

Powerful screens for bacterial virulence proteins

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Many mammalian and plant pathogenic bacteria inject virulence effector proteins into host cells by means of the type III secretion system (TTSS) (1). Effector proteins attack the host innate immune system, modify cytoskeleton and membranes, or alter vesicle trafficking (2, 3). The collective action of these proteins promotes bacterial entry into, growth and movement within, and dissemination from infected host cells/tissues. The full complement of TTSS effectors is not known for most bacterial pathogens; their identification remains a crucial step toward a comprehensive understanding of bacterial pathogenesis, host range, and pathogen evolution. In a recent issue of PNAS, Chang *et al.* (4) describe a powerful method for discovering TTSS effectors. Using this method, those authors identified two nearly complete repertoires of TTSS effectors from two pathogens (specific to different plant species) of the plant pathogen *Pseudomonas syringae*. Here, we discuss this article in the context of earlier efforts to discover TTSS effectors in plant pathogens.

In both mammalian and plant pathogenic bacteria, the initial method for identification of proteins secreted through the TTSS was to monitor extracellular proteins secreted in bacterial cultures in a TTSS-dependent manner (5–7). Unfortunately, the amounts of TTSS effector proteins secreted by plant pathogenic bacteria, although detectable by antibodies, are too low to be useful for systematic identification of effectors. A breakthrough came in 1996 with the discovery that bacterial “avirulence” (*avr*) genes encode TTSS effector proteins that function inside the plant cell (reviewed in ref. 8). These *avr* genes had previously been cloned based on their ability to convert virulent strains into avirulent ones in specific plant genotypes (9). This breakthrough, besides significantly expanding the number of known TTSS effectors in several bacteria, provided an important clue that would prove to be pivotal for the development of methods, including the one reported by Chang *et al.* (4), for genome-scale identification of TTSS effectors.

The clue was the discovery by Huynh *et al.* (10) that *P. syringae* *avr* genes are coregulated with *hrp* gene operons, which were later demonstrated to encode the components of the TTSS (7).

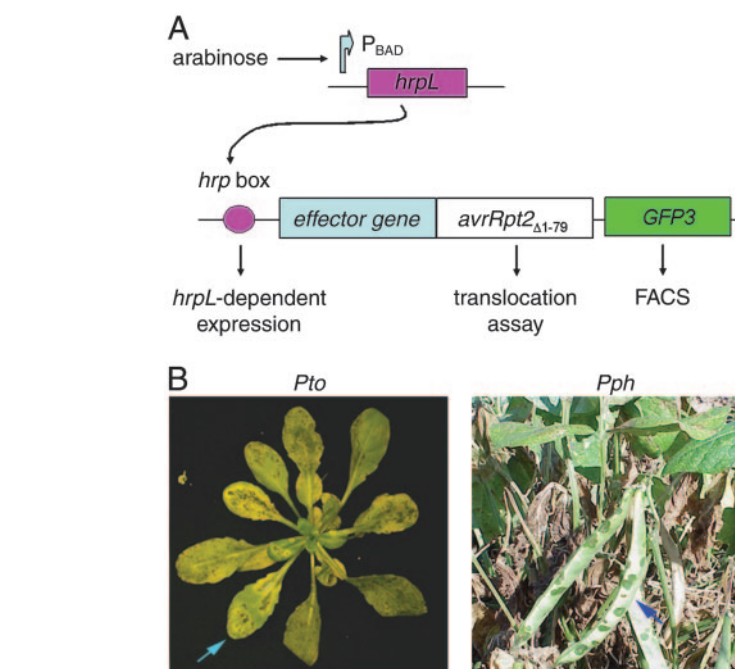


Fig. 1. A diagram of the screen strategy reported by Chang *et al.* (4). (A) *Pto* DC3000 $\Delta hrpL$ mutant is transformed with two plasmids. One plasmid expresses the *hrpL* gene from the arabinose-inducible promoter P_{BAD} . Random bacterial genomic fragments, some of which contain a *hrp* box in the promoter and 5' portions of effector genes, are cloned into the other plasmid, upstream of *avrRpt2* Δ_{1-79} , to generate translational fusions. A GFP gene is placed downstream of *avrRpt2* Δ_{1-79} . Arabinose-inducible GFP is used to rapidly select bacteria that harbor *hrp* box-containing fragments by FACS. *AvrRpt2* Δ_{1-79} is used to determine whether an *hrpL*-regulated gene encodes a TTSS effector. (B) Disease symptoms caused by *Pto* DC3000 in *Arabidopsis* leaves (necrotic specks and chlorosis) and by *Pph* 6-1448a in bean pods (water-soaked lesions; kindly provided by John Mansfield, Imperial College London, Wye, U.K.). Arrow in each image indicates a diseased leaf or pod.

This coregulation depends on several positive regulators, including the sigma factor HrpL. HrpL recognizes a consensus sequence motif (“hrp box”) present in the promoters of *hrp* and *avr* genes (11, 12) and presumably also other yet-to-be identified TTSS effector genes. Following this clue, several groups used a variety of creative strategies to predict and/or experimentally verify new members of the *hrpL* regulon and TTSS effectors in *P. syringae* strains (13–15). To date, the most complete inventory of the *hrpL* regulon is in *P. s. pv. tomato* (*Pto*) strain DC3000, the genome of which was among the first from plant pathogenic bacteria to be sequenced (16). Genome-based approaches have also been used successfully to identify TTSS effectors in other plant pathogenic bacteria, including the tomato pathogen *Ralstonia solanacearum* strain GMI1000 (17). The general applicability of genome sequence-assisted inventory of TTSS effectors depends on genome

sequence availability, which is limited for plant pathogenic bacteria.

Another advance proven to be critical for large-scale identification of TTSS effectors was the demonstration that *P. syringae* *AvrRpt2* and *Xanthomonas campestris* *AvrBs2* have a modular protein structure: the N-terminal type III secretion signal is necessary for the translocation of these proteins into the plant cell, but is dispensable for their biological activity (i.e., triggering the hypersensitive cell death response inside the host cell of a specific genotype) (18, 19). Therefore, truncated *AvrRpt2* and *AvrBs2* lacking the N-terminal signal sequence can be used as reporters to identify genes that contain a type III secretion signal. Transposon Tn5 derivatives carrying *Avr*-

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Rpt2 Δ_{1-79} or AvrBs2 Δ_{1-61} were successfully used in screenings that led to the identification of 13 effectors in *Pseudomonas s. pv. maculicola* ES4326 (an *Arabidopsis* pathogen), and 7 in *Xanthomonas c. pv. vesicatoria* strain 85–10 (a pepper pathogen) (20, 21). These elegant genetic screens, although not yet reaching saturation, should be particularly useful for identifying TTSS effectors in bacterial strains for which genome sequences are not available.

Knowledge of complete effector repertoires from a large number of strains is crucial for addressing questions related to pathogen adaptation to different host species. *P. syringae*, for example, consists of almost 50 pathovars, which collectively infect an extremely wide range of plant species (22). Each pathovar, however, infects only one or a few plant species, exhibiting a very high level of specificity. An outstanding question in plant pathology is how the host range is determined at the pathovar level. A testable hypothesis is that the number and identities of TTSS effectors determine pathovar-specific host range. Aiming to rapidly define TTSS effector repertoires for a large number of phylogenetically diverse pathovars of *P. syringae*, Chang *et al.* (4) developed a very efficient screening method that combines the power of fluorescence-activated cell sorting (FACS; ref. 23), *hrpL*-dependent expression of effector genes in *P. syringae*, and the AvrRpt2 Δ_{1-79} reporter system (Fig. 1). The most innovative aspect is the incorporation of FACS, which provided near-saturating identification of *hrpL*-

regulated genes from two test strains, *Pto* DC3000 and *P. s. pv. phaseolicola* (Pph) 6-1448a. The AvrRpt2 Δ_{1-79} -based translocation assay was used to confirm 29 TTSS effectors in *Pto* DC3000 and 19 in *Pph* 6-1448a.

At first glance, Chang *et al.* (4) appear to have identified many fewer effectors than the >40 previously reported in *Pto* DC3000 (24). They suggest that the previous procedures (e.g., secretion of candidate effector proteins after

their respective host plants (Fig. 1). *Pto* DC3000 and *Pph* 6-1448a share only 13 effectors, many of which are also found in other *P. syringae* pathovars (either experimentally confirmed or predicted) (13, 14) and in the sequence databases of *R. solanacearum* and *Xanthomonas* spp. These observations raise the alluring possibility that these effectors may have played an important role in the evolution of *P. syringae* and possibly other bacteria as plant pathogens.

The identification of effector repertoires in multiple *P. syringae* pathovars (refs. 4 and 13 and www.Pseudomonas-syringae.org) will benefit several aspects of future research on plant–bacteria interactions. For example, a major obstacle in the study of the TTSS effector functions in plant pathogenic bacteria is functional redundancy. Single mutations in effector genes often do not produce large effects on bacterial virulence. This problem may be severe in strains that harbor a large complement of effectors, such as the commonly used strain *Pto* DC3000. *Pph* 6-1448a, with a much smaller effector repertoire, may be a better choice for genetic analysis. *Pph* 6-1448a could also be used to construct an effector-minus strain into which effector genes from another pathovar could be introduced to determine what combinations of effectors would be sufficient for switching pathovar specificity. Finally, the FACS/HrpL/AvrRpt2-based method may be quickly adapted to identify TTSS effectors in other bacteria, such as *Erwinia* spp. and *Pantoea* spp., which also use the HrpL regulatory system for effector gene expression.

Effectors may have played an important role in the evolution of *P. syringae*.

expression from a strong, constitutive promoter, instead of their native promoters) may have overestimated the effector repertoire in *Pto* DC3000. Although this speculation may be true, it is also possible that not all AvrRpt2 Δ_{1-79} fusions are stably expressed, translocated, and/or biologically active inside the plant cell, so that a few effectors may have been missed when using the AvrRpt2 Δ_{1-79} fusion approach. Future studies should resolve this discrepancy.

A notable finding by Chang *et al.* (4) is the great difference in the number of experimentally confirmed effectors between *Pto* DC3000 and *Pph* 6-1448a. This difference is rather remarkable, considering that both bacteria are highly virulent in

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