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# Wake of the flood: ascribing functions to the wave of type III effector proteins of phytopathogenic bacteria

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Plant pathogenic bacteria use waves of type III effector proteins, delivered into the eukaryotic host cell, to modulate the host cell for the pathogen's benefit. This is evidenced by the flood of effector genes that have recently been uncovered from the genome sequence of several plant pathogenic bacteria. However, pathogens are unwilling to easily reveal the mechanisms by which these effectors function. Nevertheless, persistent scrutiny has led to the successful characterization of a handful of effectors and it is beginning to provide insights into how phytopathogenic bacteria cause disease on their hosts.

## Addresses

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## Abbreviations

<b>Avr</b>	avirulence gene/protein
<b>EEL</b>	exchangeable effector loci
<b>HR</b>	hypersensitive response
<b>Pph</b>	<i>Pseudomonas syringae</i> pv. <i>phaseolicola</i>
<b>Psy</b>	<i>Pseudomonas syringae</i> pv. <i>syringae</i>
<b>Pto</b>	<i>Pseudomonas syringae</i> pv. <i>tomato</i>
<b>PTP</b>	protein tyrosine phosphatase
<b>R</b>	resistance gene/protein
<b>TTSS</b>	type III secretion system
<b>Xac</b>	<i>Xanthomonas axonopodis</i> pv. <i>citri</i>
<b>Xcc</b>	<i>Xanthomonas campestris</i> pv. <i>campestris</i>
<b>Xcv</b>	<i>Xanthomonas campestris</i> pv. <i>vesicatoria</i>

## Introduction

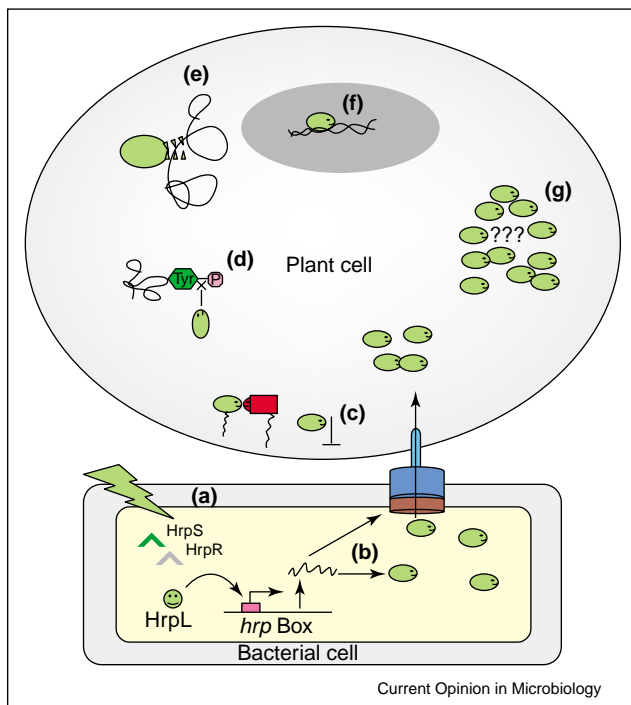
Many plant pathogenic bacteria rely on a type III secretion system (TTSS; encoded by *hrp/hrc* genes) to cause disease on their hosts (Figure 1; [1]). The protein effectors traversing the TTSS are delivered into the host cell, where they function as the virulence factors that modulate susceptible hosts to benefit the pathogen. Type III effector proteins of plant pathogens are characterized by some common traits (Figure 1). It is perhaps no surprise that the plant surveillance system in resistant plants (R proteins) relies on recognition of these same type III effector proteins. This ‘recognition’ results in the induc-

tion of a suite of defense responses, including hypersensitive cell death (HR), which renders the pathogen avirulent [2]. For more in-depth reviews on plant resistance, please refer to [2–4]. As a consequence, the type III effectors identified via their ability to trigger the specific function of a given R protein were historically referred to as avirulence proteins (Avr). The genetic analysis of R-mediated disease resistance suggested that the R protein would function as a receptor binding a ligand encoded by the Avr protein [5]. Though a couple of examples do fit this idea, most do not. This led to a proposal suggesting that the type III effector proteins (and by extension, virulence factors from other pathogens) are indirectly recognized by resistant hosts, and that R proteins monitor the homeostasis of a particular host cellular machine targeted by the pathogen [2,6,7]. Unless otherwise stated, we use ‘recognition’ to imply either direct or indirect molecular interaction between R protein and type III effectors acting as Avr proteins.

Three general virulence functions have been proposed for type III effectors: release of bacteria to the organ surface, nutrient acquisition and suppression of basal or induced host defenses. The basal host defenses are those that operate, even in susceptible plants, to limit the extent of disease in the absence of R-mediated recognition. Basal defense is genetically defined [8], and steps required for basal defense can overlap with genetically defined steps in R-mediated defense. This leads to the suggestion that the R proteins act to accelerate the overall defense response [2,8]. Experimental evidence suggests that some type III effectors are required for optimal release of bacteria to the leaf surface [9,10]. No reports have been published demonstrating a role for effectors in acquiring nutrients. By contrast, much of the data presented to date strongly suggests that type III effectors can function to suppress the host defense response. Generally, it is difficult to determine the function of a particular type III effector. This is owing to a combination of functional overlap or redundancy between the suite of type III effectors in a given strain, the probable subtle effects they may exert to increase virulence, or roles that are possibly specific to certain stages of the pathogens’ life cycle or colonization of particular hosts.

Whole genome sequencing of strains *Pseudomonas syringae* pv. *tomato* (Pto) DC3000 [11], *Pseudomonas syringae* pv. *syringae* (Psy) B728a ([http://genome.jgi-psf.org/draft\\_microbes/psesy/psesy.home.html](http://genome.jgi-psf.org/draft_microbes/psesy/psesy.home.html)), *Xanthomonas axonopodis* pv. *citri* (Xac) [12], *Xanthomonas campestris* pv. *campestris* (Xcc) [12], *Ralstonia solanacearum* [13] and that of

Figure 1



Phytopathogenic bacteria use the type III secretion system (TTSS) to deliver effectors into the plant cell. (a) The TTSS and its effectors are expressed in response to the environmental conditions in the plant apoplast. The type III effectors are then delivered into host cells via the TTSS [58]. (b) In *P. syringae*, type III effectors can be identified on the basis of shared characteristics that are common in regulation and delivery. These characteristics are: (i) induced expression by the HrpL sigma factor, (ii) presence of the *hrp*-box in their promoters, (iii) potentially serine rich amino acid sequence in the first 50 amino acids [15,18], and (iv) delivery into hosts by the TTSS. Type III effectors can travel to different subcellular locations and have different functions. (c) Type III effectors can be localized to the plasma membrane. Several effectors are predicted to undergo eukaryotic fatty acylation, such as myristoylation, and subsequently localize to the plasma membrane of the host cell [57]. Here, they may interact with host proteins also associated with the plasma membrane or interfere with extracellular defenses. Other type III effectors may localize to the cytoplasm. (d) They may dephosphorylate yet to be determined protein(s) in the host cell (HopPtoD1 encodes a protein tyrosine phosphatase). (e) Other type III effectors might act as proteases, cleaving host proteins (AvrRpt2 and AvrPphB). (f) Some type III effectors localize to the host nucleus. These proteins may modulate host gene transcription. (g) The great majority of *P. syringae* type III effectors are uncharacterized. It would not be too surprising to find some localized to chloroplasts, golgi, mitochondria or other subcellular sites.

*Pseudomonas syringae* pv. *phaseolicola* (*Pph*) race 6 (currently in progress; RC Buell, personal communication), functional screens, and several bioinformatics approaches have forced the floodgates open, leading to the discovery of many more proven and putative type III effectors [14–18]; reviewed in [19,20]. The next hurdle to overcome is unveiling their functions and determining how they benefit the pathogen. Several recent publications provide inklings as to how pathogenic bacteria use type III

effectors (Table 1; reviewed in [21]). These findings also highlight the many strategies that can be used by flummoxed researchers determined to crack the secret function of type III effectors from plant pathogenic bacteria.

### Tsunamis look like tidal waves: homology-based approaches to type III effector function

There is nothing simpler, nothing more satisfying than clicking ‘BLAST!’ and instantaneously having symbols appear that could potentially dictate how to characterize a protein. It is frustrating to wait over ten minutes for a response, but even more exasperating to see, ‘No hits found’. Yet, this has been the standard response to simple database searches using type III effector gene and deduced protein sequences. As the microbial genome projects progress, however, one can expect to be rewarded with ‘homology to protein of unknown function’ or ‘homology to hypothetical protein’ from other bacterial pathogens. While at first glance equally depressing, this increasingly common finding can provide important phylogenetic-based inference of type III effector function.

Many type III effectors from mammalian pathogens have been characterized. And, in a few cases, plant pathogen type III effectors retain homology to widely diverged proteins from animal pathogens. Their functions may be used to infer those of the plant pathogen protein, despite differences in host physiology. YopJ from *Yersinia pestis* inhibits mitogen-activated protein kinase (MAPK) and nuclear factor-kappa B (NF- $\kappa$ B) signaling and has been proposed to target SUMO-1 (small ubiquitin-like modifier) conjugated proteins for proteolysis in mammalian cells [22]. SUMO is a member of ubiquitin-like proteins that are covalently attached to proteins by a conjugation system [23]. Several cellular processes are regulated by SUMO conjugation and de-conjugation of proteins. The type III effector XopD from *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) is homologous to Ulp1 (Ubiquitin-like protein protease) which targets SUMO-1 conjugated proteins [24]. Hotson *et al.* [25] demonstrated that XopD indeed targets SUMO conjugated plant proteins for proteolysis and proposed that the pathogen de-conjugates SUMOylated proteins as a mechanism to alter signal transduction in plant cells. Whether this effector has one or several host targets remains to be determined.

YopT is a cysteine protease that cleaves lipid modified Rho GTPases, causing them to be released from the host membrane [26]. The *P. syringae* type III effector AvrPphB was predicted to be a cysteine protease on the basis of its homology to YopT. Delivery of AvrPphB into plant cells via the TTSS results in cleavage of an *Arabidopsis* serine-threonine protein kinase PBS1 [27]. Interestingly, the resistance response initiated by recognition of AvrPphB through the RPS5 R protein, is dependent on this cleavage event. This important finding suggests that it is the

Table 1

## Recently characterized type III effectors of plant pathogenic bacteria.

Effector	Function <sup>a</sup>	Confirmed activity? <sup>b</sup>	Phenotype in host <sup>c</sup>	Other phenotypes <sup>d</sup>
XopD	SUMO protease	Yes	Unknown	ND
AvrPphB	Cysteine protease	Yes	Cleaves PBS1	ND
HopPtoD2	Protein tyrosine phosphatase	Yes	Optimal growth	Suppress HR and oxidative burst in tobacco
HrpW	Pectate lyase	Binds calcium pectate	Unknown	ND
HopPtoM	Unknown	NA	Lesion formation	ND
HopPtoA1	Unknown	NA	Optimal colony size	ND
HopPtoA2	Unknown	NA	Optimal colony size	ND
AvrPto	Unknown	NA	Optimal growth [54,55]	Blocks host papillae response
AvrBs3	Unknown	NA	Pustule formation	ND
AvrRpt2	Cysteine protease	No	Release to leaf surface, optimal growth [56], RIN4 disappearance	ND
AvrPtoB	Unknown	NA	Unknown	Blocks cell death in tobacco and yeast
AvrB	Kinase???	No	RIN4 phosphorylation	Chlorosis in <i>Arabidopsis</i> [57]

Abbreviations; NA, not applicable; ND, not determined.

<sup>a</sup>The techniques used to determine function are described in the text.

<sup>b</sup>Confirmed function in either *in vitro* or *in vivo* assays.

<sup>c</sup>An effector-dependent phenotype elicited by a pathogen on its native host.

<sup>d</sup>An effector-dependent phenotype in a heterologous host, via *Agrobacterium* or expressed in transgenic plants. This does not include elicitation of resistance response.

product of the type III effector's enzymatic function that is required to trigger disease resistance in those hosts carrying *RPS5*. It remains to be determined if PBS1 is the key target of AvrPphB in susceptible (*rps5*) hosts, or one of many possible targets.

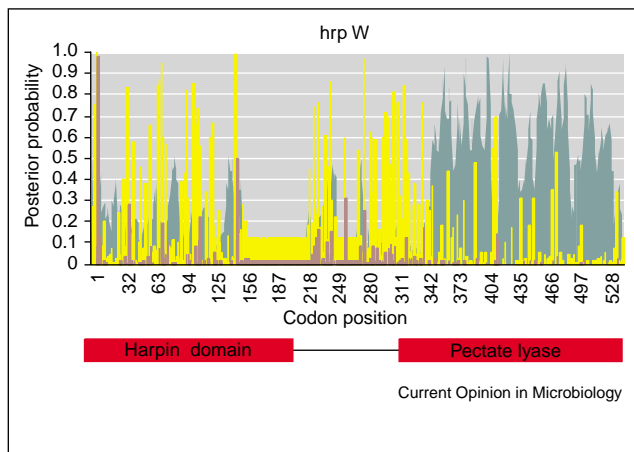
Algorithms and databases are becoming very sophisticated and more populated with characterized genes. Searches using the deduced protein sequence from *HopPtoD2* predicted a protein tyrosine phosphatase (PTP) domain in the carboxy-terminal portion of the protein; this activity was confirmed using *in vitro* assays [28\*,29\*]. *Pto* DC3000 carrying a mutation in *HopPtoD2* grew less than the wild type in both *Arabidopsis* and tomato, and induced a twofold higher level of *PR1* gene expression 48 hours post inoculation. *PR1* is a 'pathogenesis-related' gene that is transcriptionally induced during defense responses. HopPtoD2 with an inactivated PTP domain failed to restore wild-type levels of growth. When heterologously expressed in *Psy61* or *Pph* NPS3121, HopPtoD2 delayed recognition, as measured by delayed HR and delayed production of hydrogen peroxide in tobacco cells. Delay of the HR was also dependent on a functional PTP domain. These results suggest that the PTP activity of HopPtoD2 is required for suppression of basal defense responses, and is consequently important for full virulence of *Pto* DC3000.

### Surf's up! Don't forget the family

Many type III effectors are conserved between bacterial strains, and even between species. This is presumably owing to horizontal transmission as supported by the

observation that many type III effector genes are associated with mobile genetic elements, and most display some features of pathogenicity islands [30–32]. Comparing members of these gene families can reveal conserved amino acid residues and domains. Conservation of domains should reflect conserved function, presumably in virulence. Divergence could represent unselected neutral drift, or divergence driven by 'recognition' of a particular type III effector by a host R protein. The latter is hard to reconcile with the model of indirect recognition by R proteins of the consequence of an effector's virulence function and not of the effector itself (defined above). In cases where host recognition diminishes the fitness of a strain expressing a particular type III effector, then that allele might be jettisoned from that particular strain (many type III effectors are presence/absence alleles associated with mobile DNA elements). However, it might be that a given family of type III effector proteins diverged to facilitate interaction with alternate or diverged host targets. Members of these type III effector gene families would retain a common core virulence function, presumably encompassing domains under purifying selection. Other domains might also be under purifying selection to maintain an interaction with a particular host target(s). This model predicts that a given effector family might perform a specific, perhaps enzymatic function, but on various host targets. It also predicts that a given effector's fitness value will be enhanced if it acts on multiple host targets. The host would thus be under selective pressure for diversification of target allele sequences, with altered sequences, or evolution of a new R specificity to guard the alternate target.

Figure 2



HrpW has two protein domains under differential selective pressures. Each residue of the HrpW family (six proteins, X-axis) was examined for its probability of being under positive selection (Y-axis). Residues with probabilities closer to 1 are more likely to be under posterior selection; those closer to 0 are likely to be under functional constraint (yellow and purple bars represent two different models). The green bars represent the degree of similarity, with 1 being the maximum, between each of the family members at each position. A schematic representation of the predicted domains is also shown. The carboxy terminal coding region is under functional constraint (generally lower probabilities); this region has a predicted pectate lyase domain (higher conservation of amino acid sequences). Conversely, sites under positive selection are biased towards the amino terminal coding region which is also the region sufficient to trigger non-host resistance (harpin-domain) and thus under pressure to avoid recognition. Figure provided by Laurence Rohmer.

Evidence for selection has been observed among the *hrpW* gene family (Figure 2; L Rohmer, D Guttman and JL Dangel, unpublished data). The carboxy terminal coding region has homology to pectate lyases and is highly conserved among the family members. Although no published reports demonstrate this enzymatic activity, HrpW does bind calcium pectate [33]. Furthermore, the pectate lyase domain is also under functional constraint. Conversely, the amino terminal coding region, the harpin domain, is sufficient to trigger resistance responses on tobacco and thus potentially under selective pressure to avoid recognition. Consistent with this model, the HrpW amino-terminal domain is under positive selection. Even without prior knowledge of functional domains, these analyses would have accurately identified the carboxy terminal region of HrpW as an important region for its function. Thus, locating conserved regions on the basis of amino acid alignments or predicting regions under selection can be used to draw attention to putative functional domains.

Comparative analyses of entire suites of effectors will soon be realized; the genome of *Pto* DC3000 and *Psy* B728a have been sequenced and *Pph* race 6 is in progress [11]. Some comparative studies have already been

initiated [20]. Deng *et al.* [34] compared exchangeable effector loci (EELs) from nine diverse pathovars of *P. syringae*. The EEL and conserved effector locus flank the TTSS-encoding region in the chromosome. Six of the EELs contained homologs of *avrPphE* and the other three contained *hopPsyA* and its chaperone *shcA*. Phylogenetic analysis of *avrPphE* and *hrpK* indicated that effectors in the EEL were acquired before divergence of these strains; apparently before establishment of host specificity, but after acquisition of the *hrp/hrc* gene PAI (pathogenicity island). Some effector genes in the EELs were predicted to no longer encode functional proteins. This observation strengthens the hypothesis that loss of type III effector genes, presumably driven by successful host recognition, might be a mechanism by which pathogens maintain fitness.

### Look closer to see the capillary waves

Genetic knockout of type III effector genes in plant pathogens, and subsequent assay of the mutants for differences in growth relative to wild-type strains is a standard approach towards understanding function. However, several attributes of type III effectors make this approach akin to searching for a pilus in a haystack. Badel *et al.* [35,36] created strains of *Pto* DC3000 lacking either *HopPtoM* or both of the homologous genes, *HopPtoA1* and *HopPtoA2*. Neither mutant exhibited significant difference in growth *in planta* when compared with wild-type *Pto* DC3000. However, a deviation from the wild type was observed when other phenotypes were carefully examined. The strain lacking *HopPtoM* caused almost no disease lesions on susceptible tomato. The wild-type strain caused the typical lesions normally associated with bacterial speck disease. A strain lacking both *HopPtoA1* and *HopPtoA2*, resulted in a greater percentage of small colonies (250  $\mu$ m or less in diameter), on *Arabidopsis*. This growth pattern is comparable to a fully avirulent *hrcC* mutant (eliminating the TTSS) and is in contrast to wild-type *Pto* DC3000, for which most colonies were larger than 250  $\mu$ m. Growth might not be a measurable output for all type III effectors because of their potentially subtle or growth stage specific phenotypic effects. Thus it might be necessary to use more acute, yet biologically relevant, assays to define virulence functions.

Transcriptional profiling has been successfully used to illuminate many different biological functions. Hauck *et al.* [37] found that an unexpected percentage of genes predicted to encode secreted proteins were repressed in transgenic *Arabidopsis* plants expressing the type III effector *AvrPto*. Many of these genes were hypothesized to be involved in extracellular defense such as the formation of papillae, cell wall appositions that limit bacterial growth. Subsequent assays showed that these transgenic plants failed to form papillae and supported near wild-type levels of growth of the normally avirulent *hrcC* mutant.



The AvrBs3 protein of *Xcv* belongs to a family of related effectors [38–42]. Many have demonstrated virulence functions. AvrBs3 causes pustules on susceptible plants. Thirteen differentially expressed genes were identified from susceptible pepper plants challenged with either *Xcv* expressing AvrBs3 or a mutant lacking the functional AvrBs3 nuclear localization sequences [41]. Most of the genes corresponded to those proposed to have roles in cell expansion. The observed phenotype attributed to AvrBs3 and the host genes it modifies are consistent with a virulence function where this type III effector modulates cell expansion, but additional studies are necessary to demonstrate that the transcriptional induction of these pepper genes confers a benefit to *Xcv* strains expressing AvrBs3.

### Standing waves: interference at the nodes

Recognition of a particular type III effector by a host R protein induces a multitude of responses that suppress pathogen growth (see above). Pathogenic strains can express type III effectors that interfere with the R-mediated recognition of another type III effector [43–45,46\*\*]. In one case, the mechanism by which the type III effector AvrRpt2 interferes with the ability of another type III effector, AvrRpm1, to trigger the RPM1 R protein has been deciphered [46\*\*]. Expression of AvrRpt2 leads to the degradation of RIN4 [46\*\*,47\*\*]. The absence of RIN4 destabilizes RPM1, thus eliminating the resistance response to bacteria expressing AvrRpm1. In an interesting evolutionary twist, the recognition of AvrRpt2 by the RPS2 R protein is dependent on the disappearance of RIN4. This observation supports the hypothesis that R proteins ‘guard’ targets of effectors [2,6,7] although in this case direct interactions are not necessarily implied.

The observation that AvrRpt2 causes disappearance of RIN4 led to the hypothesis that AvrRpt2 encodes a cysteine protease [48]. This was supported by the observation that AvrRpt2 undergoes autocleavage *in planta* [49,50] and secondary structure prediction programs [48]. Bacteria carrying alanine substitution in any of the residues in the catalytic triad failed to elicit the RPS2-dependent HR [48]. Additionally, these mutants failed to initiate RIN4 disappearance. Whether AvrRpt2 acts directly upon RIN4 is unclear because direct interactions have not yet been shown. However, a eukaryotic protein factor is required for AvrRpt2 autocleavage and is suggested to be involved in its cleavage of RIN4 [49].

*Y. pseudotuberculosis* is an animal pathogen that expresses type III effectors demonstrated to suppress interleukin production elicited by its own TTSS structural component YopB [51\*]. Thus, pathogens have effectors that also mask non-effector elicited host responses. It is therefore logical to ask which, if any, induced responses correlated with plant defense are abrogated in response to a parti-

cular type III effector. The type III effector AvrPtoB of *Pto* DC3000 inhibits AvrPto-Pto and Avr9-Cf9 elicited HR when all three are co-overexpressed transiently in tobacco [52]. Furthermore, AvrPtoB suppresses oxidative and heat-stress induced cell death in yeast. The exact mechanism by which AvrPtoB functions in its normal host is still not defined, but these studies provide clues that can be further pursued. Two unrelated type III effectors of *Erwinia amylovora* have also been implicated in modulating the oxidative burst [53].

### The next wave: inferring effector function from structure

Determining protein structure and comparing the primary amino acid sequence with other members of the protein family can aid in identifying critical residues required for function. The AvrB protein structure was solved to 2.2 Å resolution with the intention of gaining insight into its molecular function (C Lee *et al.* unpublished data). The AvrB structure consists of a novel bilobal fold separated by a deep interlobal cleft. This cleft is highly conserved among the four AvrB family members, suggesting that each member shares a common enzymatic function involving this cleft region. The structure is a valuable tool for future studies on determining the function of AvrB. This demonstrates the use of determining protein structure in facilitating the characterization of effectors. This also highlights another use for analyzing alleles within a family of type III effectors.

### Waving goodbye: conclusions and outlook

There will be a veritable flood of data regarding type III effector protein function from plant pathogens. Plant pathology, as a discipline, has provided a broad wealth of host and pathogen genotypes from which to sample interaction phenotypes. High throughput screens will be used to further our understanding of effector function. The transcriptional profile of a given host can be determined in response to effectors. Their roles in virulence might then be predicted on the basis of commonly induced or repressed host genes, the so-called transcriptional signature.

A large-scale proteomics approach should also be considered; all or at least one member from each family can be crystallized. We have solved the crystal structures of several type III effectors and one of their chaperones (A Singer, D Desveaux, L Betts, JH Chang, ZL Nimchuk, SR Grant, J Sondek and JL Dangel, unpublished data). This, in combination with structural determination for type III effectors from animal pathogens, will provide a well-populated database for grouping type III effectors on the basis of common folds and predicted functions.

The strategies highlighted here can be used to determine functions for novel effectors. Some are relatively straightforward, for example *in vitro* phosphatase activity. These

can be easily applied to proteins predicted to have similar functions or even used in a high-throughput manner on effectors without any a priori clues to their functions. Some assays may need to be adapted to a particular effector such as one that requires a host factor in order to function. Other assays may require transgenification or be created *de novo* and corroborated by more traditional methods. It is clear that multi-disciplinary approaches will become more common.

Plant pathogenic bacteria coordinately express and deliver a suite of type III effectors into their host. As the number of uncharacterized type III effectors is growing, we are developing more sophisticated ways to scrutinize their function. Just as the pathogens have evolved coordinated effector functions to cause disease it is perhaps appropriate for us to develop coordinated experimental efforts towards elucidating effector function. The resulting flood of anticipated results will keep scientists filling up their bailing buckets for some time.

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We dedicate this article to the memory of Prof. Noel T Keen, whose generosity of spirit, intellect and reagents is a continuing inspiration.

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### Now in press

The work referred to in the text as (Lee *et al.*, unpublished data) is now in press [59]:

59. Lee CC, Wood MD, Ng K, Andersen C, Liu Y, Luginbühl P, Spraggon G, Katagiri F: **Crystal Structure of the Type III effector AvrB from *Pseudomonas syringae*.** *Structure*, **12**:in press.