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Ralstonia solanacearum: An Arsenal of Virulence Strategies and Prospects for Resistance

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Abstract

The group of strains constituting the *Ralstonia solanacearum* species complex (RSSC) is a prominent model for the study of plant-pathogenic bacteria because of its impact on agriculture, owing to its wide host range, worldwide distribution, and long persistence in the environment. RSSC strains have led to numerous studies aimed at deciphering the molecular bases of virulence, and many biological functions and mechanisms have been described to contribute to host infection and pathogenesis. In this review, we put into perspective recent advances in our understanding of virulence in RSSC strains, both in terms of the inventory of functions that participate in this process and their evolutionary dynamics. We also present the different strategies that have been developed to combat these pathogenic strains through biological control, antimicrobial agents, plant genetics, or microbiota engineering.

1. INTRODUCTION

1.1. The *Ralstonia solanacearum* Species Complex

Among plant-pathogenic bacteria, *Ralstonia solanacearum* usually refers to a cosmopolitan set of phylogenetically related strains pathogenic to a wide range of plants, often referred to in the literature as the *Ralstonia solanacearum* species complex (RSSC). Because of their ability to infect multiple plant families, as well as their wide geographic distribution, strains of the RSSC cause several devastating bacterial diseases in agriculture worldwide (14). Moreover, the persistence of these bacteria in soils and aqueous environments (e.g., ponds, rivers) makes them particularly difficult to eradicate, and, finally, international trade has facilitated their spread, notably because of the latency period before the appearance of disease symptoms. Beyond the direct agricultural losses in major agronomic species (potato, tomato, and other solanaceous crops) or locally important species (banana, ginger, peanut, eucalyptus, etc.), the bacterium is also responsible for significant indirect costs (e.g., fallow contaminated plots, restriction of trade in potentially infected plants or tubers, prohibition of watering from contaminated waterways). Infections in nurseries of ornamental plant species (geranium or rose) have also been reported (123).

Depending on the host considered, there are several types of diseases caused by RSSC strains, such as bacterial wilt of solanaceous plants, black rot of potatoes, and the so-called Moko disease on banana (91). Most of the time, the infection occurs through the plant root and the bacteria have a strong tropism to reach the xylem vascular tissue where they multiply abundantly, causing an alteration of the water transport associated with the appearance of typical wilting symptoms (101). However, there are cases of transmission of some strains by insect vectors (55, 112), and natural plant wounds, as well as those caused by agricultural practices, are major entry points for the pathogen.

1.2. Comparative Genomics and Taxonomic Reclassifications

For a long time, the bacterium was referred to as *Pseudomonas solanacearum* and then in the mid-1990s, its name was changed to *Ralstonia solanacearum*. As mentioned above, this species is in fact a species complex that was divided into four phylotypes (i.e., main phylogenetic clades) reflecting taxonomic proximity and generally associated with the geographical origin of the strains (for a detailed presentation, see 91). Thus, phylotype I grouped mainly strains from Asia, phylotype II grouped strains from the Americas, phylotype III grouped strains from Africa, and phylotype IV grouped strains from Southeast Asia. In recent years, a better knowledge of the genome of the RSSC strains and their biodiversity has led to a taxonomic reclassification. Based on average nucleotide identity, it was proposed to formally divide the RSSC into three species: *R. solanacearum*, corresponding to phylotype II; *Ralstonia pseudosolanacearum*, corresponding to phylotypes I and III; and *Ralstonia syzygii*, corresponding to phylotype IV (111) (**Figure 1**). *R. syzygii* was further divided into three subspecies: subsp. *syzygii*, corresponding to phylotype IV; and subsp. *indonesiensis* and subsp. *celebesensis*, each corresponding to still restricted groups of strains with particular ecological characteristics (111, 112).

Considering that all these pathogenic strains of the RSSC share obvious similarities in many aspects of their life traits, it is debatable whether these subdivisions of the complex into distinct species are appropriate, but there are now consistent analyses with genomic metrics to justify it (118). Despite the increase in genomic data, with around 380 genomes publicly available in 2022, the number of high-quality complete sequenced genomes in the RSSC is still relatively small, resulting in obvious undersampling in some clades (e.g., phylotype IV), and it is therefore possible that the architecture of the current phylogeny may still evolve. There are correlations

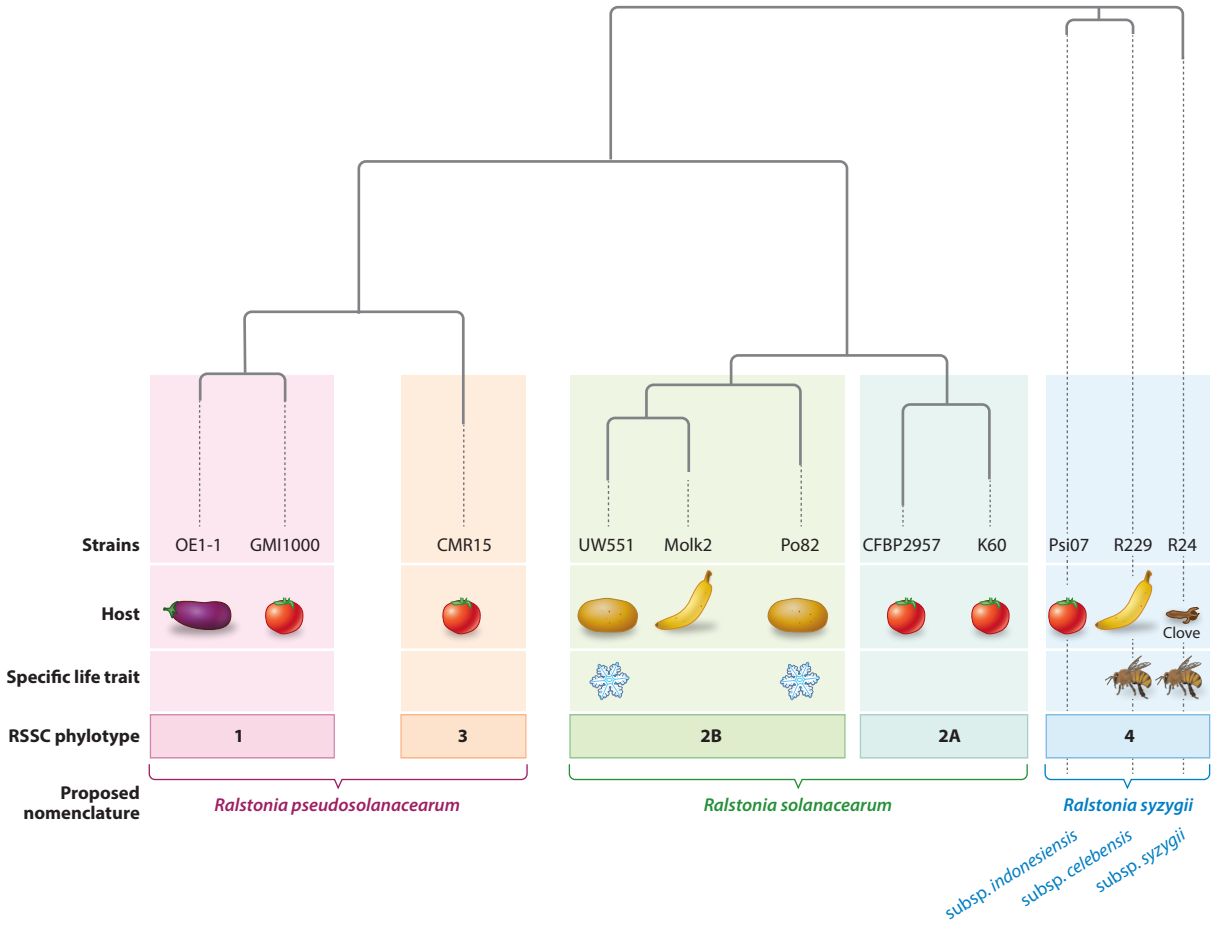

Figure 1

The *Ralstonia solanacearum* species complex (RSSC). Phylogenetic tree of the RSSC showing some representative reference strains, the hosts from which they have been isolated (host here refers to the original host the strain was isolated from, not necessarily the host range of the strain), and some specific traits (adaptation to cool temperatures and transmission by insect vectors). The former distinction into four phylotypes and the novel species nomenclature (111) are indicated. Host symbols: eggplant, tomato, potato, banana, and clove. Figure adapted from Reference 118 with permission of *Microbial Genomics*.

between clades and certain life traits (e.g., insect transmission, adaptation to cool temperatures, a tendency to specialize on certain hosts; see **Figure 1**), but no genes responsible for these traits have yet been identified by comparative genomics approaches, only candidates (3, 13, 120). One of the most interesting aspects of recent phylogenetic analyses concerns the evolutionary dynamics of these strains. It appears that phylotypes II and IV would be the most ancestral of the RSSC, whereas phylotype I, which has the lowest genetic diversity, is possibly the one that has most recently emerged and expanded (118).

2. HOST INVASION AND VIRULENCE: MECHANISMS

Since the beginning of molecular genetics, many studies have focused on identifying and characterizing genetic determinants contributing to the pathogenicity and virulence of *R. solanacearum*, with several model strains. For ease of reading, we do not distinguish between the three different

species in the remainder of this review, except when necessary, and stick with the generic term *R. solanacearum* to refer to the RSSC strains. We also refer to the term virulence as the degree of damage caused to a host by parasite infection, assumed to be negatively correlated with host fitness, whereas pathogenicity refers to the qualitative capacity of a parasite to infect and cause disease on a host (110).

2.1. Type III Secretion–Dependent Pathogenesis

From the multitude of reverse genetics studies performed to decipher the molecular basis of pathogenesis, it is clear that *R. solanacearum* virulence is multifactorial and that the type III secretion system (T3SS) is essential for pathogenicity, as the mutants defective for type III secretion are completely unable to cause disease on any host (36, 66, 93). The general protein secretion pathway (type II) is also important for infection but has been less characterized (36). Depending on the strain, 50–75 type III effector (T3E) proteins transit the T3SS and are translocated into plant cells (66, 73, 109). The fine orchestration allowing the translocation of such a large number of effectors in *R. solanacearum* is still poorly understood but several chaperones (or secretion helper proteins) with a key role in this process have been characterized (71, 72), allowing differentiation between early and late secretion T3E (73).

Over the past two decades, numerous studies have focused on the functional analysis of *R. solanacearum* T3Es and the characterization of their plant targets (for a review, see 66). For now, the majority of characterized functions fall primarily under the roles of suppressing plant defense mechanisms, although recent reports reveal other possibilities (such as altering plant metabolism to benefit the pathogen) (135, 137). Enzymatic functions of some T3Es have been established (e.g., acetyltransferase, proteases, ubiquitin ligases), and for many of them, repertoires of plant proteome interactors have been established (40). So far, the PopP2 effector is the best characterized at the molecular level, both in terms of its interaction with its virulence targets and its recognition by the plant immune system (68, 115, 136). However, although sophisticated mechanisms of disarming plant defense pathways are being uncovered, a comprehensive view of type-III-dependent pathogenesis is still lacking, including the relative contribution of these effectors in disease. Several effectors are reported to contribute significantly to pathogenicity (66), but no T3E mutant results in an avirulent phenotype comparable to a T3SS-defective mutant, demonstrating a clear functional overlap among T3Es. The only known exceptions to this behavior are effectors that enable the enlargement of the host range (6) or mutations in T3Es that allow the pathogen to escape recognition by the plant's immune system and thus restore pathogenicity (e.g., see 82, 83, 88). Furthermore, the same effector can have multiple functions, which may differ depending on the host. Hence, the effector RipAB has been shown to downregulate the calcium signaling pathways in potato (151) and inhibit the action of TGA transcription factors in *Arabidopsis* (107). Thus, there is also probable redundancy at the level of plant pathways/functions targeted by T3Es, making it difficult to have a clear view of the events that conclusively lead to the susceptibility of the plant to the pathogen.

2.2. Infection Process and Parasitic Fitness In Planta

The transitional steps between the free-living *R. solanacearum* in the soil and its initial interaction with plant roots are still poorly understood. However, several specific mechanisms of the infectious phase have been documented, of which we summarize the main aspects below.

2.2.1. Root invasion. Plant colonization begins with the attachment of *R. solanacearum* on roots, prior to entry at sites of lateral root emergence or elongation (16). Bacteria depend on chemotaxis

to locate and infect plant hosts (143), particularly through the perception of amino acids and malate (51). These compounds are present in root exudates and the inability to perceive these signals greatly reduces infection (51). It should be noted that the genome of *R. solanacearum* has an average of 20 cell membrane–associated receptors, called methyl-accepting chemotaxis proteins, the role of which in the infectious process is not yet characterized. The flagellum-driven cell motility is required for the pathogen to move toward the plant roots, whereas both type-IV pili and flagella are crucial for optimal plant colonization and disease development (21).

The study of the early infectious process benefited from in vitro plant inoculation systems as well as transcriptomic analyses of infected root tissues. Several studies showed that bacteria trigger developmental abnormalities in the roots of infected hosts, some of which may benefit bacterial invasion of plant tissues (reviewed in 138). Some of these morphological alterations appear to be mediated by auxin, which seems to play a key role locally in the balance between susceptibility and resistance to the pathogen (35, 138, 149), but the mechanisms involved are not yet clear.

2.2.2. Biofilm formation and resistance to stress in planta. Once in the plant, the pathogen faces hostile environmental conditions, with the establishment of plant defense reactions. This must be indeed critical in the early stages of infection when the bacterial population is still reduced. Nevertheless, bacteria have an arsenal of enzymes to effectively reduce these defense mechanisms. Thus, exposure to reactive oxygen species (ROS) that accumulate in the apoplast as part of the primary plant defense response is counteracted by the production of multiple ROS-scavenging molecules (1, 124). *R. solanacearum* can degrade plant phenolic defense compounds such as hydroxycinnamic acid to facilitate the early stages of infection (74). The pathogens also possess an enzyme capable of degrading another antimicrobial compound, salicylic acid, which also serves as an activator of defense responses (76). Similarly, bacterial detoxification of nitric oxide was proposed to reduce the host plant's ability to perceive the presence of the pathogen (128).

A more general way for bacteria to protect themselves under stressful conditions is through the formation of biofilms (77). *R. solanacearum* was shown to form biofilms on tomato leaf mesophyll cells (85) and xylem vessels (127). As in other bacteria, several secreted molecules and surface determinants [e.g., exopolysaccharide (EPS), extracellular DNA, pili structures, lectins] contribute to the process of cell aggregation and biofilm formation (84, 85, 127). Interestingly, *R. solanacearum* also produces extracellular nucleases to facilitate its dispersal from biofilm structures. The effect of these nucleases was also observed on the extracellular matrix containing polysaccharides and DNA that are produced at the root tips of plants to trap bacteria. The secretion of DNases to degrade such extracellular traps was shown to facilitate infection (126).

2.2.3. Other determinants of parasitic fitness in planta. The implementation of the -omics methodologies has contributed to the identification of more determinants required for bacterial colonization of plant tissues. First, the realization of transcriptomes from bacteria isolated during infection has allowed the identification of genes whose expression is specifically induced under this condition (24, 56). This led to the discovery of the major role of nitrate respiration during growth in the xylem (22) or the protection against osmotic stress encountered during infection through trehalose synthesis (78), both bacterial traits strongly induced in planta. This also demonstrated that T3SS expression is observed even under late infection conditions (24, 56), which led to evidence of complex gene regulation patterns that coordinate T3SS expression with other virulence determinants (94). Other methods such as TnSeq (transposon mutant sequencing) have recently been developed in RSSC strains and have revealed fitness factors that promote growth of bacteria in planta, more precisely in the xylem sap (37, 121). These studies have shown the importance of several classes of genes such as those involved in cell wall and membrane biogenesis as well as amino acid and lipopolysaccharide biosynthesis, but the characterization of many interesting candidates remains to be done.

2.3. Nutritional Virulence

It is now well established for many pathogens that the ability to assimilate nutrients during host infection is crucial for pathogenesis, and the term nutritional virulence is used to emphasize that resource uptake during infection is integral to the virulence program. Several observations highlighted the metabolic adaptation of the pathogen to the environment encountered in its host; for example, there are redundancies of metabolic pathways, allowing the pathogen to activate the specific expression of some of these pathways by virulence regulators to adapt to the absence of cofactors of enzymatic reactions absent in the plant (97, 102). Second, it has been reported that the T3E RipI interacts with plant glutamate decarboxylases to alter plant metabolism and support bacterial growth (137), thus revealing a link between a T3E and the directed biosynthesis of nutrients by the pathogen once inside its host. Third, it became increasingly clear that the metabolic potential of *R. solanacearum* is controlled by key virulence regulators and that this regulation plays a crucial role in the plant, probably at several stages of the infection process (47, 61, 94, 96, 98, 144).

Concerning nutrient utilization in planta, particular attention was paid to changes in the xylem sap of infected tomato plants during *R. solanacearum* growth (39, 75). The knowledge of the complete metabolic network of the bacterium also allowed the investigation of which sources of carbon and nitrogen are preferentially used (11, 98). At the quantitative level, it is essentially amino acids (notably glutamine), and not sugars, that support the strong growth of *R. solanacearum* observed in vascular tissues (39). Contrary to an often-accepted view that xylem is poor in nutrients, the upward flow of sap maintains sufficient carbon substrate to sustain high bacterial multiplication (11). Interestingly, several metabolites appear to be enriched in xylem sap from diseased plants (75). This is the case of putrescine, a molecule abundantly produced by *R. solanacearum* (39, 75, 98), which probably plays different roles not yet clarified during the interaction with the plant (38).

2.4. Regulation of Virulence

Virulence of *R. solanacearum* is governed by a complex regulatory network comprising multiple transcriptional regulators and responding to different environmental signals (36). We focus hereafter on PhcA, which is a central regulator of this network.

2.4.1. Regulation of virulence is dependent on the Phc quorum-sensing system. *R. solanacearum* has several systems of production/perception of autoinducing chemical signals to coordinate diverse cooperative activities: Two of them rely on homoserine lactone derivatives (36, 140), one relies on anthranilic acid (119), and one is activated by either methyl 3-hydroxymyristate (3-OH MAME) or methyl 3-hydroxypalmitate (3-OH PAME) (52, 58, 77, 129). The latter, called the Phc system, controls the expression of a large majority of virulence traits through the master transcriptional regulator PhcA (36). Mutations in *phcA* result in a phenotypic conversion (a phenotype used to coin the name) characterized by hypermotility and a deficiency in producing EPSs, among multiple other alterations. This state of PhcA inactivation actually defines the stage of low (or unconfined) population bacteria. At higher densities, activation of PhcA leads to the induction of multiple virulence factors (36, 52, 93). In fact, the transcriptomic profiling of a *phcA* mutant showed that the expression of more than a thousand genes is affected by this regulator (61, 86, 94), so obviously much more than the virulence genes stricto sensu.

More than 40 regulatory genes controlling the expression of virulence factors or other important bacterial traits have been characterized in *R. solanacearum* (36, 97), and most of them are, directly or not, connected to or dependent on PhcA, resulting in an intricate network that is difficult to untangle genetically (97). The activation of the Phc system itself is also complex, subject to feedback from multiple signals, including surface molecules such as lectin or EPSs (50, 86).

2.4.2. PhcA and the pathogen's growth/virulence dilemma. Beyond the need to extract nutritional resources during plant infection, plant pathogens face a resource allocation dilemma: They must use these resources to grow and produce virulence functions essential for infection. Metabolic flux analyses have highlighted that the cost for the production of virulence factors strongly impacts bacterial growth and has major consequences in terms of metabolic restriction under specific environmental conditions (98). It was shown that this growth/virulence trade-off is orchestrated by the regulatory protein PhcA and thus in response to the 3-OH MAME/PAME quorum-sensing signals. A *phcA* mutant, which is almost avirulent, has indeed a much higher metabolic potential and multiplication rate than the wild type (61, 98). This may be due to the fact that PhcA exerts broad catabolic repression toward several types of carbon substrates, but the cascade of regulatory events leading to the strong growth gain observed in the mutant remains to be elucidated.

3. EVOLUTIONARY HISTORY OF VIRULENCE FACTORS

3.1. Genome-Wide Comparisons and Genes Under Selection

The increasing availability of high-quality RSSC strain genomes has allowed the refinement of genomic comparisons and the ability to draw hypotheses about the evolution of pathogenicity determinants or their association with specific strain phenotypes. One of the first conclusions from these comparisons is the remarkable conservation of major genes contributing to pathogenicity in all RSSC strains. This is also true for the virulence regulatory genes and the PhcA network, sometimes with high rates of identity conservation (95). There are few notable exceptions such as the repertoires of secreted proteins (e.g., through T3SS or type VI secretion systems), which are known to be relatively variable between bacterial strains (109), or certain metabolic functions, e.g., the denitrification pathway, which is complete only in *R. pseudosolanacearum* and not in other RSSC species (22). Some other cases are more specific and are not simply based on the presence/absence of gene(s) but on allelic differences that could result from evolutionary divergences between phylogenetic clades or from selection constraints linked to particular life traits. One example concerns proteins of the RipF family that form the T3SS translocation pore, which, unlike the other highly conserved T3SS components, have had a particular evolutionary history specific to each RSSC phylotype, without yet knowing their biological implications (92). Another example is the finding that RSSC strains selectively employ 3-OH MAME or 3-OH PAME as their Phc quorum-sensing signal. Intriguingly, this 3-OH MAME/3-OH PAME selectivity cannot be correlated to phylogeny, geographic origins, or host range of the strains (58). Such selectivity of quorum-sensing signals among different species of the RSSC probably has an ancient origin, but, here again, the reason behind that remains enigmatic.

Studies have sought to determine which genes of *R. solanacearum* are subject to selection by calculating each gene's Tajima's D, a population genetics test statistic that identifies genes that do not evolve neutrally (17, 32). On a genome-wide scale, 50–90 genes have been identified as evolving under non-neutral selection, including genes that are likely to be under selection by the plant immune system, encoding either T3Es (17, 92, 104) or conserved proteins under selection to escape recognition by plant pattern recognition receptors (32). As expected, among the genes subject to strong selection are the T3SS translocator RipF proteins and those encoding for Phc quorum-sensing synthases and receptors (17). In addition, it has been shown that recent recombination events affected contact-dependent inhibition loci, whose products mediate self-/nonself-recognition, suggesting that microbial competition plays a significant role in *R. solanacearum* evolution (104). To date, these evolutionary analyses should be completed because the populations analyzed are not yet representative of the biodiversity of the RSSC. Most

certainly, the coevolution of RSSC strains with the diversity of phages that infect them is also a major factor shaping the population dynamics in the species complex (42).

A recurrent question is whether the host range of *R. solanacearum* has structured the virulence factor content and especially its T3E repertoire. It is clear that variations in the T3E repertoire can have an influence on the type of host infected (6, 66). It is difficult, however, to draw robust correlations between the ability to infect a given host and the presence of specific genes explaining this property, and none of the candidates identified has yet been functionally validated (3).

3.2. Selection of Adaptive Mutations In Planta

Beyond the classical approaches of reverse genetics, another way to better understand the mechanisms that shape the increase and decrease of virulence has been achieved by seeking to identify adaptive mutations under infection conditions. In the case of pathogens, studies of the adaptation of bacteria to their hosts mainly involved serial passage experiments, a form of experimental evolution in which an ancestral individual is transferred from one host to another under defined conditions to select for evolved populations, which have a better fitness in the host than the ancestor (80).

An evolution experiment of *R. solanacearum* was conducted using a single ancestor clone propagated for approximately 350 generations on eight different plant species, including susceptible and resistant host species (45). The selection scheme applied in this experiment consisted of inoculating the pathogen into the plant vascular system and then propagating the populations recovered from xylem sap beyond the infection zone. In planta competition analyses of evolved clones with their ancestor showed that the pathogen can increase its fitness on both susceptible and tolerant hosts, although the magnitude of the adaptive process appears variable depending on the host. This has led to the identification of genes in which mutations providing adaptive gains have occurred, such as the global regulator *efpR*, whose function appears to be somewhat related to *phcA*, although the functional links between the two are not clear (95, 96). Several of these genes under selection appear to be related to improved adaptation to the stress conditions that bacteria encounter in the plant. Moreover, significant shifts in gene expression were observed in evolved clones and associated with deregulation or mutation of several transcriptional regulators, which suggests a rewiring of the regulatory network, resulting in novel gene expression patterns (41). It should be kept in mind that this evolutionary experiment selected for better growth phenotypes in the xylem, which represents only one part of the complete life cycle of *R. solanacearum*. Bacteria must encounter specific constraints in the soil or early stages of infection, such as competition with other bacteria (104, 134), and the selection imposed upon entry into the plant (i.e., the size of the founding population of the infection) is bound to have a major impact on the structuring and evolution of the pathogen populations.

4. WHAT PROGRESS HAS BEEN MADE IN THE SEARCH FOR RESISTANCE STRATEGIES?

The great diversity of strains in the RSSC and variety of virulence factors and T3E repertoires, as well as the long persistence of *R. solanacearum* in the environment, contribute to the difficulties in developing effective control strategies. Research using chemical methods (pesticides and nonpesticides), physical treatments (solarization, biofumigation), and cultural practices (crop rotation, multi-crops) has previously been described (34, 147), but these methods have been less extensively studied this past decade. Biological control is now more commonly developed and studied, with a wide variety of plant-derived natural products and biological control agents (BCAs) (5, 147). Work on the impact of existing control methods on the diversity and composition of soil microbial

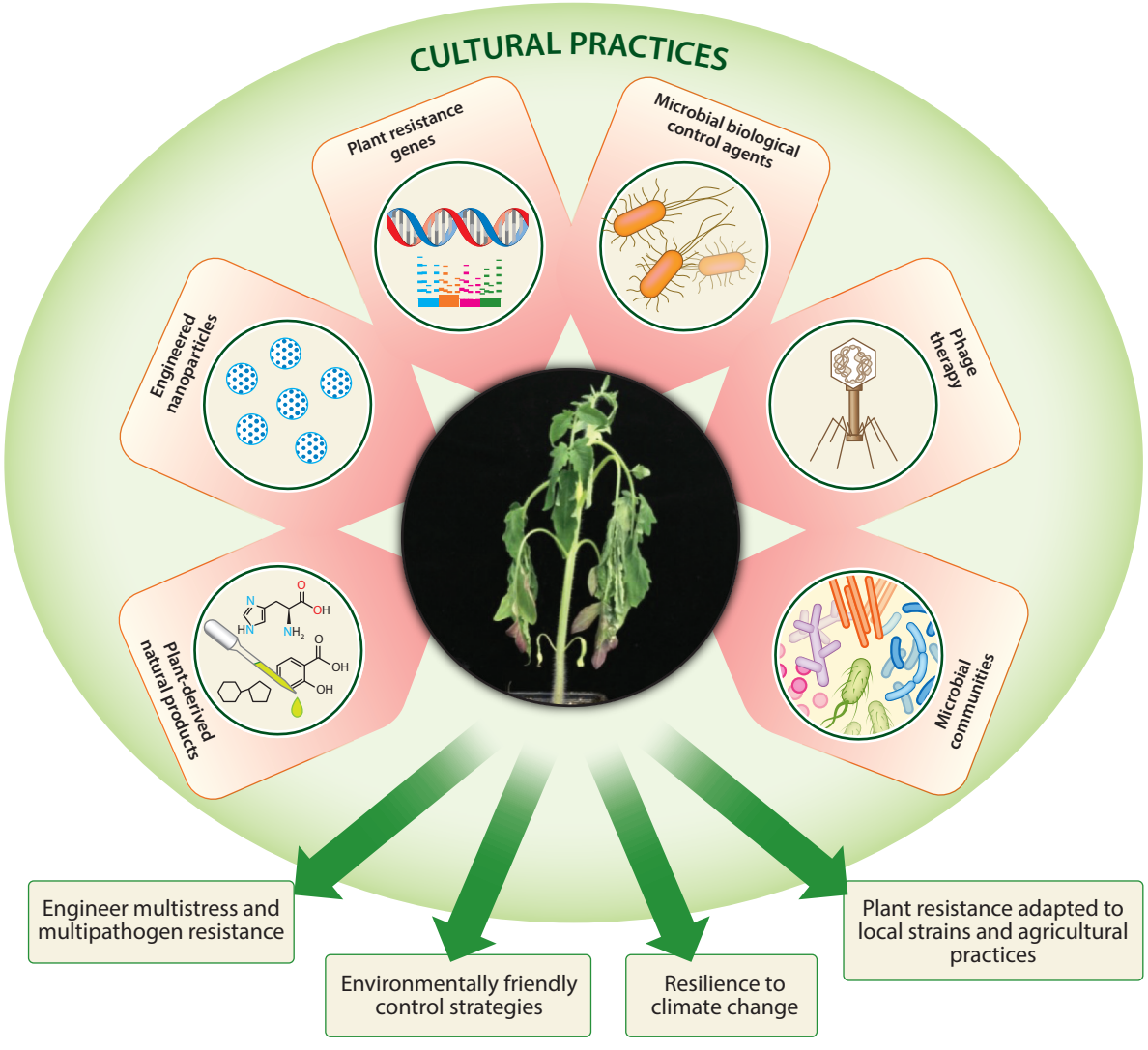

Figure 2

Control strategies for bacterial wilt and future challenges for sustainable resistance. Many methods are being developed and need to be combined and integrated with cultural practices (*in the green circle*). These strategies must be designed from a sustainable disease management perspective (*green arrows*).

communities, or on the role of the microbiota itself in disease control, has developed significantly. Control strategies for bacterial wilt and future challenges for sustainable resistance summarized in **Figure 2** are detailed below.

4.1. Plant-Derived Natural Products

A good understanding of the conditions that promote or accompany disease establishment can be useful in developing practical strategies for bacterial wilt management. For example, following the finding that xylem sap from *R. solanacearum*–infected tomato is rich in trehalose, the application

of exogenous trehalose was shown to reduce bacterial wilt disease and trigger systemic disease resistance, possibly through a damage-associated molecular pattern response pathway (78, 79). Phenolic caffeic acid, whose secretion is increased in tobacco root exudates after *R. solanacearum* inoculation, was applied exogenously in pot and field experiments, resulting in reduced disease symptoms (70). Several other plant-derived natural products, such as coumarins, have also been evaluated positively for their antibacterial activity (141). The application of 7-methoxycoumarin mitigated tobacco bacterial wilt in pot experiments (48). Salicylic acid inhibits *R. solanacearum* growth and represses several bacterial wilt virulence factors (76). Another interesting and alternative option is to carry out a targeted strategy to fight the pathogen by finding ways to block the T3SS, a major pathogenicity determinant of *R. solanacearum*. Thus, targeted screening for type III secretion inhibitors was performed and identified salicylidene acylhydrazides as inhibitors of T3SS expression, and their effect resulted in limited growth of *R. solanacearum* in planta (105). Among natural substances of vegetal origin, essential oils, produced by aromatic plants, contain a wide range of volatile molecules, and effects have been studied against many phytopathogenic fungi, oomycetes, and bacteria as well as weeds (108). Essential oils have been evaluated for their protective effects against *R. solanacearum* for more than 20 years (25, 103). For most of these plant-derived molecules, the study of their mode of action in natural conditions in the field remains to be done. Similarly, research on facilitated formulation and application methods, as well as on their potential environmental toxicity, must be continued to allow their use as green pesticides (108).

4.2. Engineered Nanoparticles

Metal-based nanoparticles (NPs) have been studied for their roles as potent antimicrobial agents, such as silver (Ag-NPs), copper oxide (CuO-NPs), iron oxide (FeO-NPs), zinc oxide (ZnO-NPs), magnesium oxide (MgO-NPs), and silicon oxide (SiO_2-NPs) (33, 125, 131). Previously synthesized with chemical and physical methods, the search for eco-friendly alternatives led to the development of biogenic (or biological) synthesis with plant derivatives, bacteria, yeasts, fungi, macro- and microalgae, and waste materials (125). Although several works evaluated antibacterial activity of NPs in vitro, some recent studies investigated NP-mediated bacterial wilt resistance in planta. Hence, silica NPs were shown to enter tomato leaves through stomatal pores and accumulated mainly in tomato shoots, limiting wilt disease in a dose-dependent manner (131). Another work studied the in vivo antibacterial effect of several metal oxide NPs (CuO, FeO, and ZnO) against *R. solanacearum* and their impacts on the soil bacterial communities (57). The impact on bacterial wilt was variable, as CuO-NPs significantly reduced tomato wilt disease, whereas this was not the case for FeO-NP and ZnO-NP applications. NPs were also positively or negatively affecting the abundance of certain bacterial species in the rhizospheric soil and thus altered the bacterial community structure (57). ZnO-NPs synthesized via flower extract of *Matricaria chamomilla* significantly reduced tomato bacterial wilt (60). Additionally, these studies, as with most similar works, showed that NP treatments had a positive role in plant growth promotion (57, 60, 131). Nonetheless, effects observed are for the moment variable and dose and host dependent, and the environmental impact of NPs must still be better evaluated (33).

Chitosan [α-(1–4)-2-amino-2-deoxy-β-D-glucan], the second most abundant biopolymer in nature after cellulose, is a deacetylated form of chitin, and interest in it has increased due to its broad-spectrum antimicrobial activity (113). NPs of chitosan and a derivative, N-(benzyl) chitosan, have been tested against several microbes, including *R. solanacearum*, and have demonstrated antimicrobial action (9). These nontoxic biopolymers could be interesting alternatives for biocontrol strategies, through the use of NPs or directly, as shown by a recent study that successfully

evaluated the action of chitin and chitosan extracted from black soldier fly pupal exuviae against tomato bacterial wilt (59).

4.3. Biological Control Agents

A plethora of BCAs, consisting mainly of bacteria but also some fungi, have been evaluated to limit the development of bacterial wilt over the past decade. These beneficial microorganisms have been mainly isolated from the rhizosphere of healthy plants and have biocontrol properties through various interactions such as competition for nutrients and space, antibiosis, and parasitism or by inducing plant resistance (117, 147). The different modes of action of microbial BCAs (MBCAs) with their associated specificity and risks were recently reviewed (62). To date, the behavior of around 150 bacterial strains has been evaluated in the presence of *R. solanacearum*, either in vitro for their antibiosis activity or in planta, with studies reporting a role in disease mitigation, often associated with a plant growth promotion effect (117, 147). Among all this diversity of bacteria, *Pseudomonas* spp. (mainly *Pseudomonas fluorescens* and *Pseudomonas putida*) and *Bacillus* spp. (mainly *Bacillus cereus*, *Bacillus subtilis*, and *Bacillus amyloliquefaciens*) were reported to be the most effective for bacterial wilt control (19). This is due to their ability to limit pathogen multiplication by antagonism with the production of broad-spectrum antimicrobial compounds, through niche competition and induced systemic resistance (ISR) (81). ISR refers to the development of the self-protective capacity of the plant against a broad spectrum of pathogens after appropriate priming with nonpathogenic organisms, particularly with saprophytic rhizosphere bacteria and fungi (122). This mechanism was studied by quantitative analysis of defense-related proteins, which allows the detection of peroxidase and polyphenol oxidase isoform patterns after treatment of tomato plants inoculated with *R. solanacearum* in the presence of *P. fluorescens* strain VSMKU3054 (122). To address the important question of the effect of the BCA genotype, in vitro and in vivo screening were performed with eight *Pseudomonas* strains against *R. solanacearum*. This work showed that the antimicrobial effects were specific to the *Pseudomonas* strain used, highlighting one *Pseudomonas protegens* strain as the most effective, with protection dependent on the *R. solanacearum* strain tested in vivo (20). Interesting findings from the evaluation of MBCAs include their ability to exhibit a variety of complementary effects in controlling disease development. This is the case for *B. amyloliquefaciens*, which secretes hydrolytic enzymes, EPSs, and antimicrobial compounds and strongly suppresses the incidence of the disease in tobacco plants by reducing the *R. solanacearum* population dynamic in the soil while reshaping the rhizosphere microbiome (2). EPS leads to the formation of a bacterial biofilm, which helps the bacteria successfully colonize the host, contributing to the establishment of a protective environment synergistically with inhibition of *R. solanacearum* growth via the secretion of antimicrobial compounds (i.e., lipopeptides and polyketides) (2). A last example of a bacterial genus largely described for its biocontrol properties is *Streptomyces*, which are Gram-positive filamentous bacteria from the actinomycetes family (90). Several strains of *Streptomyces*, such as the AN090126 strain, have been studied for their role in reducing bacterial wilt, but also for their pleiotropic effects, acting against several other pathogens, including fungal pathogens, through the production of secondary antimicrobial metabolites and various volatile organic compounds, leading to broad-spectrum antimicrobial activities (67).

4.4. Phage Therapy

Bacteriophages are specific viruses of bacteria that subvert the metabolism of their hosts to replicate (15). Many of them have a host range restricted to one or a few related bacterial species, which makes them BCAs with limited environmental risks. This has motivated the

intensification of research on bacteriophage-based treatments with the publication of patents, but field experiments are still rare and have not yet led to commercial products (5). To date, around 15 phages, with either lytic or lysogenic infection cycles, have been evaluated in in planta assays. Lytic phages, which do not integrate into the bacterial genome, are more efficient at destroying bacteria (5, 53). Experiments were conducted in both the greenhouse and the field to evaluate the effects of phage therapy in the rhizosphere of the tomato plant in the presence of *R. solanacearum*. Phages were most effective when applied in combination, specifically infecting *R. solanacearum*, and the phage combinations tested had no significant effect on the composition and diversity of the rhizosphere microbiome (132). The use of bacteriophage cocktails to treat plants or seeds appears to be a promising alternative and should be increased under field conditions, but the evolution of phage resistance to RSSC defense systems and their potential impact on the rhizosphere microbiota need to be further studied to promote these precision biocontrol tools (18, 42, 132).

4.5. Control of the Microbial Community Composition

It is now widely accepted that collective plant-associated microbes, known as plant microbiota, play an important role in plant growth and health (12, 87). Studies aimed at deciphering how microbial communities drive plant resistance to bacterial wilt have greatly increased in recent years. A key example is a tomato study that showed that transplantation of rhizosphere microbiota from resistant plants ('Hawaii 7996' line) reduced disease symptoms after inoculation of *R. solanacearum* on susceptible plants (Moneymaker line) (64). Comparative rhizosphere metagenome analyses demonstrated that flavobacteria were abundant in the rhizosphere microbiome of resistant plants, and a specific isolate was shown to reduce bacterial wilt symptoms in susceptible tomato plants inoculated with *R. solanacearum* (64).

Several other studies on the tomato–*R. solanacearum* pathosystem have highlighted major findings related to the specificities of root-associated bacterial communities that could limit bacterial wilt:

- Taking into account the distribution of trophic links by direct engineering of competitive interaction networks for resources of plant-associated bacterial communities could both limit the pathogen multiplication through optimized competition and promote positive interactions of native bacterial communities (134).
- Competition for iron via secreted siderophore molecules seems to be a good predictor of microbe–pathogen interactions and plant protection, via the selection of bacteria with siderophore-inhibitory effects on the pathogen (43).
- Microbiota manipulation following long-term bio-organic fertilization leads to disease suppression induced by changes in the composition and functionality of rhizosphere bacterial communities (29).
- Precision agriculture must be favored by searching for specific protective microbes enriched in the microbiota of healthy soil (146).
- Changes in the composition of the rhizosphere microbiota could predict disease outcome before the onset of symptoms, allowing the identification of new BCAs (44). It has also been shown that reducing soil acidification to a slightly acidic level (pH 6.0–6.5) appears to mitigate bacterial wilt of tobacco and enrich the rhizosphere with potentially beneficial bacteria (148). A better understanding of the complex plant–*R. solanacearum*–microbiota interactions, including consideration of soil properties and plant genotype, is certainly a promising target for seeking innovative strategies to promote soil suppressiveness.

4.6. What About the Plant Resistance Genes?

The search for plant resistance genes to bacterial wilt historically started with classical genetic linkage mapping, highlighting quantitative sources of resistance in several crops, mainly in solanaceous plants. Studies in model plants have also allowed the proposal of alternative strategies, both through a better knowledge of gene-for-gene resistance and through new and global approaches, such as using plant natural diversity.

4.6.1. Quantitative sources of resistance. Use of resistant cultivars can be considered the most economical and sustainable method for disease control, but truly effective cultivars are still lacking (54, 147). The genetic analyses of resistances to bacterial wilt showed they were mainly polygenic and strain specific (54, 93). Quantitative trait loci (QTLs) identified in model and cultivated plants were reviewed by Peeters et al. (93). Since then, new quantitative resistance mechanisms have been identified in potato (46), pepper (31, 69), peanut (106, 150), and tomato (10) and more deeply characterized in eggplant (114). In potato, five QTLs were identified in response to *R. solanacearum* strain MAFF327001 using genome-wide markers (46). In pepper, numerous QTLs were identified in response to three different isolates of *R. solanacearum* (69), including a cluster of resistance (*R*) genes and three defense-related genes (31). In peanut, bulk segregant analysis combined with QTL mapping using F2 and F8 populations led to the discovery of the *qBW-1*, an interesting candidate QTL associated with a disease-resistance gene (150). Another recent study on peanut using 521 recombinant inbred populations identified one major QTL, *qBWA12*, underlying nine *R* genes (106). For all these studies, further research is needed to formally validate their contribution to bacterial wilt control. Recent work on tomato, using whole-genome sequence data analysis, suggested that 'Hawaii 7996,' a cultivated tomato line with one of the most stable resistances, carried additional loci contributing to bacterial wilt resistance, in addition to the major historical QTLs *Bwr-6* and *Bwr-12* previously characterized (10). Another major difficulty to consider is that most of the identified QTLs are strain specific, and thus pyramiding of these quantitative traits must be considered to achieve durable, broad-spectrum resistance (100, 114).

4.6.2. Exploiting gene-for-gene resistance. In parallel to classical genetics, several biotechnological approaches have been deployed, including using the well-described *Arabidopsis thaliana* immune receptor EFR (ELONGATION FACTOR-THERMO UNSTABLE RECEPTOR). The transgenic expression of *EFR* in other plant species, e.g., in crops but also the model plant legume *Medicago truncatula*, conferred quantitative resistance to several bacterial pathogens, including *R. solanacearum* (65, 99). This technology was used for the first time in field trials in Florida (USA), with significantly reduced incidence of tomato bacterial wilt (63). As for EFR, the same approach to generate resistance against bacterial wilt was proposed using the SICORE receptor, which recognizes the csp22^{Rsol}, an *R. solanacearum* elicitor peptide (133). Another option to circumvent the current limitations of transgenic crops is to deploy the cultivation of plants grafted with new sources of resistance. This has been evaluated in particular with grafted tomato with resistant rootstocks (117).

How to identify new and sustainable sources of resistance? One possibility is to look for these sources in naturally asymptomatic plant varieties, as has been done with bittersweet (*Solanum dulcamara*) or other wild, resistant Solanaceae (82, 116). Extensive work has also been done on the model plant *A. thaliana*, leading to a deep and fine characterization of the *RRS1* locus. First described in 1998, studies conducted by several research teams allowed the functional validation of *RRS1*, followed by studies on the nucleotide-binding domain and leucine-rich repeat receptor pair RRS1/RPS4, which confers resistance to different pathogens and specifically recognizes the T3E PopP2 in *R. solanacearum* (68, 115). As for *EFR*, the *A. thaliana RRS1* and *RPS4* genes were

successfully transferred into a crop, *Brassica rapa* var. *perviridis*, in which they conferred effective resistance to bacterial wilt (89). The *RRS1-R* story illustrates how the understanding of molecular mechanisms of immunity can be potentially transferred to applied research, although there is a risk that this type of gene-for-gene resistance will be quickly bypassed by the pathogen.

4.6.3. Genome-wide association mapping. More recently, genome-wide association (GWA) mapping, an alternative and complementary strategy based on the exploitation of natural genetic variation in plant–pathogen interactions, has emerged with the development of next-generation sequencing technologies and statistical methods in quantitative genetics (28). Genome-wide association studies (GWAS) have allowed the identification of numerous QTLs associated with *R. solanacearum* infection using the GMI1000 wild-type strain or derivative mutants in key virulence factors (4, 7, 8, 26, 27). First, these works confirmed that the *RPS4/RRS1-R* locus, previously identified with traditional linkage mapping, was the main QTL of resistance detected with GMI1000 (8). GWA is therefore a powerful method that accelerates the fine mapping of polygenic resistance associated with bacterial wilt. Despite the complexity of the responses that take place after *R. solanacearum* inoculation, these GWAS studies also allowed the identification of many candidate genes, followed by the functional validation of those that were shown to act in either resistance or susceptibility to bacterial wilt (7, 8, 26, 27). So GWA appears as a stepping stone to highlight new candidate genes, both in the search for new resistance genes and the identification of susceptibility genes, which have previously demonstrated their potential to be used in resistance breeding (49, 130). GWA mapping has also worked well with crops (28), so it could be particularly relevant to solanaceous plants attacked by *R. solanacearum*. This is true in a global warming context and should be implemented by studying interactions under different environmental conditions (7, 8, 30), using either wild-type strains, taking into account the epidemiology and local genetic diversity (139), or mutant strains to develop specific directed approaches (26, 27). This could be an interesting alternative in the future, as public acceptance is still needed before commercial use of genetically modified crops. Combining GWAS with traditional linkage mapping could be an attractive solution to improve the choice of candidate genes for breeders.

4.6.4. Global approaches in the context of global warming. The search for new sources of resistance must now involve global approaches that take into account the plant's response to combined stresses, and this is even more true in the context of climate change. Beyond pathogen attack, plants have to face many abiotic stresses, among which temperature is one of the main factors affecting resistance to pathogens. It is now clearly demonstrated that most of the identified resistances are altered by high temperature (7, 8, 30, 54, 145). Numerous comparative transcriptomic and metabolomic analyses have been performed on the plant–*R. solanacearum* interaction with different host plants (138). Many plant transcriptional factors have been studied for their involvement in the regulation of immunity to *R. solanacearum*. For example, the pepper WRKY transcription factor CaWRKY40 is involved in both resistance to *R. solanacearum* and tolerance to high temperature and high humidity (23, 142). A major challenge in the search for effective control methods is the many biotic (e.g., plant genotype, natural microbial communities) and abiotic (e.g., environment, soil properties, agricultural practices) factors that can influence disease establishment.

5. CONCLUSIONS AND VISIONS FOR THE FUTURE

Twenty years have passed since the sequencing of the first *R. solanacearum* genome. Today, *R. solanacearum* is no longer studied as a single entity but as a complex of species, and the molecular bases for speciation and/or acquisition of specific properties by certain strains should now,

with the densification of genomic data, be more easily identified (36, 118). However, we still know little about the structuring of RSSC natural populations or their dynamics in the field, either at the level of plots or territories (139). In relation to this great diversity of the RSSC, many studies have also tried to elucidate the mechanisms related to host specificity, but no overall explanation is yet available beyond the presence/absence of specific T3Es in specific strains. It remains to be determined whether there are polygenic factors, other than T3Es, conditioning the host range, as is conceivable. This knowledge will surely help to develop targeted and combined strategies to control bacterial wilt.

In parallel, a better understanding of the disease cycle and host plant targets have been powerful levers to propose new resistance strategies or evaluate the limits of the existing ones (**Figure 2**). To overcome the limitations of each method, an integrated, multifaceted disease control strategy should be implemented whenever possible by developing precision agriculture at the local scale with a strong consideration of environmental conditions. In this context, based on the many advances made in the past decade, a list of parameters can be integrated: (*a*) the geographical scale, the physico-chemical properties of the soil, and the crop and its genotype; (*b*) the combination of quantitative resistances using thermotolerant sources of resistance when possible; (*c*) the selection of BCAs with the broadest spectrum, by using either cocktails of BCAs with the best combination or a single BCA as a precision tool to target the pathogen; (*d*) all these methods must preserve the local microbiota network; and (*e*) the development of these sustainable and environmentally friendly methods must be accessible to farmers. Thus, many possibilities already exist to better control bacterial wilt. The future lies in an amplified link between basic research and fieldwork for a personalized agriculture leading to locally adapted solutions targeted at indigenous strains of *R. solanacearum*.

FUTURE ISSUES

1. More strains need to be isolated and sequenced to cover the genetic diversity of the *Ralstonia solanacearum* species complex.
2. A long-standing issue is to decipher the molecular basis of host range specificity, including the search for polygenic adaptation mechanisms.
3. Another challenge is to disentangle the respective contribution of multiple virulence determinants and effectors in disease establishment.
4. New sources of plant resistance must be sought by combining genome-wide association and traditional linkage mapping.
5. The most effective combinations of biocontrol strategies will need to be identified.
6. Strategies that enhance the beneficial impact of the microbiota must be developed.
7. We need to promote precision agriculture at a local scale.
8. Finally, it is necessary to integrate the context of global warming into disease control strategies.

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