other bacteria and those without bacteria (110). The advantage for the bacteria to induce ice nucleation might be to initiate frost damage to the plants so that bacteria can have access to the nutrients of the plants (29). Ice nucleating bacteria such as *P. syringae* makes Ina (ice nucleation active) proteins, which translocate to the outer bacterial cell wall on the surface of bacteria to act as the nuclei for ice formation (65). Lindow & colleague found that the population of the ice nucleation active bacteria can be significantly reduced by inoculation of $ice^-$ bacteria in which the ice gene was deleted, therefore, $ice^-$ bacteria can be used to reduce frost damage to crop plants. Competition for the limited nutrients on the leaf surface has been suggested as a possible mechanism for this effect (160, 161). In fact, $ice^-$ mutants of *P. syringae* and *P. fluorescence* were the first recombinant organisms that were deliberately released into the environment (71). Freeze-dried cells of an ice nucleation active *P. syringae* strain were used commercially for the production of artificial snow (65). Addition of ice nucleation active bacteria such as *P. syringae* to food stuffs can lead to an improved frozen food product at a higher freezing temperature with reduced freezing time and energy cost (37).

### *P. syringae* as epiphytes

In 1959, J. E. Crosse reported that the causal agent of bacterial canker and leaf spot of stonefruit tree, *P. syringae* pv. morsprunorum, can be isolated in large amounts from asymptomatic cherry leaves (71). J. E. Crosse also found that larger numbers of the *P. syringae* pv. morsprunorum in the phyllophere were more highly associated with susceptible cultivars than more resistant cultivars. It is generally believed that the epiphytic populations of the bacteria serve as the source of inoculums for disease (71).

Haefele et al. showed that motility contributes to epiphytic fitness (66). Steve Lindow and colleagues used random Tn5 mutagenesis to identify genes that contribute to epiphytic fitness. Among eighty two mutants with reduced epiphytic growth, only three were auxotroph. Other mutants showed defects in motility, osmotolerance, desiccation tolerance, growth rate in batch culture, and extracellular polysaccharide production (103). Epiphytic bacteria do not occur in uniform, but rather aggregate at certain sites on the leaf. This suggests that quorum-sensing may contribute to epiphytic growth (119, 120). In fact, a transcriptional activator that is required for quorum sensing signal molecular N-acyl-homoserine lactone (AHL) production, is also required for epiphytic fitness of *P. syringae* supporting the role of quorum sensing in epiphytic growth (134).



### Pathogenic *P. syringae*

*P. syringae* cause diseases on a wide variety of host plants. The disease symptoms caused by *P. syingae* include leaf spot, bacterial blight and stem canker etc. *P. syringae* is divided into over 40 pathovars based on host specificity at the plant species level (71, 89).

### Genome organization of *P. syringae*

*P*. *syringae* pv. tomato DC3000 was the first *P*. *syringae* strain completely sequenced (26). DC3000 encodes 5763 ORFs in its 6.5 megabase genome. It has two plasmids in addition to a circular chromosome. 298 genes are implicated in virulence functions. 1159 genes, including 811 genes with unknown function, are not shared with soil bacterium *P. putida* and human pathogen *P. aeruginosa,* and, therefore, unique to DC3000 (26).

*P*. *syringae* pv. syringae B728A was the second *P. syringae* strain sequenced (52). In contrast to DC3000, B728A does not have plasmids. The genome size is 6.1 megabases. B728A is a pathogen with a very pronounced epiphytic growth phase (71). Because epiphytic growth makes the pathogen more exposed to environmental stress conditions, this pathogen developed genes involved in ectoine synthesis, DNA repair, copper and antibiotic resistance which makes this pathogen more tolerate to thes.e stresses. Other genes among a total of 976 unique genes without counterparts in DC3000 include syringopeptin, syringomycin, arginine degradation and production of ice nuclei (52).

The third and final sequenced *P*. *syringae* strain sequenced at the time this thesis was written is *P*. *syringae* pv. phaseolicola 1448A (83). 1448A is a causal agent of halo blight disease found on common bean. This pathogen has been used a lot in field studies and early studies for interactions between avirulence (*avr*) genes and resistance *R* genes (introduced below). 1448A belongs to race 6, which is virulent on all common bean varieties; therefore race 6 has been used as a recipient strain to identify type III effector or Avr proteins based on avrulence phenotypes. 1448A encodes 5353 ORFs on its circular chromosome and two plasmids. The genome size of 1448A is 6.1 megabases. Among 298 genes from DC3000 that were implicated in virulence, 240 of them are present in 1448A. Joardar compared the genome of 1448A to DC3000, *P.*

*putida* KT2440 , *P. aeruginosa* PAO1, *P. fluorescence* Pf-5 and identify 3567 ORFs that comprise the *Pseudomonas* core genome and 365 ORFs that are unique to *P. syringae* (83).

**Virulence factors of *P. syringae***

In Fig. 1, I show the virulence factors present in DC3000 based on its genome sequence as an example of virulence factors commonly found in *P. syringae*. The central part of the pathogenic process for *P. syringae* is the injection of bacterial proteins called type III effector proteins into the plant cell by the syringe-like type IIII secretion apparatus. Once inside plant cells, these effectors modify plant physiology, suppress plant defense and collectively cause plant disease (50). DC3000 produces methyl jasmonate mimicking a phytotoxin called coronatine (16). Coronatine has an unusual structure and two distinct components: Coronafacic acid and coronamic acid. This polyketide toxin induces chlorosis on a variety of plant species (16). Recently, it was shown that bacterial PAMPs (Pathogen-associated molecular patterns) trigger stomata closure, while coronatine causes the reopening of the stomata to allow bacteria invasion and, therefore, contribute to the virulence of DC3000 (118). DC3000 produces phytohormone IAA. The exact role of the IAA produced by the bacterial pathogen is not well understood, but it has been shown that IAA may suppress the hypersensitive response (136), affect bacterial growth and bacterial toxin syringomycin biosynthesis by *P. syringa*e pv. syringae (116).. Siderphores as small molecular weight and high affinity iron chelators contribute to the bacterial growth in iron limiting environments. Cell wall degrading enzymes such as pectin lyase secreted by the type II secretion system may be involved in symptom development by releasing plant nutrients for bacterial growth). Adhesins, such as type IV pili and filamentous hemagglutinin likely play a role in the

adhesion of bacteria to the host and epiphytic fitness (26, 137, 139). Extracellular polysaccharides (EPS) may provide an exclusion to ROS (reactive oxygen species) produced by plant host. Interestingly, EPS from *Burkholderia cenocepacia*, an opportunistic pathogen in compromised human hosts, inhibited ROS production of human neutrophils (30). Catalase, superoxide dismutases (SODs) and other ROS (reactive oxygen species) scavengers likely provide protection to ROS as well (26). ABC exporters may protect DC3000 by exporting antimicrobial produced by plant host.

**Type III secretion system**

TTSS is a very important research topic because it is required for most plant bacterial pathogens including *P. syringae.*, *Erwinia spp*., *Pantoea spp*., *Ralstonia solanacearum* and *Xanthomonas spp*. and many animal pathogens such as *Shigella spp*., *Yersinia spp.*, Enteropathogenic *Escherichia coli* and *Salmonella spp*. to cause diseases. TTSSs have also been identified in symbiotic bacteria such as *Rhizobium spp*., *Bradyrhizobium japonicum* and *Mesorhizobium loti* (114). Thus, TTSSs are used by gram-negative bacteria living in close association with eukaryotes where the effectors injected improve the interactions between these organisms.



**Regulation**

According to gene organization and the regulatory system controlling TTSS gene expression, the TTSSs in the phytopathogenic bacteria are divided into two groups. Group I TTSS includes *Erwinia spp*., *Pantoea agglomerans* and *P. syringae* and group II TTSS includes *Xanthomonas spp*. and *R. solanacearum* (6).

**Regulation of the group I TTSS**

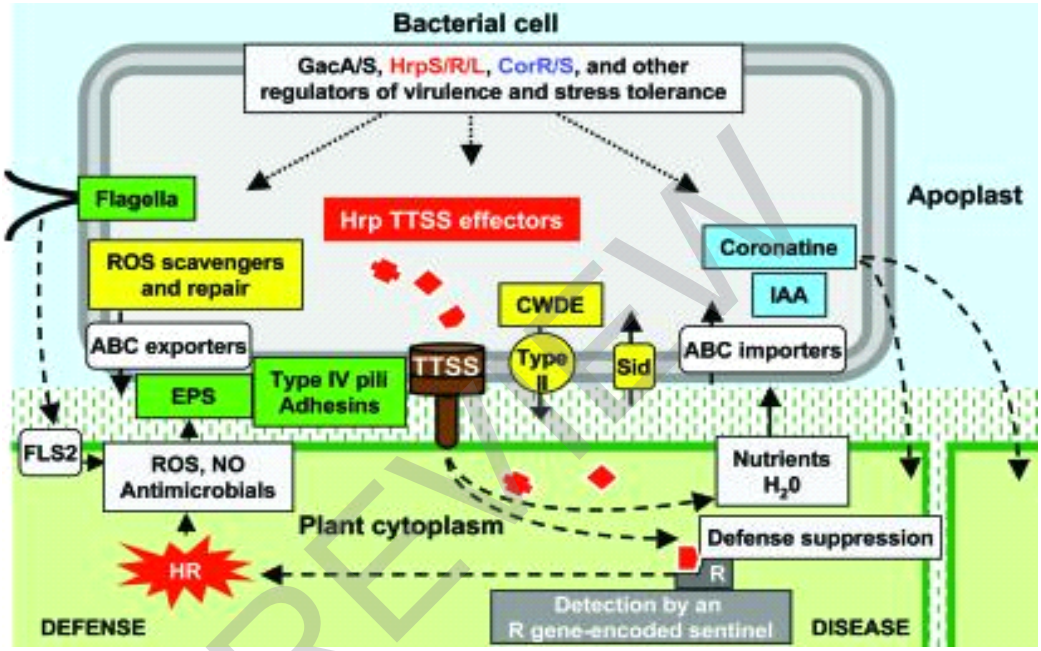




Fig. 1. Overview of *P. syringae* virulence factors. The central pathogenic process is the injection of multiple effector proteins into plant cells by the TTSS. The effectors may suppress defenses and promote nutrient and water accumulation in the apoplast. Flagella provide motility and favor bacterial epiphytic survival and entry into leaves. Exclusion or tolerance of reactive oxygen species (ROS) and other antimicrobials is likely promoted by extracellular polysaccharides (EPS), scavengers, and ABC exporters. Favoring virulence are type IV pili and possibly adhesins, coronatine synthesis and regulation, plant cell-wall-degrading enzymes (CWDE) and other variously secreted proteins, iron-scavenging siderophores (Sid), indoleacetic acid (IAA), and probably numerous ABC transporters and other nutrient uptake systems. From Buell et al. (26)