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Pathogen profile

Ralstonia solanacearum, a widespread bacterial plant pathogen in the post-genomic era

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SUMMARY

Ralstonia solanacearum is a soil-borne bacterium causing the widespread disease known as bacterial wilt. *Ralstonia solanacearum* is also the causal agent of Moko disease of banana and brown rot of potato. Since the last *R. solanacearum* pathogen profile was published 10 years ago, studies concerning this plant pathogen have taken a genomic and post-genomic direction. This was pioneered by the first sequenced and annotated genome for a major plant bacterial pathogen and followed by many more genomes in subsequent years. All molecular features studied now have a genomic flavour. In the future, this will help in connecting the classical field of pathology and diversity studies with the gene content of specific strains. In this review, we summarize the recent research on this bacterial pathogen, including strain classification, host range, pathogenicity determinants, regulation of virulence genes, type III effector repertoire, effector-triggered immunity, plant signalling in response to *R. solanacearum*, as well as a review of different new pathosystems.

Taxonomy: Bacteria; Proteobacteria; β subdivision; Ralstonia group; genus *Ralstonia*.

Disease symptoms: *Ralstonia solanacearum* is the agent of bacterial wilt of plants, characterized by a sudden wilt of the whole plant. Typically, stem cross-sections will ooze a slimy bacterial exudate. In the case of Moko disease of banana and brown rot of potato, there is also visible bacterial colonization of banana fruit and potato tuber.

Disease control: As a soil-borne pathogen, infected fields can rarely be reused, even after rotation with nonhost plants. The disease is controlled by the use of resistant and tolerant plant cultivars. The prevention of spread of the disease has been achieved, in some instances, by the application of strict prophylactic sanitation practices.

Useful websites: Stock centre: International Centre for Microbial Resources—French Collection for Plant-associated Bacteria CIRM-CFBP, IRHS UMR 1345 INRA-ACO-UA, 42 rue Georges Morel, 49070 Beaucouzé Cedex, France, http://www.angers-nantes.inra.fr/cfbp/. Ralstonia Genome browser: https://iant.toulouse.inra.fr/R.solanacearum. GMI1000 insertion mutant library: https://iant.toulouse.inra.fr/R.solanacearumGMI1000/GenomicResources. MaGe Genome Browser: https://www.genoscope.cns.fr/agc/microscope/mage/viewer.php?

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A CLASSIFICATION INTO FOUR PHYLOTYPES

In pace with the development of molecular tools, the classification of *R. solanacearum* has undergone many changes during the past 10 years. In 2005, Fegan and Prior proposed a new hierarchical classification based on the analysis of the sequence of the internal transcribed spacer (ITS) region, the *hypersensitive response and pathogenesis B* (*hrpB*) and *endoglucanase* (*egl*) genes (Fegan and Prior, 2005). The analysis of 140 *R. solanacearum* strains isolated from all over the world revealed a subdivision of the species into four phylotypes, which were correlated with the geographical origins of the strains. Phylotype I included strains originating primarily from Asia, phylotype II those from America, phylotype III those from Africa and phylotype IV those from Indonesia, Australia and Japan. Phylotype IV also contained the two close relatives of *R. solanacearum*: *Ralstonia syzygii* and the blood disease bacterium (BDB) strains. A multiplex polymerase chain reaction (PCR) based on sequence information from the ITS region has been developed to rapidly identify the phylotype to which a strain belongs (Fegan and Prior, 2005). Each phylotype can be further subdivided into groups of strains, named sequevars, or sequence variants, according to the *egl* nucleotide sequence. More than 50 sequevars have been defined so far.

This classification was confirmed by comparative genomic hybridization of a set of 18 strains, representing the biodiversity of *R. solanacearum* on a microarray representative of the GMI1000 reference strain genome (Guidot *et al*., 2007). Genomic data for nine new *R. solanacearum* strains also confirmed this classification (Remenant *et al*., 2010, 2011, 2012). Thanks to the overwhelming phylogenetic data on phylotype II strains, it has been

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suggested that this phylotype should be divided into two subgroups IIA and IIB (Castillo and Greenberg, 2007; Cellier *et al*., 2012). It cannot be excluded that deeper sampling in the other phylotypes may also result in a similar refinement of classification.

The geographical isolation and nonhost preference have been the main drivers of the separation of *R. solanacearum* strains into four phylotypes (Castillo and Greenberg, 2007; Wicker *et al*., 2012). Using coalescent genealogy reconstruction, Wicker *et al*. (2012) suggested that *R. solanacearum* originated from the Australian/Indonesian region in which phylotype IV strains are found. A subgroup of the ancestral strains from this region probably spread throughout present Austral-Eastern Africa and Madagascar, and differentiated later into phylotypes III and I (predicted with an East African/Asian origin). Another subgroup of ancestral strains migrated to Brazil and differentiated later into the subgroups IIA and IIB, at a time similar to that of the phylotype I/III differentiation, possibly before the fragmentation of Gondwana (Castillo and Greenberg, 2007; Wicker *et al*., 2012).

A SPECIES COMPLEX, LET'S KEEP IT SIMPLE

A species complex is defined as a cluster of closely related isolates whose individual members may represent more than one species. The term 'species complex' was first applied to *R. solanacearum* by Gillings *et al*. (1993) to reflect the phenotypic and genotypic variability within the species. Taghavi *et al*. (1996) then confirmed the concept of the *R. solanacearum* species complex by including *R. syzygii* and BDB strains into the *R. solanacearum* phylogeny. Studies of DNA–DNA similarity revealed that the relatedness between *R. solanacearum* isolates is often just under the 70% threshold level commonly expected within a species (Roberts *et al*., 1990). By comparing different strains with the phylotype I strain GMI1000 by microarray hybridization, the most divergent strains still have 68%–69% of their genes hybridizing with the GMI1000 oligonucleotides (Guidot *et al*., 2007). More recently, Remenant *et al*. (2010, 2011) used average nucleotide identity (ANI) to evaluate genetic distances between the eight sequenced genomes from this species complex. From the results, based on the ANI > 95% cut-off (Konstantinidis and Tiedje, 2005; Konstantinidis *et al*., 2006), the authors suggested that the *R. solanacearum* species complex should be restructured into three different species: one containing phylotypes I and III, a second containing phylotype II, and a third containing phylotype IV, including *R. syzygii* and BDB strains (Remenant *et al*., 2011). ANI provides a more robust and accurate measurement of the genetic distance than does DNA–DNA hybridization (Konstantinidis and Tiedje, 2005). However, as pointed out by Konstantinidis and Tiedje (2005), ANI should not be considered as the sole argument for species definition. Ecological niche occupation, which is a justifiable measurement of the phenotypic potential of a bacterial strain, is another important argument for species definition (Konstantinidis and Tiedje, 2005; Konstantinidis *et al*., 2006). A simple inspection of the ecological niches occupied by strains from the *R. solanacearum* species complex indicates that all strains share phenotypic potential, as they are all soil-borne and plant xylem-infecting bacteria. In addition, *R. solanacearum* strains from the four phylotypes are able to infect tomato plants and cause the same symptoms (Remenant *et al*., 2010). Another important consideration is the (likely allopatric) divergence of related strains accompanied by important genome reduction and ecological specialization, as is the case for *R. syzygii*, the BDB strains and *R. solanacearum* phylotype IV strains (i.e. PSI07) (Remenant *et al*., 2011). Despite a high ANI score (>98%), we could argue that these strains should not be the same species, in the same way as *Burkholderia mallei* and *B. pseudomallei* are justified as different species (Konstantinidis *et al*., 2006).

The renaming proposition of Remenant and colleagues is interesting, but should be re-evaluated in the light of more whole-genome sequence data to clearly evaluate whether there is a continuum or clustered genetic relatedness in this species complex. For instance, the sequence of strain ACH0732, which is not clearly associated with a specific phylotype (Fegan and Prior, 2005), would be especially informative.

HOST RANGE

Host range specificity in *R. solanacearum* is unclear. The diverse strains in the *R. solanacearum* species complex exhibit an unusually large host range, being able to infect more than 250 plant species in 54 monocot and dicot botanical families (Elphinstone, 2005) (Fig. 1). Host specialization has been reported for some strains, for example the Moko strains infecting banana and *Heliconia* and the brown-rot strains infecting potato and tomato. However, host specialization in the *R. solanacearum* species complex has rarely been described in detail. For example, pathogenicity tests under controlled conditions found that most Moko strains are also virulent to susceptible tomato and potato (Cellier *et al*., 2012). Many studies have been conducted to tentatively identify the genes associated with host specificity. For this purpose, the methodology of comparative genomic hybridization on pangenomic microarrays representative of *R. solanacearum* genes was used to compare gene repertories of hundreds of strains for which pathogenicity traits had been defined (Cellier *et al*., 2012; Guidot *et al*., 2007). These studies were conducted with potato pathogenic strains and banana pathogenic strains, but did not find any genes associated with these pathogenicity traits (Cellier *et al*., 2012; Guidot *et al*., 2007).

In order to better characterize the specificity of the interaction between *R. solanacearum* and solanaceous plants, a recent analysis has been conducted in controlled conditions to study the pathogen interaction between a collection of three Solanaceae

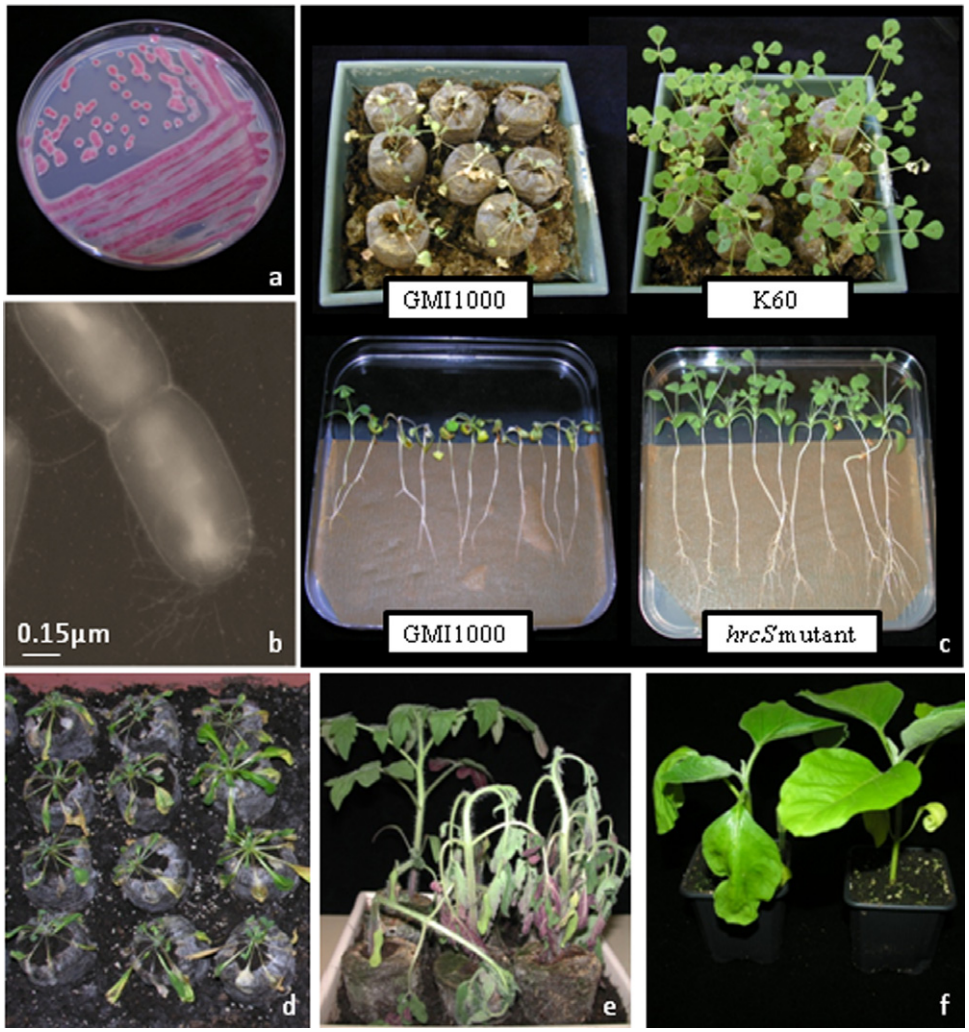


Fig. 1 Macro- and microscopic views of *Ralstonia solanacearum* and illustration of associated symptoms on plant bioassays. (a) *Ralstonia solanacearum* growing on complete BG medium (Boucher *et al*., 1985). The pink colour of the colonies is caused by the presence of triphenyl tetrazolium chloride in the medium. (b) Electron microscopy image of *R. solanacearum* rod-shaped cells under division displaying pili structures (by the late Jacques Vasse). (c) Symptoms of bacterial wilt on *Medicago truncatula* plants. Inoculation in Jiffy pots with two wild-type strains (top part). Gnotobiotic inoculation with a wild-type strain and an *hrp* mutant (bottom part). (d) Symptoms of bacterial wilt on *Arabidopsis thaliana* plants. (e) Symptoms of bacterial wilt on tomato plants. (f) One eggplant with symptoms of bacterial wilt, and a healthy control plant.

(tomato, eggplant and pepper) representative of the bacterial wilt resistance genetic resources and a collection of 12 strains representative of the known phylogenetic diversity of *R. solanacearum* (Lebeau *et al*., 2011). Interestingly, although all plants belong to the same family, they interact differently with the 12 *R. solanacearum* strains. Six interaction phenotypes were defined and were used to name pathoprofiles based on the aggressiveness of the strains on the host plants. Intermediate phenotypes correspond to latent infection of the plants (bacterial colonization of the xylem tissue with few or no wilting symptoms). No pathoprofile is phylotype specific and none of the plants of this collection were resistant to all tested *R. solanacearum* strains (Lebeau *et al*., 2011).

EMERGENCE OF STRAINS WITH A NEW HOST RANGE

Ralstonia solanacearum is described as a highly flexible organism capable of rapid adaptation to environmental changes and new hosts and of counteracting plant resistance. However, the characterization of emerging strains in *R. solanacearum* is difficult and has rarely been reported. The most studied recently emerging strains are the phylotype IIB-4NPB (nonpathogenic on banana) strains in Martinique (Wicker *et al*., 2007, 2009). These strains belong to the phylotype IIB-4 group in which Moko disease-causing strains also cluster, but they are not pathogenic to banana. The epidemiological data demonstrate that phylotype IIB-4NPB

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strains constitute an emerging population in Martinique. This genetic group was absent in *R. solanacearum* collections from the French West Indies until the first strain was isolated in 1999. These strains show a previously unknown host range in *R. solanacearum*, including cucurbits, ornamental plants and Solanaceae. Importantly, they seem to have expanded their host range from *Anthurium*–Cucurbitaceae in 1999–2002 to *Anthurium*–Cucurbitaceae–Solanaceae in 2002–2003 (Wicker *et al*., 2007). Moreover, they were recovered from solanaceous wild species and several weeds, as well as in the water, throughout Martinique, demonstrating their rapid spread over the island. The factors that have favoured this emergence of strains with novel host specificity are still unclear. The banana/vegetable rotations in Martinique fields probably have a role to play. Indeed, the isolation of IIB-4NPB strains from wilted tomatoes or wilted cucurbits was only reported on fields that followed a previous banana crop (Wicker *et al*., 2009). Interestingly, IIB-4NPB strains were also isolated in Brazil from cucurbits (Cellier *et al*., 2012). Because Brazil is a Moko disease area, it is also possible that IIB-4NPB strains emerged in Brazil and established in Martinique through movement of contaminated ornamental material, such as *Anthurium*.

GENERATION OF BIODIVERSITY

In bacteria, polymorphism created by mutations is redistributed among strains by recombination and horizontal gene transfer. The major contribution of recombination in the evolutionary dynamics of *R. solanacearum* has recently been demonstrated by Wicker *et al*. (2012). The authors conducted multilocus sequence analysis (MLSA) with nine loci on a worldwide collection of 89 *R. solanacearum* strains representative of the four phylotypes and the 51 *egl*-based sequevars, and concluded that recombination has played a major role in *R. solanacearum* genome evolution. They detected phylotype IV as a gene donor for the majority of the recombination events. Interestingly, phylotype I, which is known to affect the largest number of hosts, appeared to be the most recombinogenic lineage. The only clonal group was phylotype IIB. These findings contrast with the conclusions reached by Castillo and Greenberg (2007). Using MLSA and estimations of linkage disequilibrium between eight loci in 58 strains from the four phylotypes, Castillo and Greenberg (2007) concluded that *R. solanacearum* is an essentially clonal organism. However, most (24 of 58) of the strains analysed by Castillo and Greenberg (2007) belonged to phylotype IIB (phylotype II, group A in Castillo and Greenberg, 2007), which is a clonal group according to Wicker *et al*. (2012). This could be part of the reason why Castillo and Greenberg (2007) arrived at this conclusion.

Another important mechanism in the evolution of *R. solanacearum* genomes is horizontal gene transfer (Coupat *et al*., 2008; Fall *et al*., 2007; Guidot *et al*., 2009; Remenant *et al*., 2010). Analysis of the genomic sequences of nine *R. solanacearum* genomes revealed numerous genomic islands, many surrounded by mobile elements, such as insertion sequences (ISs) or bacteriophages, suggesting a horizontal acquisition (Remenant *et al*., 2010, 2011, 2012). Hierarchical clustering based on the variable genes within the genomic islands among 18 *R. solanacearum* strains indicated that they were acquired by ancestral strains and were then transmitted vertically within phylotypes (Guidot *et al*., 2007). Methods based on phylogenetic reconstruction of gene families with prokaryote homology detected 151 genes (13.3%) of foreign origin in the *R. solanacearum* GMI1000 genome (Fall *et al*., 2007). The small plasmid carrying the type IV secretion system detected in the genome of the CMR15 strain was possibly acquired from *Xanthomonas euvesicatoria*, another tomato pathogen prevalent in Cameroon (Remenant *et al*., 2010). Interestingly, recombination 'hotspots' were detected in the GMI1000 genome correlating with the presence of Chi-like signature sequences (Fall *et al*., 2007).

The frequency of gene transfer between phylogenetically distant bacteria is expected to be low. Nonetheless, horizontal gene transfer between strains from the four phylotypes has been shown to be possible in the laboratory (Coupat *et al*., 2008; Guidot *et al*., 2009). Coupat *et al*. (2008) demonstrated that 80% of *R. solanacearum* strains are naturally transformable by plasmids and/or genomic DNA, and that large DNA fragments ranging from 30 to 90 kb can be transferred between strains. The potential to exchange virulence genes by horizontal gene transfer could play a major role in rapid pathogenicity evolution of *R. solanacearum* strains. The role of horizontal gene transfer in enhancing the aggressiveness on tomato of *R. solanacearum* strains has been demonstrated experimentally (Coupat-Goutaland *et al*., 2011).

PATHOGENICITY DETERMINANTS

The main pathogenicity determinant in *R. solanacearum* is the type III secretion system (T3SS) (Boucher *et al*., 1985; Coll and Valls, 2013), a syringe-like membrane appendix that injects the so-called 'effector proteins' (type III effector proteins, or T3E hereafter) into the plant cell cytosol to favour infection (Erhardt *et al*., 2010; Tampakaki *et al*., 2010). Mutants defective in any of the >20 *hrp* or *hrp-conserved* (*hrc*) genes, encoding structural or regulatory proteins of this molecular syringe, are nonpathogenic (Boucher *et al*., 1985). Exopolysaccharide (EPS), a loose slime of heterogeneous composition (Orgambide *et al*., 1991), also plays an important role in *R. solanacearum* pathogenicity. EPS strongly contributes to the occlusion of the xylem vessels that eventually causes the plant wilting symptoms. EPS is also important for plant colonization (Araud-Razou *et al*., 1998; Denny and Baek, 1991; Husain and Kelman, 1958; Kao *et al*., 1992). In addition to these two major virulence determinants mentioned, *R. solanacearum* produces an array of other factors that also contribute to colonization and/or to symptom appearance. These have been exhaus-

tively reviewed (Genin and Denny, 2012) and include, among others, type II-secreted plant cell wall-degrading enzymes, motility or attachment appendages, aerotaxis transducers, cellulases and pectinases. For instance: type 4 pili, involved in twitching motility, biofilm formation and root attachment, and the flagella, responsible for swimming motility, have been shown to contribute to virulence on tomato (Kang *et al*., 2002; Tans-Kersten *et al*., 2001). Interestingly, motility and attachment seem to play a role during plant invasion, as most mutants affected in these capacities are hypopathogenic when inoculated in the soil, but behave like wild-type strains when inoculated directly in plant stems (Meng *et al*., 2011; Yao and Allen, 2007). A recent report has suggested that aggregation caused by Flp pili also plays a role in pathogenicity, as a mutant deficient in these pili displays wild-type swimming or twitching motility, but is impaired in its ability to cause wilting of potato plants (Wairuri *et al*., 2012).

REGULATION OF VIRULENCE GENES

Bacterial plant pathogens possess sophisticated regulatory circuits to finely control the energy-consuming expression of virulence determinants. An exhaustive comparative review on virulence regulatory modules in different bacterial pathogens can be found elsewhere (Mole *et al*., 2007). The pathways controlling the transcription of the main *R. solanacearum* virulence genes are well known (Genin, 2010; Genin and Denny, 2012; Schell, 2000). The LysR family transcriptional regulator PhcA plays a central role, as it regulates directly or indirectly many of these genes (Fig. 2). PhcA activates EPS and cellulase-encoding genes and represses swimming motility, T3SS and siderophore expression (Genin and Denny, 2012). PhcA represses the transcription of a pectinase-encoding gene via PehR, although it also activates slightly the expression of other pectinase genes (Fig. 2). Interestingly, transcription of the global regulator *PhcA* is controlled by a *Ralstonia*-specific cell density-dependent mechanism that involves 3-hydroxy palmitic acid methyl ester (3-OH PAME) produced by the inner membrane protein PhcB (Flavier *et al*., 1997; Genin and Denny, 2012). At low cell densities, *PhcA* expression is repressed by PhcR by a post-transcriptional mechanism (Fig. 2). When the amounts of 3-OH PAME build up as a result of confinement or bacterial densities above 10^7 cells/mL, the molecule activates the two-component system PhcS/PhcR. PhcR is then phosphorylated and *PhcA* expression is de-repressed (Clough *et al*., 1997; Schell, 2000). One of the main outcomes of *PhcA* activation in high-cell-density conditions is the production of large amounts of EPS. This control is exerted through the induction of *XpsR*, which activates directly the transcription of the *eps* operon (Huang *et al*., 1995). Interestingly, it has been shown that the two-component regulatory system VsrA/VsrD is also required to fully activate *xpsR* transcription and, consequently, EPS synthesis (Huang *et al*., 1998; Schell *et al*., 1994). In addition, VsrD affects swimming motility directly by repressing the transcription of the flagellum genes (Fig. 2). Another two-component system, VsrB/VsrC, has also been described to control EPS synthesis and to repress the transcription of the pectinase *pglA*, adding another layer of control on EPS synthesis (Huang *et al*., 1995). It is remarkable that members of both systems (*vsrB* and *vsrD*) were identified in a genetic screen amongst 153 *R. solanacearum* K60 genes induced during growth in tomato (Brown and Allen, 2004). XpsR is thus a central switch in EPS regulation, as it integrates inputs from both VsrAD and PhcA to regulate directly the *eps* promoter, and is required for both its negative control by EpsR and its positive control by VsrC (Garg *et al*., 2000; Huang *et al*., 1995).

Regulation of the T3SS exemplifies that, during evolution, horizontally transferred operons can co-opt transcriptional regulators present in the recipient bacterium (Cases and de Lorenzo, 2001).

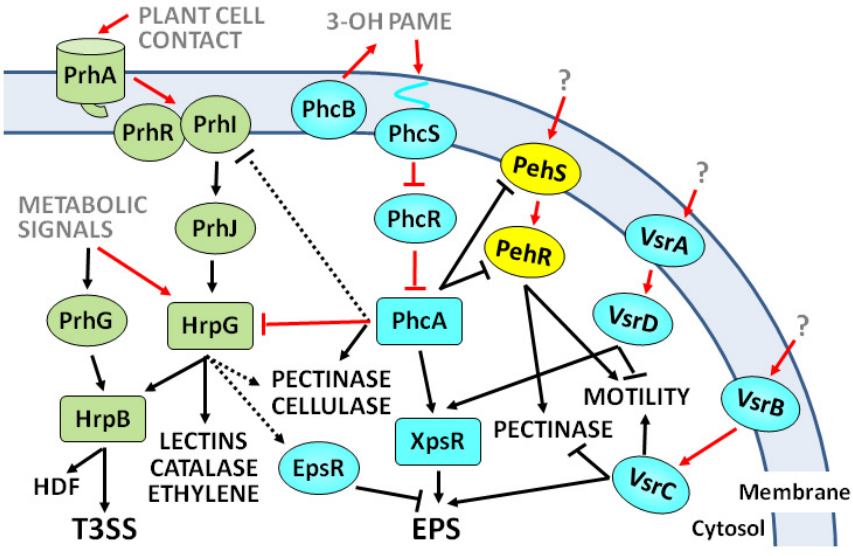


Fig. 2 Major pathways controlling the expression of *Ralstonia solanacearum* virulence genes. Ovals and squares indicate regulatory proteins, the latter representing the main regulatory hubs. Grey, regulatory inputs sensed by the bacterium; black, pathogenic activities controlled by this regulatory network. Arrows and T-bars indicate activation or repression, respectively. Black lines denote control at the transcriptional level and red lines indicate post-transcriptional effects. Full lines represent major effects and dotted lines slight transcriptional influences (modulation). For detailed explanations, see text. EPS, exopolysaccharide; HDF, Hrp-dependent factor; 3-OH PAME, 3-hydroxy palmitic acid methyl ester; T3SS, type III secretion system.

For instance, although the genes encoding the T3SS are highly conserved across species, the pathways controlling their transcription in *R. solanacearum* and *Xanthomonas* spp. are totally unrelated to those found in *Pectobacterium* spp. and *Pseudomonas* spp. (Tang *et al*., 2006). In *R. solanacearum*, HrpB, an AraC-type regulator, and HrpG, its upstream OmpR-like two-component response regulator, control *hrp/hrc* gene expression. HrpB triggers directly the transcription of T3SS genes, probably binding to the so-called *hrpII* box found in promoter regions (Cunnac *et al*., 2004a, b; Genin *et al*., 1992), and its expression is controlled by HrpG (Brito *et al*., 1999) (Fig. 2). HrpG and HrpB are both genetically and functionally conserved in *Xanthomonas* spp. (Li *et al*., 2011; Wengelnik and Bonas, 1996; Zou *et al*., 2006), but unique to *R. solanacearum* are the upstream regulators that trigger the specific induction of HrpG when the bacterium detects a plant cell wall component (Aldon *et al*., 2000). PrhA is the outer membrane receptor that perceives this signal (Aldon *et al*., 2000) and transfers it to the membrane-associated proteins PrhI and PrhR to trigger *hrp/hrc* expression through the consecutively induced transcriptional regulators PrhJ, HrpG and HrpB (Brito *et al*., 2002) (Fig. 2). *In vitro* transcriptomic studies have revealed that the *hrp* regulators control additional functions other than the T3SS and most of its associated effectors. It has been shown that HrpB is also involved in the regulation of chemotaxis and the biosynthesis of various low-molecular-weight chemical compounds, such as the Hrp-dependent factor (HDF), which may induce a cell density-dependent LuxR system (Delaspre *et al*., 2007; Occhialini *et al*., 2005). HrpG has been found to control an even larger set of T3SS-unrelated genes independent of HrpB (Fig. 2). These encoded genes are probably involved in plant–pathogen interactions, including adhesion factors (lectins), the only predicted catalase enzyme in the genome and an ethylene-forming enzyme that produces this plant hormone (Valls *et al*., 2006). HrpG has also been found to affect slightly known virulence determinants, such as pectinolytic and cellulase activities, some of which are common targets of PhcA (Fig. 2). A recent study has also shown that HrpG is involved in the control of the last step of methionine synthesis (Plener *et al*., 2012). It has been proposed that HrpG promotes the production of MetE, which synthesizes methionine without the need of vitamin B12, as a way to ensure the biosynthesis of this amino acid in the vitamin-poor environment encountered *in planta*. Thus, HrpG occupies a central node in pathogenicity regulation as, in addition to controlling a panoply of virulence genes, it integrates both plant cell-dependent induction and metabolic cues that affect the transcription of the T3SS (Fig. 2). Examples of the latter metabolic signals are the repression of T3SS genes by casamino acids and their induction during growth in minimal medium (Arlat *et al*., 1992; Genin *et al*., 2005). Recently, PrhG has been identified in strain GMI1000 as a HrpG paralogue that also activates HrpB expression. Interestingly, *prhG* is induced during growth in minimal medium, but not by plant cells, so that this regulator controls the expression of T3SS under minimal medium conditions (Plener *et al*., 2010). Additional inputs influencing the expression of T3SS have also been described, but the regulators involved and their mechanisms of action remain poorly understood. Examples of these are LrpE, a leucine-rich repeat protein found to negatively regulate the expression of *hrp* genes by three- to five-fold (Murata *et al*., 2006). Similarly, the absence of any of the three genes encoded in the *prKLM* operon in strain OE1-1 decreases *prhG* expression and, consequently, that of *hrpB* and the *PopA* effector by 10-fold (Zhang *et al*., 2011). However, these genes do not encode transcriptional regulators and must influence the *hrp* regulon indirectly by an unknown mechanism.

As mentioned previously, the global regulator PhcA modulates T3SS gene expression and does so at two different levels: by slightly inhibiting *prhI/R* transcription and by strongly inhibiting *hrpB* gene expression through an unknown mechanism acting on HrpG (Genin *et al*., 2005; Yoshimochi *et al*., 2009a, b). Interestingly, EPS production is also slightly downregulated by HrpG through an increase in the levels of *EpsR* (Valls *et al*., 2006) (Fig. 2). These examples illustrate that cross-talk occurs between regulatory cascades at various levels. All of this knowledge has led to the corollary that, in *R. solanacearum*, the infection process takes place in two steps (Brito *et al*., 2002; Mole *et al*., 2007; Schell, 2000): First, early in colonization, expression of the T3SS is induced by plant cell contact and PhcA is not induced because of the low bacterial density, allowing swimming motility to be active. In the second step, when bacterial numbers increase inside the xylem, the PhcA regulator is expressed, triggering EPS and cellulase production and repressing T3SS and the siderophore. However, recent *in planta* expression data using *promoter::reporter* fusions integrated in the bacterial chromosome and quantitative reverse transcription-polymerase chain reaction (RT-PCR) have challenged this model (Monteiro *et al*., 2012a, b). These studies showed that the *hrpB* and T3E transcripts are abundant at late stages of plant colonization, when bacterial numbers are high and plants are already wilted. More recently, transcriptome analyses have confirmed this finding, showing that one-half of the *hrpB*-regulated genes are induced in bacteria recovered from the xylem of wilting tomato plants (Jacobs *et al*., 2012). All of these observations illustrate our limitations to predict the behaviour of bacterial virulence genes in real field conditions and suggest the existence of still unknown inducing signals.

T3E REPERTOIRE

Over the last 10 years, T3E biology has boomed. Different methods have been applied to define the set of T3Es in *R. solanacearum*: (i) the search for orthologues of already known T3Es; (ii) the identification of T3Es through gene regulation studies (Cunnac *et al*., 2004a, b; Occhialini *et al*., 2005); (iii) the search for atypical protein

motifs indicating a potential function in eukaryotic host cells (Angot *et al*., 2006); and finally (iv) a functional screen for type III injected protein (Mukaihara *et al*., 2010). Altogether, these efforts generated a comprehensive list of T3Es for two closely related phylotype I strains (Mukaihara *et al*., 2010; Poueymiro and Genin, 2009). This latter work could now be expanded with the availability of several new genomic sequences spanning the four phylotypes representing the *R. solanacearum* diversity (Gabriel *et al*., 2006; Remenant *et al*., 2010, 2011; Salanoubat *et al*., 2002; Xu *et al*., 2011). This would allow the evaluation of common and specific Rip ['*Ralstonia* injected proteins' (Cunnac *et al*., 2004b; Mukaihara *et al*., 2010)]. As already pointed out when the first *R. solanacearum* genome was published (Salanoubat *et al*., 2002), there are some striking features specific to this bacterium. First, it seems that the T3E repertoire is larger than in other plant pathogenic bacteria; second, *R. solanacearum* seems to be the recipient of a diverse set of T3Es probably acquired by horizontal gene transfer. Indeed, the GALA T3E (Kajava *et al*., 2008) and the pentatricopeptide repeat (PPR)-containing T3E (Salanoubat *et al*., 2002), harbouring typical eukaryotic features, such as the F-box domain (Ho *et al*., 2008) and PPR motifs (Delannoy *et al*., 2007), could originate from an ancestral eukaryotic donor. However, many T3Es could have originated from horizontal gene transfer from other pathogenic bacteria, as homologues exist in various other animal and plant bacterial pathogens (Poueymiro and Genin, 2009). This is very likely to be the case for the *Xanthomonas* spp.-specific transcriptional activator-like (TAL) T3Es (Fall *et al*., 2007). Recently, a detailed characterization of these *R. solanacearum* TAL-like T3Es showed that they are indeed nuclear targeted and can function as transcriptional activators in plant cells (Li *et al*., 2013). Another particular feature of the *R. solanacearum* effector repertoire is the abundance of duplicated T3E genes. Indeed, several T3E genes are present as gene families (Poueymiro and Genin, 2009; Remigi *et al*., 2011; Sole *et al*., 2012). Interestingly, most of these gene families are conserved among the different *R. solanacearum* strains (Remigi *et al*., 2011; Sole *et al*., 2012) (see also the MaGe genome browser displaying the sequenced *R. solanacearum* strains: https://www.genoscope.cns.fr/agc/microscope/mage). These gene families, arising from gene duplications in a common ancestor, are likely to undergo functional diversification to provide adaptation on different host plants, as has been shown for the GALA family (Remigi *et al*., 2011).

EFFECTOR-TRIGGERED IMMUNITY (ETI)

Historically, the first biological function identified for T3Es was their contribution to ETI (Jones and Dangl, 2006). The T3E PopP1 induces a cultivar-specific hypersensitive response (HR)-like response on petunia (Arlat *et al*., 1994; Lavie *et al*., 2002). Furthermore, PopP1 (Robertson *et al*., 2004) and, recently, PopP1 together with AvrA (Poueymiro *et al*., 2009) have been shown to contribute to HR-mediated resistance in tobacco. Indeed, on tobacco root inoculation, the double *PopP1 AvrA* mutant in GMI1000 causes wilting and is indistinguishable from the K60 tobacco pathogenic strain (Poueymiro *et al*., 2009). The closely related T3E PopP2 has been shown to be responsible for RRS1-R-mediated resistance in the Nd-1 *Arabidopsis* ecotype (Deslandes *et al*., 1998). PopP2 interacts directly with RRS1-R in the nucleus of plant cells, leading to an asymptomatic and much reduced bacterial colonization (Deslandes *et al*., 2002, 2003). PopP2 has been shown to interact with the *Arabidopsis thaliana* protein RD19, redirecting its localization from lytic vacuoles to the nucleus, where both physically interact. Although its exact role has yet to be defined, RD19 is required for PopP2/RRS1-R-mediated resistance (Bernoux *et al*., 2008). Furthermore, it has been shown that this T3E, belonging to the widespread YopJ/Avrxv family, displays acetyltransferase activity. This activity results in the autoacetylation of PopP2 required for an effective PopP2/RRS1-R resistance (Tasset *et al*., 2010). *Ralstonia solanacearum* could harbour other T3Es triggering plant immunity. Indeed, *Agrobacterium tumefaciens*-mediated expression of AWR5 induces HR-like symptoms in *Nicotiana tabacum* (Sole *et al*., 2012). Interestingly, in the same T3E family, the multiple mutant *awr(1–5)* displays an increased pathogenicity on *Arabidopsis* Col-0, suggesting the possibility that at least one of these AWRs is actually recognized by an *R* gene, triggering a weak ETI in this host (Sole *et al*., 2012).

T3E VIRULENCE FUNCTIONS

Other T3Es have been characterized on the basis of their contribution to disease. Although the exact molecular mechanisms have yet to be described, different T3Es have different contributions to disease on different host plants. On tomato, mutants in the T3Es *RSp0304* (*HopD1* homologue) and *AWR2* show decreased disease progression (Cunnac *et al*., 2004b). The AWR T3E family is collectively needed on both tomato and eggplant for full pathogenicity. Interestingly, AWR2 can restore the wild-type phenotype of the multiple *awr(1–5)* mutant on eggplant (Sole *et al*., 2012). For the GALAs, another well-studied T3E family, it has been demonstrated that they are collectively required on tomato and *A. thaliana* for a full disease phenotype (Angot *et al*., 2006). More recently, this result has been shown to be more complex, with a redundancy of *GALA2*, *GALA3*, *GALA6* and *GALA7* on *Arabidopsis*, whereas only *GALA7* and *GALA3* seem to be able to restore full virulence of the quadruple mutant *gala2 gala3 gala6 gala7* on tomato (Remigi *et al*., 2011). In this same family, it has also been shown that the single *gala7* mutant (and none of the other single mutants) is avirulent on the legume host *Medicago truncatula* (Angot *et al*., 2006). GALA T3Es could potentially control host protein stability as they probably form E3-ubiquitin ligases inside the host cell (Angot *et al*., 2007). Although structurally different, several other

T3Es have similarities with ubiquitin ligases. This is the case for *RSc1349*, the homologue of the *Shigella flexneri IpaH* ubiquitin ligase (Singer *et al*., 2008), and the MolK2-specific *RSMK00763*, the T3E homologue of the *Pseudomonas syringae AvrPtoB* (Poueymiro and Genin, 2009). On *M. truncatula*, GALA7 and AvrA are both required for the early infection steps of intact roots (Turner *et al*., 2009). GALA7, but not AvrA, is also required for disease development when colonization is facilitated by cutting the root tips (Turner *et al*., 2009). Another early/late disease development differential role has been shown for the T3SS-secreted harpin PopA. Indeed, in the Japanese strain OE1-1, constitutive early expression of this T3E prevents the natural root infection of *N. tabacum*, but not bacterial multiplication inside stem-inoculated plants (Kanda *et al*., 2003).

Classical plant pathoassays have enabled the identification of only a few T3Es with a virulence function (Cunnac *et al*., 2004b). This low yield of T3Es with virulence functions compared with the large T3E repertoire (Poueymiro and Genin, 2009) could be explained by functional redundancy (Angot *et al*., 2006; Sole *et al*., 2012), but also by the fact that some T3Es have only a marginal contribution to virulence, and hence are undetectable with classical wilt scoring. For this purpose, a novel assay based on mixed inoculations was developed (Macho *et al*., 2010). The principle is to compare the ability of two strains to multiply in the host when they are co-inoculated, compared with their ability to multiply when inoculated individually. Interestingly, two T3Es, *Rsp0304* and *PopP2*, have been shown to be involved in efficient bacterial multiplication in three host plants: eggplant, tomato and bean (Macho *et al*., 2010).

COMPLEX MECHANISMS UNDERLIE BACTERIAL WILT RESISTANCE AND TOLERANCE

The genetic analysis of resistance to bacterial wilt has been developed on both model and cultivated plants. In *A. thaliana*, the study of the interaction between two ecotypes, Nd-1 and Kil-0, and two strains of *R. solanacearum*, GMI1000 and BCCF402 (both phylotype I strains), respectively, revealed the involvement of *RRS1-R*. *RRS1-R*, a Toll/interleukin-1 receptor-nucleotide-binding site-leucine-rich repeat (TIR-NBS-LRR) gene with a WRKY C-terminal domain, has been described as a single recessive resistance gene against strain GMI1000, through the direct recognition of the PopP2 effector (Deslandes *et al*., 1998, 2002, 2003). Interestingly, it has been demonstrated recently that the gene-for-gene interaction RRS1-R–PopP2 is also involved in Kil-0 tolerance (Van der Linden *et al*., 2013). Indeed, Kil-0 does not exhibit wilting symptoms after its inoculation with strain BCCF402 of *R. solanacearum*, despite a high bacterial multiplication *in planta*. The catalytic triad and the autoacetylated lysine are conserved in the BCCF402 *popP2* allele, but some allelic variations in both BCCF402 *PopP2* and Kil-0 *RRS1-R* could account for altered protein interactions and/or signal transduction in the plant cell. It cannot be excluded either that this RRS1-R-dependent tolerance in Kil-0 could be dependent on other plant or bacterial factors. A quantitative resistance mechanism has also been described in *A. thaliana* against *R. solanacearum* (Godiard *et al*., 2003). Among the three quantitative trait loci (QTLs) identified, one is associated with *ERECTA*, an LRR receptor-like kinase (LRR-RLK) involved in development (Godiard *et al*., 2003; Torii *et al*., 1996). This suggests that cross-talk can occur between resistance to *R. solanacearum* and developmental pathways (Godiard *et al*., 2003). In the model legume *M. truncatula*, recombinant inbred line (RIL) population A17 × F83005.5 enabled the identification of three QTLs for resistance to *R. solanacearum* strain GMI1000 (Vailleau *et al*., 2007). The fine mapping of the major QTL located on chromosome 5 of *M. truncatula*, *MtQRRS1*, allowed the identification of a 64-kb region with a cluster of seven putative *R* genes among 15 candidate genes (Ben *et al*., 2013) Tomato cultivar 'Hawaii 7996' has been described as resistant to bacterial wilt (Danesh *et al*., 1994; Mangin *et al*., 1999; Thoquet *et al*., 1996a, b; Wang *et al*., 2000). The polygenic resistances identified are phylotype and strain specific (Carmeille *et al*., 2006). Recently, Wang *et al*. (2013a) have identified two major QTLs for resistance to *R. solanacearum* (*Bwr-6* and *Bwr-12*) using the tomato cross 'Hawaii 7996' × 'West Virginia 700'. The *Bwr12* QTL is specific for resistance to phylotype I, whereas the *Bwr-6* QTL is associated with resistance to phylotype I and II strains. Interestingly, the presence of both QTLs has an additive effect, displaying enhanced resistance. In tobacco, Qian *et al*. (2012) identified four QTLs associated with resistance to noncharacterized 'naturally occurring strains' from bacterial wilt-affected areas of China. In eggplant, four QTLs have been identified in a RIL population that exhibited resistance to different strains of *R. solanacearum* (Lebeau *et al*., 2013). Among them, the *ERs1* QTL has been described as a major dominant source of resistance towards three phylotype I strains of *R. solanacearum* (Lebeau *et al*., 2013). The further characterization of the genetic components underlying these different resistances to *R. solanacearum* will prove to be very useful for the plant breeding community.

PLANT SIGNALLING IN RESPONSE TO *R. SOLANACEARUM*

A link has been described between *A. thaliana* secondary cell walls and the outcome of the interaction with *R. solanacearum* (Hernandez-Blanco *et al*., 2007). Indeed, a mutation in any of the three secondary cell wall-specific cellulose synthase genes led to a complete resistance to the bacterium. Furthermore, abscisic acid (ABA) signalling has been demonstrated to be involved in this cellulose synthase-dependent enhanced resistance (Hernandez-Blanco *et al*., 2007). The role of the cell walls in *A. thaliana* as barriers against *R. solanacearum* colonization has also been studied through the *wat1* (*walls are thin1*) mutant (Denance *et al*.,

2012). The *WAT1* gene is required for secondary cell wall deposition, and the corresponding mutant shows enhanced resistance to *R. solanacearum* (Denance *et al*., 2012). Comparing two different inoculation methods of *R. solanacearum* in *A. thaliana* leaves, by piercing in the central leaf vein or infiltrating a bacterial suspension in the mesophyll, the authors concluded that *wat1* resistance is localized to the vascular system. Moreover, salicylic acid (SA) has been identified as a key element of *wat1*-mediated resistance to *R. solanacearum* (Denance *et al*., 2012). Several other studies have identified WRKY transcription factors as important players in modulating the plant response towards *R. solanacearum* attack. Indeed, *A. thaliana* plants lacking a functional *WRKY27* gene exhibited an enhanced tolerance to *R. solanacearum* strains GMI1000 and Rd-15 (Mukhtar *et al*., 2008). Similar situations have been observed previously for the ethylene-insensitive *EIN2-1* gene (Hirsch *et al*., 2002) and the *NWS1* gene (Feng *et al*., 2004), which appeared to be required for full virulence of the bacteria. Recently, two WRKY transcription factors of pepper have been identified as important positive and negative contributors to *R. solanacearum* resistance. The overexpression of *CaWRKY40* in tobacco enhanced resistance to *R. solanacearum*, whereas the silencing of *CaWRKY40* in pepper attenuated the resistance (Dang *et al*., 2012). However, *CaWRKY58*-overexpressing tobacco plants showed an enhanced susceptibility to the same strain, and *CaWRKY58*-silenced pepper plants displayed an enhanced resistance (Wang *et al*., 2013b). In another study, Feng *et al*. (2012) identified the ABA signalling pathway as important for the biological control of bacterial wilt in *A. thaliana*. This ABA-dependent defence mechanism was shown to be independent of SA, jasmonic acid and ethylene in the biological control exerted by an *hrpB* mutant of *R. solanacearum* (Feng *et al*., 2012).

GNOTOBIOTIC PATHOSYSTEMS TO STUDY ROOT INFECTION

As *R. solanacearum* infects plants via their roots, gnotobiotic artificial inoculation systems constitute useful tools to enable access to the host roots. Such bioassays, in which axenic plantlets are inoculated with *R. solanacearum*, have been described on tomato (Monteiro *et al*., 2012b; Vasse *et al*., 1995), petunia (Zolobowska and Van Gijsegem, 2006), *M. truncatula* (Vailleau *et al*., 2007) and *A. thaliana* (Digonnet *et al*., 2012). They enable the study of the early steps of infection, step-by-step colonization and the observation of 'root symptoms'. *Ralstonia solanacearum*–plant interaction at the root level was first studied in tomato (Monteiro *et al*., 2012b; Vasse *et al*., 1995). The authors observed bacterial penetration via the root extremities and at the axils of the secondary root (Vasse *et al*., 1995). Ten years later, gnotobiotic experiments on petunia allowed the identification of new root lateral structures (RLSs) after the inoculation with *R. solanacearum* (Zolobowska and Van Gijsegem, 2006). These structures are T3SS dependent and were demonstrated to be highly efficient colonization sites. However, the involvement of specific T3Es in the formation of these RLSs has not been proven (Zolobowska and Van Gijsegem, 2006). In the *M. truncatula* gnotobiotic pathosystem, it has been described that *R. solanacearum* penetrates via the root tips (Turner *et al*., 2009). Bacterial inoculation leads to an arrest of root hair elongation and a reduction in root growth, associated with a browning and a loss of viability of the root tip epidermal cells (Turner *et al*., 2009). Two T3Es, GALA7 and AvrA, have been demonstrated to be partially involved in the induction of the root epidermal cell death phenotype (Turner *et al*., 2009). For the interaction involving *A. thaliana* and *R. solanacearum*, which has been well described and characterized at the whole plant level (Deslandes *et al*., 1998), an axenic plant bioassay has been developed recently to study the early steps of root colonization by *R. solanacearum* (Digonnet *et al*., 2012). The authors showed that the bacteria penetrate *A. thaliana* plantlets at the root apex. Bacteria induced a plasmolysis of epidermal and cortical root cells, accompanied by pectin degradation. The bacteria then move preferentially via intercellular spaces to invade the vascular cylinder directly (Digonnet *et al*., 2012). Contrary to the *M. truncatula* system (Vailleau *et al*., 2007), an important bacterial surface colonization can be seen alongside the inoculated plantlets. Thanks to these gnotobiotic systems, it has been shown that *R. solanacearum* disturbs normal root growth on host infection. Preferential zones of bacterial penetration are the root tips, correlating with the zone in which plant exudates are produced, attracting *R. solanacearum* (Yao and Allen, 2006). In the future, these systems will prove to be important to better characterize the early events of infection and the chronology of plant colonization.

PERSPECTIVES

In this short review, we have tried to encapsulate all the recent research on *R. solanacearum*, ranging from strain diversity to gene regulation, T3E biology and plant resistance. We would like to finish by emphasizing the fact that this plant pathogenic bacterium is a research model with interesting specificities, i.e. root infection, vascular colonization, large T3E repertoire and broad host range. We believe that further research will shed light on the specificities as well as the general infection strategies common between *R. solanacearum* and other parasites.

In this post-genomic era, the following challenges lie ahead: further strain sampling for a better description of the species complex; deciphering of the role of T3Es during infection; analysis of the *in planta* expression of bacterial genes (RNAseq); metabolic modelling towards a systems biology model; and experimental evolution to study adaptation to different plant hosts.

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