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PLANT PATHOGENIC *RALSTONIA* SPECIES

Abstract. *Ralstonia solanacearum* is an important phytopathogen that attacks many crops and native plants in warm and moist climates. In contrast, the closely related *Ralstonia syzygii* and blood disease bacterium attack primarily clove and banana, respectively, in and around Indonesia. This review should be useful both for scientist unfamiliar with these organisms and specialists in the field. It briefly examines the taxonomy of these organisms and summarizes the phenotypic and genetic diversity known to exist within the *R. solanacearum* species complex. The strategies used to detect and identify these pathogens are discussed. General aspects of the pathogens' life cycle, the disease symptoms on representative hosts, and approaches to disease control are described. The biochemical and genetic mechanisms underlying pathogenesis, including results from recent genomic analyses, and host responses to infection are summarized.

1. INTRODUCTION

Bacterial wilts of tomato, pepper, eggplant and Irish potato caused by *Ralstonia solanacearum* were among the first diseases that E. F. Smith proved to be caused by a bacterial pathogen. *R. solanacearum* is now known as one of the world's most important phytopathogenic bacteria due to its lethality, persistence, wide host range, and broad geographic distribution (Elphinstone, 2005). Although most troublesome in the tropics and subtropics, *R. solanacearum* is a continuing threat in temperate climates. Consequently, *R. solanacearum* is one of the more intensively studied phytopathogenic bacteria, and bacterial wilt of tomato is a model system for investigating mechanisms of pathogenesis.

Research on *R. solanacearum* up to the early 1950's, much of it in journals that are now difficult or impossible to access, was summarized by Kelman in a seminal monograph (Kelman, 1953). Since then, short reviews have usually focused on selected aspects of *R. solanacearum* pathobiology (Boucher, Gough, & Arlat, 1992; Buddenhagen & Kelman, 1964; Genin & Boucher, 2004; Hayward, 1991; Hayward, 1995; Hayward, 2000; Schell, 2000). In contrast, the international conferences held since 1985 generated six books that summarized most of the contemporaneous *R. solanacearum* research (Persley, 1986a; Hartman & Hayward, 1993; Hayward & Hartman, 1994; Prior, Allen, & Elphinstone, 1998; Allen, Prior, & Hayward, 2005). Although valuable for scientists specializing in this pathogen, their detail and depth make these reviews and books less useful for non-specialists. Consequently, I have endeavored to provide a wide-ranging review of *R. solanacearum* biology and pathology suitable for a broad audience.

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2. TAXONOMY

Taxonomic groups should follow the natural divisions that are apparent after characterizing the phenotype and genotype of related organisms and determining their relationship to known taxa. Until recently, however, the knowledge necessary to discern natural groups was often unavailable and many groups were 'incorrectly' classified in phylogenetic terms. This was certainly true for the organisms now considered members of the *Burkholderia* group, which were long classed as non-fluorescent *Pseudomonas* species (Anzai, Kim, Park, Wakabayashi, & Oyaizu, 2000; Palleroni, 1984). However, recent genetic analyses have revealed many new relationships and prompted renaming many bacteria in this and other groups (one Web site with official nomenclature is http://www.bacterio.cict.fr/).

Rank	Name	Comments
Class:	β proteobacteria	one of four classes of 'purple bacteria'
'Family'	*Burkholderia* group	has nine genera (three shown)
Genus:	*Ralstonia*	has five species (three shown)
Species:	*pickettii*	human pathogen; common outgroup
	solanacearum complex	plant pathogen, wide host range
	syzygii	plant pathogen of cloves in Indonesia
	Burkholderia	has human and plant pathogens and symbionts
	Cupriavidus (*Wautersia*)	
	metallidurans	many orthologous genes in *R. solanacearum*
	necator	originally *Alcaligenes eutrophus*
	taiwanensis	nodulating, nitrogen-fixing *Mimosa* symbiont

Fig. 1. Abbreviated taxonomic list of the *Burkholderia* group.

2.1. Introduction to the Burkholderia Group

Figure 1 presents an abbreviated taxonomic list for the *Burkholderia* group, showing only the plant-associated or other relevant species. A complete list is available on several Web sites (e.g., TaxBrowser on the NCBI site (http://www.ncbi.nlm.nih.gov/)).

The relevant taxonomy of this group began in the early 1970's when nucleic acid hybridization revealed that fluorescent *Pseudomonas* species comprising RNA homology group I are unrelated to the non-fluorescent pseudomonads in group II (Hayward, 2000; Palleroni, 1984). However, group II organisms were not officially separated until Yabuuchi et al. (1992) established the genus *Burkholderia*, which included *B. cepacia*, *B. pickettii*, and *B. solanacearum* among its seven species. A few years later, the latter two species were transferred into the new genus *Ralstonia* along with *Alcaligenes eutrophus*, which was renamed *R. eutropha* (Yabuuchi, Kosako, Yano, Hotta, & Nishiuchi, 1995). Very recently, 16S rDNA sequence analyses and phenotypic differences (Table 1) stimulated Vaneechoutte et al. (2004) to divide the genus *Ralstonia* into two groups: the *R. pickettii* lineage and the *R. eutropha* lineage. In addition to three species found in human clinical samples, the genus *Ralstonia* now

contains the plant pathogens *R. solanacearum* and the newly renamed *R. syzygii*. Species in the *R. eutropha* lineage were reassigned to the new genus *Wautersia*, with *W. eutropha* as the type species. Unexpectedly, only a few months later, Vandamme and Coeyne (2004) showed that *W. eutropha* is a later synonym of *Cupriavidus necator*, which prompted renaming the genus and eliminating *eutropha* as a specific epithet. The phylogenetic relationships of *Ralstonia* and *Cupriavidus* species based on a 16S rDNA gene sequence comparison are shown in Figure 2.

Table 1. Phenotypic differences between genus *Ralstonia* and genus *Cupriavidus* (adapted from Vaneechoutte et al., 2004).

Character	*Ralstonia*	*Cupriavidus*
flagellation	polar, 1-4[a]	peritrichous
colistin resistance	yes	no
viability on TSA at 25° C[b]	<6 days	>9 days
acid from glucose	yes	No

[a] In motile species. *R. syzygii* is nonmotile (Roberts, Eden-Green, Jones, & Ambler, 1990). Wild-type *R. solanacearum* is most motile in a rich broth medium at about 10^8 cfu/ml (Clough et al., 1997).
[b] Not applicable to *R. syzygii*, which does not grow on TSA (tryptic soy agar).

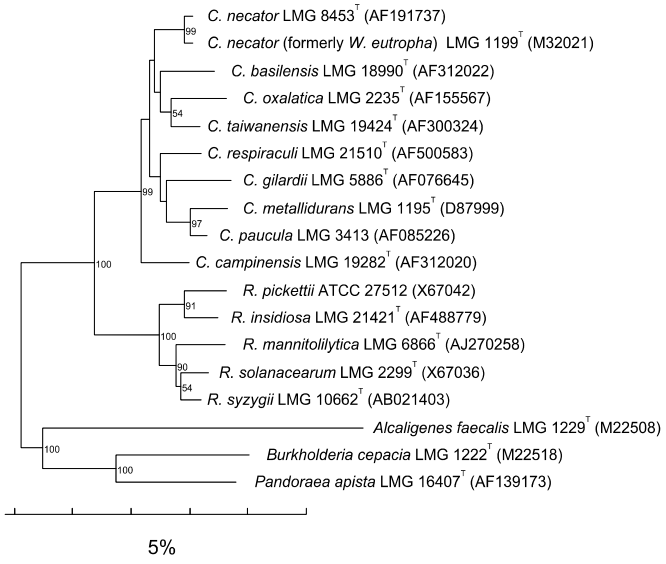

Fig. 2. Phylogenetic tree based on 16S rDNA sequence similarity. Reproduced from Vandamme & Coenye, 2004 (with permission).

2.2. Ralstonia Species

Ralstonia contains only five recognized species, many fewer than other genera in the *Burkholderia* group. *R. insidiosa*, *R. mannitolilytica*, and *R. pickettii* have been isolated from human clinical samples, and the latter two can be pathogenic (Coenye, Goris, De Vos, Vandamme, & LiPuma, 2003; De Baere et al., 2001; Ralston, Palleroni, & Doudoroff, 1973). *R. pickettii* has often been used as an outgroup in genetic studies of *R. solanacearum*, but *R. mannitolilytica* is more closely related to *R. solanacearum* (Fig. 2) (De Baere et al., 2001; Vandamme & Coenye, 2004; Vaneechoutte, Kampfer, De Baere, Falsen, & Verschraegen, 2004). They will not be discussed further.

This chapter focuses on *R. solanacearum*, which causes lethal wilting diseases on many crops and wild plants. Many of its most important hosts are in the nightshade family (*Solanaceae*), hence the specific epithet. This pathogen was briefly known as *Bacillus solanacearum* and then for most of the last century as *Pseudomonas solanacearum* (Saddler, 2000). *R. solanacearum* is an aerobic, Gram-negative rod with a polar flagella tuft. It is oxidase positive, arginine dihydrolase negative, and accumulates poly-β-hydroxybutyrate intracellularly. Most strains denitrify and also produce a diffusible brown pigment on rich medium. It does not grow at 4 or 40°C, and there is little or no growth in 2% NaCl. Its G + C content is 66.5-68 mol%. Numerical taxonomy, serology, and phylogenetic analyses, revealed that the blood disease (BD) bacterium, an Indonesian banana pathogen, is closely related to *R. solanacearum*, but its taxonomic standing remains undefined (Baharuddin, Rudolph, & Niepold, 1994; Coenye et al., 2003; Eden-Green, 1994; Taghavi, Hayward, Sly, & Fegan, 1996; Villa, Tsuchiya, Horita, Opina, & Hyakumachi, 2005).

Ralstonia syzygii, previously known as *Pseudomonas syzygii* (Roberts et al., 1990), is a pathogen of clove trees in Indonesia. Sumatra disease of cloves was first reported from western Sumatra over 80 years ago, and the pathogen may have evolved there. Numerical taxonomy (Eden-Green, 1994; Roberts et al., 1990) and sequence analysis of rDNA and other genes (De Baere et al., 2001; Poussier et al., 2000b; Poussier, Prior, Luisetti, Hayward, & Fegan, 2000a; Taghavi et al., 1996; Villa et al., 2005) showed that it is closely related to *R. solanacearum*, but is more distant than the BD bacterium. Unlike *R. solanacearum*, strains of *R. syzygii* grow poorly or not at all on standard media, are less nutritionally versatile, do not grow at 37°C, are more sensitive to NaCl, and are nonmotile and nonflagellate (Roberts et al., 1990). Its G + C content is 66-67 mol%, and DNA-DNA homology studies show that *R. syzygii* should be retained as a species rather than being incorporated into *R. solanacearum* or established as a subspecies (Roberts et al., 1990; Vaneechoutte et al., 2004).

3. DIVERSITY IN THE *R. SOLANACEARUM* SPECIES COMPLEX

R. solanacearum is present on all continents and many islands between the Tropics of Cancer and Capricorn. As a group, it causes disease on over 200 plant species in over 50 families (Hayward, 1994b; Kelman, 1953). In light of its geographic and pathogenic diversity, Ivan Buddenhagen, a veteran of the campaign to control bacterial wilt (BW) on banana in Central America, opined that there are many bacterial wilts and there are many '*Pseudomonas solanacearums*' They have originated and evolved in widely different places and they have different capabilities with both native flora and introduced hosts, and presumably with different soils and environmental conditions (Buddenhagen, 1986, p. 126).

The most recent genetic studies have proven that Dr. Buddenhagen was correct, and the BW pathogens are currently considered to be members of a species complex.

3.1. Races

Early attempts to codify the diversity present in the *R. solanacearum* species complex resulted in separate race and biovar systems, which greatly influenced thinking about *R. solanacearum* pathobiology during the last four decades. Buddenhagen et al. (1962) recognized three races based largely on host range at the plant species level using strains collected in the Americas (North, Central and South) and Caribbean up to the early 1960's. Unfortunately, this research was only published as an abstract, so a full description of this work is now unavailable. Buddenhagen later admitted that a formal paper was never prepared because their subsequent work revealed more variation among strains than originally observed (Buddenhagen & Kelman, 1964; Buddenhagen, 1986). Nevertheless, most *R. solanacearum* biologists and regulatory agencies have used (and sometimes misused) the system up to the present day.

Race 1 strains were originally described as affecting tobacco, tomato, many solanaceous weeds, some other weeds, and diploid bananas (e.g., *Musa* with AA and BB genotypes) (Buddenhagen, Sequeira, & Kelman, 1962). Over time, the acceptable host range was gradually expanded to include many other plants, including but not limited to groundnut, potato, pepper, eggplant, olive, ginger, strawberry, geranium, and *Eucalyptus*. One long recognized problem with the definition of race 1 is that some strains are highly virulent on tomato and eggplant but low in virulence on tobacco (Granada & Sequeira, 1975; Kelman & Person, 1961) and most of these induce a hypersensitive response (HR; a rapid defensive reaction (Klement, 1982)) when infiltrated into tobacco leaves (Granada & Sequeira, 1975; Robertson, Wechter, Denny, Fortnum, & Kluepfel, 2004). In contrast, strains virulent on tobacco almost always cause necrosis that appears 48-72 h after infiltration. Therefore, tobacco is not a good host for differentiating races. Another problem is that race 1 strains are

phenotypically diverse and not part of a natural taxonomic group (Table 3) (Hayward, 1964; Hayward, 1994a).

Race 2 strains were described as pathogenic on either triploid bananas (AAA, AAB, ABB genotypes), *Heliconia* species or both (Buddenhagen et al., 1962; Thwaites, Eden-Green, & Black, 2000). Buddenhagen and Sequeira established that *R. solanacearum* is endemic on *Heliconia* species native to Central America and, although very rarely pathogenic on triploid *Musa* cultivars, the establishment of large commercial plantations apparently provided the biological filter necessary to select the rare strains that cause BW of banana (Moko disease) (Buddenhagen, 1986; Sequeira, 1993; Sequeira, 1998). Subgroups of strains from Central and South America were recognized based on pathogenicity, cultural characteristics, and whether they were insect transmitted (Fegan, 2005; French, 1986). When artificially inoculated, race 2 strains isolated from diseased triploid banana are highly virulent to *Musa acuminata* and *M. balbisiana* and are often at least moderately virulent to either eggplant, pepper, potato or tomato (French & Sequeira, 1970; Janse, 1991; Raymundo, Orlina, Lavina, & Opina, 2005). When infiltrated into tobacco leaves, race 2 strains typically induce a HR (Janse, 1991; Lozano & Sequeira, 1970), but some strains can be moderately virulent on tobacco (French & Sequeira, 1970).

Race 3 strains originally were described as pathogenic on potato and tomato but weakly virulent on other solanaceous crops (Buddenhagen et al., 1962). Race 3 was considered to have a narrow host range compared to race 1 (Persley, 1986b), and it has sometimes been referred to as the 'potato race' (Buddenhagen & Kelman, 1964; French, 1994; Hayward, 1991). Race 3 is thought to have evolved in the Andes mountains, and it appears to be well adapted to cool-temperate climates (Elphinstone, 1996; French, 1986; Hayward, 1991). Most strains are not pathogenic on tobacco and when infiltrated into tobacco leaves only induce yellowing within the inoculated zone (Lozano & Sequeira, 1970). Recently, race 3 (biovar 2) strains have naturally infected cultivated geraniums (*Pelargonium* spp.) in Central America and Africa, and asymptomatic cuttings were subsequently shipped to the United States and Europe (Janse et al., 2004; Swanson, Yao, Tans-Kersten, & Allen, 2005; Williamson, Nakaho, Hudelson, & Allen, 2002). In Europe, bittersweet nightshade (*Solanum dulcamara*), a semi-aquatic weed, is also an asymptomatic host (Elphinstone, 1996). A variety of solanaceous and nonsolanaceous herbaceous weeds can harbor race 3strains, but grasses seem to remain free of the pathogen (Janse et al., 2004; Pradhanang, Elphinstone, & Fox, 2000a; Tusiime, Adipala, Opio, & Bhagsari, 1998). In addition, artificial inoculation of race 3 (biovar 2) strains from Columbia and Peru showed them to be moderately or highly virulent on tomato, eggplant, *Datura stramonium*, *Cyphomamdra betacea*, and *Solanum nigrum* (Marín & El-Nashaar, 1993; Swanson et al., 2005; Thurston, 1963). Therefore, race 3 has a much larger host range than was originally envisioned (Buddenhagen et al., 1962).

Race 4 strains are particularly aggressive on ginger (Anon., 2004; Persley, Batugal, Gapasin, & Vander Zaag, 1986). I found no paper in the primary literature designating such strains as race 4, but this terminology came into general use in the mid 1980's (Buddenhagen, 1986; Persley et al., 1986) and is now the defacto standard (Anon., 2004; Denny & Hayward, 2001). *R. solanacearum* strains that cause a rapid wilting of edible ginger (*Zingiber officinale*) and ornamental gingers have been isolated in Australia, China, Hawaii, India, Indonesia, Japan, Malaysia, Mauritius, the Philippines, and Thailand (Alvarez et al., 2005; Hayward, 1994b; Kumar, Sarma, & Anandaraj, 2004). A host range test in a naturally infested Australian field determined that race 4 (biovar 4) strains also wilted tomato, pepper, eggplant and some native weeds (e.g., *Solanum nigrum* and *Crassocephalum crepidioides*) (Pegg & Moffett, 1971). The few race 4 strains tested are low in virulence on tobacco (Pegg & Moffett, 1971; Quinon, Aragaki, & Ishii, 1964; Zehr, 1970a) and when infiltrated into tobacco leaves they induce a slow necrosis similar to that caused by race 1 strains virulent on tobacco (Janse, 1991). The host range of race 4 (biovar 3) strains from India has not been reported. Some race 1 strains may cause a slow wilting of ginger, but other races are not pathogenic (Hayward, 1994b; Janse, 1991; Quinon et al., 1964; Zehr, 1970b).

Race 5 is known only from China, where it causes wilt of mulberry trees (*Morus alba*) (He, Sequeira, & Kelman, 1983). Artificial inoculations show that race 5 strains are weakly virulent on eggplant and potato, and not virulent on tomato, pepper, groundnut or tobacco. Strains from mulberry were originally designated as race 4 due to the confusion surrounding the unofficial prior designation of ginger strains as race 4.

Several caveats regarding race designation are worth mentioning. First, although pathogenicity assays with *R. solanacearum* are relatively easy, artificial inoculations are influenced by a many variables (e.g., host cultivar, growth conditions, inoculum concentration, and inoculation method) and may overestimate the natural host range. In addition, because host range assays require substantial time and resources, only a few strains have usually been included in any one experiment. There are no *in vitro* tests that reliably predict the race or host range of *R. solanacearum*. Although Janse (1991) reported that whole cell fatty acid analysis supports differentiation of races 1, 2, and 3, confirmation of this correlation using a larger, more diverse set of strains has not been reported. Thus, it understandable why Cook et al. (1989, page 113) stated that Ahost range is often an ambiguous and unreliable taxonomic character.@

A second caveat is that the *R. solanacearum* race system is confusing to most scientists not familiar with this pathogen. For almost all other host-pathogen systems, races are determined by the matrix of resistance responses generated when two or more strains are tested on a set of differential cultivars of a single host species (i.e., cultivar-level specificity or gene-for-gene specificity) (Keen, 1990). In contrast, the unavailability of highly resistant cultivars of crop plants precludes cultivar-level

specificity testing of *R. solanacearum*. Only recently did several authors (Alvarez, 2005; Prior & Fegan, in press) finally state the obvious: that the races of *R. solanacearum* resemble pathovars in other species of phytopathogenic bacteria.

After considering the ambiguities and problems with the existing *R. solanacearum* race system, I propose that it be abandoned. It generally has not lived up to its original purpose of 'aiding in the evaluation of the pathogenic potential' of *R. solanacearum* (Buddenhagen et al., 1962). On the contrary, the race system has become increasingly unwieldy and unreliable as a predictor of pathogenic potential as the known diversity of this species complex has increased. Consequently, mistakes could be made when the race system is used by regulatory agencies to define quarantine agents. Even worse, the race system's imprecision and inaccuracies impede our developing an accurate picture of *R. solanacearum* pathobiology.

3.2. Biovars

In contrast to the pathocentric race system, the biovar system originally proposed by Hayward (1964) groups strains by their ability to acidify media containing one each of three disaccharides or three sugar alcohols (Table 2). The system was later expanded to include additional substrates, production of nitrite from nitrate, and production of gas from nitrate (Hayward, 1994a). This is a special-purpose classification system that is only useful once a strain has already been identified as *R. solanacearum* using other methods. Nevertheless, because biovar determination is easy, inexpensive, and reproducible, it has been widely adopted as an essential trait in strain characterization.

Table 2. Differentiation of *Ralstonia solanacearum* biovars[a].

Test	*Biovars*					
	1	*2*[b]	*2-T*	*3*	*4*	*5*
Acidification of medium						
Mannitol	-	-	-	+	+	+
Sorbitol (Glucitol)	-	-	-	+	+	-
Dulcitol (Galactitol)	-	-	-	+	+	-
myo-Inositol[c]	+	+	+	+	+	+
D-Ribose[c]	d	-	+	+	+	+
Trehalose	+	-	+	+	+	+
Lactose	-	+	+	+	-	+
Maltose	-	+	+	+	-	+
D-(+)-Cellobiose	-	+	+	+	-	+
Nitrite from nitrate	+	+	+	+	+	+
Gas from nitrate	-	-	-	+	+	+

[a] +, 80% or more strains positive; -, 80% or more strains negative; d, 21-79% of strains positive.
[b] Some biovar 2 strains from Chile and Columbia (RFLP 27) are negative for inositol, positive for trehalose, and most are negative for nitrite production (Hayward, 1994a).
[c] For biovars 1, 3, 4, and 5 the results are from nine or fewer strains and should be considered preliminary

Four biovars (numbers 1 to 4) were recognized initially and they have accommodated almost all *R. solanacearum* strains isolated. Some strains isolated from potato in the Amazon basin can be differentiated from the archetypal biovar 2 using the extended panel of carbohydrates (French, Aley, Torres, & Nydegger, 1993; Hayward, 1994a). This new group is known as 2-T (T for tropical) in recognition of its lowland origin or N2 (new biovar 2). Consequently, the original biovar 2 is sometimes now referred to as biovar 2-A (A for Andean) in recognition of its highland origin (see below). Biovar 5 was established to accommodate strains isolated from mulberry in China.

There is no general correlation between biovars and races, but biovar 2 strains are almost always race 3 (and the reverse is always true) and biovar 5 strains are usually race 5 (and vice versa). Until recently, race 4 strains were biovar 4, but strains highly virulent on ginger in India are biovar 3 (Kumar et al., 2004). Race assignment to biovar 2-T strains is problematic, because they exhibit a wider host range than do the archetypal biovar 2 (race 3) strains. Biovar 1 and 3 strains are isolated from many different plants in many locations, so little can be predicted about the biology of such strains based only on biovar typing.

Biovars are based on a few traits, each of which probably requires only one or a few genes (some of which may have been acquired by horizontal gene transfer (Gabriel et al., in press)), and preliminary work indicates that one gene is essential for utilization of both sorbitol and dulcitol (Denny, unpublished results). Therefore, spontaneous mutation, in nature or in storage, could change a strain's reaction profile and confuse biovar assignment. For example, a single frame-shift mutation could change a biovar 3 strain into one classified as biovar 5. However, experience has demonstrated that biovars are stable in culture. In addition, recent phylogenetic studies indicate that biovars 3, 4, and 5 are part of a separate genetic lineage distinct from the other biovars (see below) and these strains also can utilize more varied carbon sources than can those in other biovars (Hayward, 1994a; Palleroni & Doudoroff, 1971). Consequently, biovar type appears to be relatively stable in nature.

3.3. Phylogenetic Groups

Although pathogenicity and biovar typing have genetic underpinnings, these traits are one or more steps removed from the DNA encoding them. Strictly genetic characterization has dramatically increased our knowledge and understanding of the diversity, relationships, and evolution of the *R. solanacearum* species complex. The trend over the last decade has been to more finely divide this group of related organisms as more strains from around the globe are studied in greater depth, and it may not be long before there is a proposal to split the complex into two or more subspecies or species.

The seminal phylogenetic work by Cook et al. (1989) used classical restriction fragment length polymorphism (RFLP) analysis to examine 62 *R. solanacearum* strains representing 4 races and 5 biovars isolated in the Americas, Asia and Oceania. Southern blots of restriction endonuclease-digested genomic DNAs were probed with each of nine DNA fragments cloned from *R. solanacearum* that hybridized to regions encoding biosynthesis of tryptophan, the high molecular mass extracellular polysaccharide (EPS1), the core lipopolysaccharide, three regions of the HR and pathogenicity (*hrp*) gene cluster, and three regions of unknown function. Twenty eight unique RFLP groups (later called multilocus genotypes (MLGs)) were recognized and a similarity coefficient matrix showed that they formed two distinct groups. These divisions were apparent in the RFLP patterns with each probe individually, suggesting that the loci sampled had coevolved as a part of the same genome.

Follow-up studies examined 102 additional *R. solanacearum* strains that better represented the same races and localities and also included biovar 2-T strains (Cook & Sequeira, 1994). RFLP data generated using eight of the original nine DNA probes produced a total of 44 MLGs and hierarchical cluster analysis confirmed the presence of the same Divisions I and II (Table 3). Division I contains all strains characterized as race 1 biovars 3, 4, 5, and they are mostly from Asia and Oceania. Division II contains strains characterized as race 1 biovar 1, race 2, and race 3 biovar 2, and they are mostly from the Americas. Division II also has many biovar 2-T strains (which produced many new MLGs) and comprises two sub-clusters: the biovar 2 and 2-T strains are in one subcluster and almost all the biovar 1 strains are in the other subcluster. Strains from banana (race 2) are in both subclusters. Most strains from potato are in MLG 26, despite being isolated from many locations around the world, which strongly suggests that a clonal population has been distributed by humans in latently infected potatoes.

A variety of modern techniques have been used since the early 1990's to investigate the phylogeny of *R. solanacearum*. These include total genomic RFLP fingerprinting (Gillings & Fahy, 1993; van der Wolf et al., 1998), polymerase chain reaction (PCR)-RFLP (Gillings, Fahy, & Davies, 1993; Poussier et al., 2000b; Poussier, Vandewalle, & Luisetti, 1999), amplified fragment length polymorphism (AFLP) (Poussier et al., 2000b; van der Wolf et al., 1998), tRNA-anchored PCR (Seal, Jackson, & Daniels, 1992) and repetitive element (rep)-PCR (Horita & Tsuchiya, 2001; Thwaites, Mansfield, Eden-Green, & Seal, 1999; van der Wolf et al., 1998).

DNA sequencing of desired loci that have been PCR-amplified from many strains has become increasingly popular as the price of this method has fallen (Fegan & Prior, 2005; Fegan, Taghavi, Sly, & Hayward, 1998; Li et al., 1993; Pastrik, Elphinstone, & Pukall, 2002; Poussier et al., 2000b; Poussier et al., 2000a; Prior & Fegan, in press; Taghavi et al., 1996; Villa et al., 2003; Villa et al., 2005). Computer software is used to identify polymorphic nucleotides, calculate genetic distances and perform cluster analyses. Relationships between strains that are distantly related can be determined by

analyzing loci that evolve slowly, such as the 16S ribosomal RNA gene (rDNA). Conversely, relationships between more closely related strains require targeting loci that are evolving more rapidly. Genes that encode important proteins may evolve at a moderate rate, but non-coding intergenic regions should evolve most rapidly. The *R. solanacearum* loci examined to date include genes encoding an endopolygalacturonase (*pehA*), an endoglucanase (*egl*), the AraC-type regulatory protein in the *hrp* gene cluster (*hrpB*), the DNA mismatch repair system (*mutS*), the intergenic region adjacent to the *lpxC* gene, and the 16S-23S intergenic spacer region (also called the ITS region).

Regardless of the method, all of the genetic analyses support the existence of two major groups that are essentially the same as Divisions I and II described by Cook et al. (1994). Studies from the mid 1990's found only the same two divisions, because they only examined strains representing populations similar to those sampled previously. Later studies included strains isolated in Africa and/or Indonesia, which had not been examined previously, and the greater diversity resulted in at first three and then four major divisions being recognized (Table 3). The most recent study compared the 16S rDNA, *egl*, and *hrpB* sequences from the most representative set of strains studied to date and consistently found four major divisions that correlated with geographic origin (Villa et al., 2005). The data also indicate that the group of strains originating in the Americas has two subclusters (phylotypes IIa and IIb) (Prior & Fegan, in press; Villa et al., 2005). The same relationships are observed regardless of the locus examined, suggesting that these regions have co-evolved as part of a conserved *R. solanacearum* genome. This is not the case for all loci, because the genome of strain GMI1000 is a mosaic of genes apparently acquired from related and unrelated bacteria (see below) (Salanoubat et al., 2002).

The new classification scheme proposed by Fegan and Prior (2005) denotes the major groups as phylotypes, defined as a monophyletic cluster of strains revealed by phylogenetic analysis of sequence data (Table 3). A set of multiplex PCR primers is available to determine a strain's phylotype (Fegan & Prior, 2005). Each phylotype is comprised of a number of sequevars, or sequence variants, and single strains that have a highly conserved sequence within the target locus. Sequevars are comparable to RFLP MLGs and the two schemes often produce the same or similar groups of strains (Table 3). However, both schemes suffer from the same potential problem, which is that the recognized clusters may be dependent on the particular gene(s) sequenced or the set of polymorphic fragments examined (e.g., sequevar groups might be different when based on *egl* and *hrpB* sequence data (Villa et al., 2005)). Additional sequevars probably will be recognized as more strains are examined (especially strains not isolated from diseased plants), but few if any new phylotypes are likely to be described (Fegan & Prior, 2005). This classification scheme has a distinct advantage over other measures of genetic diversity, such as RFLPs, because sequences entered into an on-line database can easily be 'reused' in future, increasingly comprehensive phylogenetic studies.

Table 3. Diversity within the *Ralstonia solanacearum* species complex assessed using different methods[a].

Phylotype & Origin	*RFLP div.*	*16S group*	*Sequevar (egl)*[b]	*RFLP MLG*[c]	*Biovar or species*	*Host of origin*	*Comments*
I Asia and Oceania	I	1	12-18	8-14	3	many	Race 1
				11, 15-18, 42	4	many	
				22, 23	4	Ginger	Race 4
				19, 20	5	Mulberry	Race 5
					2-T	Pepper, Tomato	Japan (one strain from each)
IIa Americas	II	2A	3, 4	24, 25	1	Musaceous	Race 2
			1[e], 2	26[e], 27, 34	2	Potato mostly	Race 3
			none yet	29-31, 33, 36, 37, 39	2-T	Potato, Eggplant	only single strains sequenced
IIb Americas			5, 7	1-7, 38	1	many	Race 1, type strain
			6	28, 46	1	Musaceous	Race 2
III Africa	nd	nd	19-23	nd	1	many	unrelated to Bv.1 in Div. II
					2-T	Potato	
IV Indonesia and Asia	nd	2B		nd	1	Clove	unrelated to Bv. 1 in Div. II
			11		2	Clove, Potato	Bv. 2 also in Australia
			8		2-T	Potato	also in Japan & the Philippines
			10		2-T, BDB[d]	Musaceous	
			9		1, *R. syzygii*	Clove	

[a] Sources of data are as follows: Phylotype (Fegan & Prior, 2005; Prior & Fegan, 2005; Villa et al., 2005); RFLP (restriction fragment length polymorphism) Division (Cook et al., 1994); 16s rDNA group (Poussier et al., 2000b; Taghavi et al., 1996); RFLP MLG (multilocus genotype) (Cook et al., 1994). Biovars and host ranges are compiled from many references. nd, no data.

[b] Sequevar designations are from analysis of the *egl* gene. Analysis of other genes might not cluster strains into the same groups.

[c] Cook et al. (1994) did not define MLGs 32 or 35 and merged MLGs 16 and 21 with MLGs 11 and 8, respectively.

[d] BDB, Blood Disease Bacterium.

[e] Strains isolated from potato worldwide are most often in sequevar 1 (MLG 26).

The robustness of the phylotypes strongly suggests that they reflect true evolutionary lineages within the *R. solanacearum* species complex. These lineages presumably arose when progenitors became geographically isolated and subsequently adapted to different environments and potential host plants. Therefore, unlike the races and biovars, phylogenetic analyses and phylotyping classification should make possible a stable and meaningful taxonomy that defines subspecific groups of *R. solanacearum* that are at least related to geographic origin. Available DNA-DNA hybridization data (Palleroni & Doudoroff, 1971; Roberts et al., 1990) indicate low homology (<70%, the threshold for speciation) between some strains in different biovars, but the data are too preliminary to conclude that the species complex should be divided into subspecies or new species. There is also insufficient information on the biological, epidemiological, and ecological properties of strains that would make the phylotype system more useful to plant breeders, plant pathologists and quarantine officials (Fegan & Prior, 2005).

4. DETECTION AND IDENTIFICATION

Detection and identification are conceptually and methodologically intertwined processes. When studying the pathobiology and epidemiology of *R. solanacearum* it is usually necessary to first detect its presence (based on a tentative identification) before isolating and rigorously identifying a strain. Substantial international effort has been focused on developing better detection methods, because soil and water samples typically have low populations of *R. solanacearum* and not all cells may grow *in vitro*. General aspects of detection methods will be reviewed and their relative advantages and disadvantages discussed. Technical details and access to the earlier literature are available elsewhere (Anon., 2004; Alvarez, 2005; Denny et al., 2001; Elphinstone, Hennessy, Wilson, & Stead, 1996; Saddler, 2000; Seal & Elphinstone, 1994).

Pure cultures of *R. solanacearum* are not difficult to identify. Cultural and physiological tests can quickly rule out related organisms (Anon., 2004). There are also commercially available fatty acid methyl ester (FAME) analyses and BIOLOG™ kits, but they will not be described here (see (Black & Sweetmore, 1993; Janse, 1991; Li & Hayward, 1993; Stead, Sellwood, Wilson, & Viney, 1992)). There are also many suitable serological and nucleic acid based methods (see (Alvarez, 2005; Seal et al., 1994)), but only those useful for detecting the pathogen are discussed below.

4.1. General Considerations

The first consideration is that the purpose of a diagnosis should dictate its thoroughness (Black & Elphinstone, 1998). If only advice concerning an ongoing pest management problem is needed, then presumptive evidence of *R. solanacearum* will probably suffice. In contrast, quarantine issues dictate an unequivocal, well documented identification of the pathogen. Second, no single detection method is

suitable for all samples or meets all the requirements for sensitivity, specificity, and speed. Plant tissue, seeds, soil, and water each present different challenges that vary with the detection method and increase as the pathogen population decreases. Third, the amount of material sampled affects relative sensitivity. For example, method A may detect 10x more cells per unit volume than method B, but if the latter uses 100x more sample volume then it may be the more sensitive overall. Fourth, the sampling protocol prior to detection can influence the results. For example, it is a statistical certainty that a random sample of 200 tubers from a ton lot will contain at least one infected tuber when 10% of the lot is infected, but the probability of a sample having an infected tuber drops to only 63.3% when 0.5% of the lot is infected (Elphinstone et al., 1996). Fifth, cost effectiveness will be important for diagnostic labs that process many samples, but the least expensive method may be different in developed and less developed countries (Seal, 1998). Availability, reliability and price of reagents and equipment are more important in developing countries, whereas labor costs are generally higher in developed countries.

4.2. Traditional Methods

A variety of pathogens can cause wilting, so symptoms alone are not definitive for BW. However, plants wilted by *R. solanacearum* have $>10^8$ colony forming units (cfu)/g of tissue, so a milky white ooze often forms on the cut surface of a stem left after decapitating a wilted plant as root pressure forces out xylem fluid. Ooze also may accumulate on the cut surface of infected tubers or rhizomes. Even if ooze does not form spontaneously, a 'streaming test' may be positive (Fig. 3) (Allen, Kelman, & French, 2001). Other wilt-inducing pathogens do not produce comparable ooze. The ooze is usually an almost pure culture of *R. solanacearum*, which can be cultured on standard, low ionic strength bacteriological media. Detecting *R. solanacearum* in soil and water samples is more difficult, because the pathogen population is usually small ($<10^4$ cfu/g soil or ml water) and saprophytic bacteria are present in equal or greater numbers. Such samples are best cultured on a semi-selective medium, such as modified SMSA (Denny et al., 2001; Elphinstone et al., 1996), which usually suppresses contaminants well enough to permit detection of *R. solanacearum* down to 100 to 500 cfu/g soil or ml water, and about 10-fold lower in tissue extracts (Elphinstone et al., 1996; Poussier, Cheron, Couteau, & Luisetti, 2002; Pradhanang, Elphinstone, & Fox, 2000b). Other semi-selective media may work better in some locations or with particular soils (see (Denny et al., 2001; Pradhanang et al., 2000b)). Surprisingly, none of the common serological or nucleic acid based techniques described below has a substantially lower threshold of detection. Even when other methods are used for detecting *R. solanacearum*, culturing is required to confirm pathogen viability and provide a pure strain for confirmation of identity and pathogenicity bioassays.

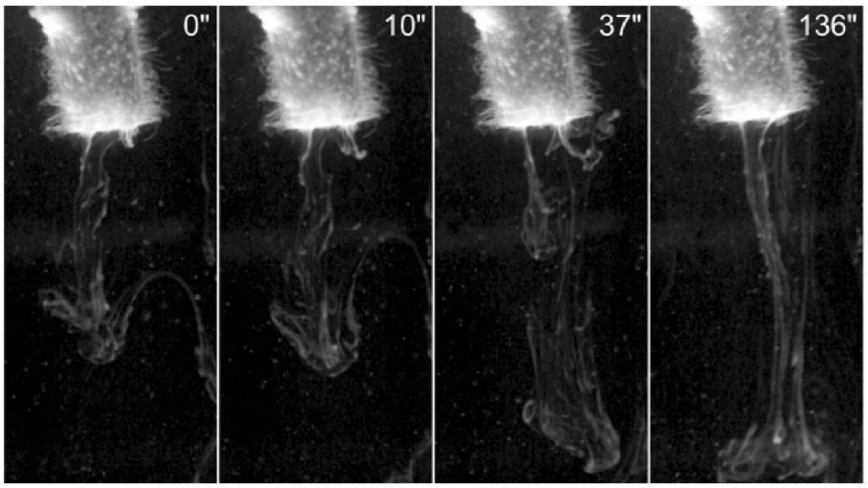

Fig. 3. Streaming test to observe bacteria oozing from an infected tomato stem in water.

It is also possible to use a susceptible plant as the enrichment 'medium'. Typically, a crude extract from tissue or soil is introduced into the stem of young tomato plants (3 to 4 leaf stage) that are incubated at 25-30^{o}C for up to 4 weeks. Plants are monitored for symptom development and any unwilted plants are sampled for latent infection by plating tissue extracts on SMSA. Tomato seedlings also can be transplanted into infested soil. Stem inoculation reliably detects *R. solanacearum* when it is present at $\geq 10^4$ cfu/ml of sample extract (Elphinstone et al., 1996; van der Wolf et al., 2000), whereas for the transplantation test there must be $>10^5$ cfu/g soil (Pradhanang et al., 2000b).

4.3. Serological Methods

Serological methods are popular for primary screening of samples because they are generally quick and reliable. These methods have thresholds of detection that range from 10^3-10^4 bacteria/ml of sample. Each serological method has different strengths, but they all suffer from problems with either specificity, sensitivity or both. Polyclonal antisera (PAb) usually react more strongly with *R. solanacearum* than do monoclonal antibodies (MAb), but this greater sensitivity is counterbalanced by reduced specificity. False positives become more common as the proportion of contaminants in samples increases, whereas false negatives occur when the pathogen lacks the targeted epitope(s). Serological methods also have the disadvantage that they do not discriminate between live and dead cells. Knowledge that a sample has living *R. solanacearum* is essential before making a decision to quarantine or destroy a shipment of produce.

Rapid, virtually fool-proof serological kits are commercially available (e.g., Agdia (Elkhart, IN, USA), Neogen Europe Ltd. (Auchincruive, Scotland), and the Central Science Laboratory (Sand Hutton, York, UK)). Most kits use a MAb to *R. solanacearum* coated onto colored latex beads that are preloaded onto a dipstick or horizontal flow unit. Results can be read in several minutes: positive samples produce two colored bands, negative samples produce a single control band, and no band indicates a method failure. The Pocket Diagnostic™ kit from the CSL is approved by the United States Department of Agriculture for testing of tissue from symptomatic potatoes and geraniums. A rapid agglutination test kit also is available form Neogen Europe. However, speed and convenience come at a price, because each test costs $3 to $6 USD.

Enzyme-linked immunosorbent assays (ELISA) are still used in many labs, because they are relatively inexpensive, easy, fairly fast, and tolerate foreign material in the sample (e.g., plant tissue, soil). Direct ELISA, indirect ELISA, and double antibody sandwich (DAS) assays have been developed. ELISA kits are commercially available from Agdia and Neogen Europe. A variation on this theme is to bind the samples to a nitrocellulose membrane, which can be stored for long periods before and after the ELISA (Priou, Aley, & Gutarra, in press). This method has been used in many countries for pathogen surveys and testing propagative tissues (Elphinstone, 2005; Priou et al., in press). ELISA has a relatively high detection threshold; under ideal conditions, PAb cannot detect fewer than 10^4 bacteria/ml sample and MAb are about 100-fold less sensitive (Pradhanang et al., 2000b; Seal, 1998). Values are 10- to 100-fold higher when using samples from plants or soil. Sensitivity can be improved substantially by adding the sample to broth medium and incubating 24 to 72 h prior to running the ELISA on the enriched sample (Elphinstone et al., 1996; Pradhanang et al., 2000b). Caruso et al. (2002) used a non-selective medium and reported detecting *R. solanacearum* at 1 to 10 cfu/ml of potato extract and Priou et al. (in press) used a new semi-selective medium and detected about 100 cfu/g of naturally infested soil.

Alternatives to ELISA are immunofluorescence-antibody staining (IFAS) and immunofluorescence-colony staining (IFCS) (Elphinstone et al., 1996; Janse, 1988; van der Wolf et al., 2000). These methods have most of the same advantages and disadvantages as ELISA, but require special microscopes that may not be readily available. Both have a detection threshold of 10^2 to 10^4 bacteria/g soil or ml tissue extract (Elphinstone et al., 1996; Seal, 1998; van der Wolf et al., 2000). The specificity of IFAS can be improved by simultaneously running fluorescent *in situ* hybridization (FISH) using a probe to the 23S rDNA gene sequence (Wullings, van Beuningen, Janse, & Akkermans, 1998). The specificity of IFCS can be verified by recovering viable bacteria from the stained colonies for either direct PCR testing or subculturing on SMSA and subsequent IFAS testing (van der Wolf et al., 2000).

4.4. Nucleic Acid-Based Methods

Nucleic acid-based methods to detect *R. solanacearum* rely on hybridization of probes or PCR primers to targeted pathogen sequences. FISH is the only current method that still uses labeled probes, because greater sensitivity can be achieved with amplification of target sequences by PCR. Direct PCR, nested PCR, co-operational PCR, real-time PCR and multiplex PCR reactions have all been developed to detect *R. solanacearum*. When the target sequence is present in multiple copies (e.g., tRNA genes) and reaction conditions are ideal, PCR can detect a single pathogen cell or its equivalent in purified DNA. With this level of sensitivity, though, extra care is needed to avoid false positives due to sample contamination by *R. solanacearum* cells or DNA.

Proper selection of target sequences is essential when developing a PCR assay. The target should be conserved within the species or the desired subspecies group (e.g., phylotype or race) and absent in all other bacteria. Targeting more than one pathogen sequence is also recommended to guard against unexpected failures in specificity. The primers should be tested on as many *R. solanacearum* strains and related species as possible to determine the likelihood of false positive or negative reactions. The primers also should be tested in multiple laboratories to assess the effect of small differences in sample preparation and reaction conditions.

PCR methods are theoretically more specific and sensitive than serological approaches. However, similar to serological methods, PCR detects both living and dead cells, because DNA can remain intact for long periods after death. More importantly, unlike serological methods, PCR is often partially or completely inhibited by compounds introduced into the reaction along with the sample, resulting in false negatives. For example, extracts from potato tubers interfere strongly with PCR (Elphinstone et al., 1996; Seal, 1998; Weller, Elphinstone, Smith, Boonham, & Stead, 2000a). Extracts from other plants, seeds, soil and water usually have different, unidentified PCR inhibitors. Multiplex PCR addresses this issue by including primers to co-amplify the sequence of an internal standard (e.g., a conserved plant or eubacterial rDNA gene) that is consistently present in (or is added to) all samples (Glick, Coffey, & Sulzinski, 2002; Pastrik et al., 2002; Schönfeld, Heuer, van Elsas, & Smalla, 2003; Weller et al., 2000a). The internal standard tests the 'amplification competence' of each sample to reveal those that may give a false negative.

A variety of approaches can reduce or eliminate PCR inhibitors. Diluting a sample will sometimes reduce the inhibitor concentration enough to allow amplification (Weller et al., 2000a), as can washing the bacteria by centrifugation, filtration or during immunocapture (Dittapongpitch & Surat, 2003; Poussier et al., 2002).

However, the more reliable solution to this problem is to extract DNA from the sample. Extraction buffer additives, in particular polyvinyl pyrrolidone (PVP) and polyvinyl polypyrrolidone (PVPP), can help to remove PCR inhibitors (Llop, Caruso, Cubero, Morente, & Lopez, 1999; Poussier et al., 2002). In the few reports where different extraction methods were compared, the commercial QIAamp DNA (QIAGEN, Inc) and the Easy-DNA (Invitrogen) kits were more reliable and produced DNA that worked better in PCR than other commercial kits and conventional recipes (Pastrik & Maiss, 2000; Poussier et al., 2002). PCR buffer additives, such as bovine serum albumin and trehalose (Ozakman & Schaad, 2003; Poussier et al., 2002), may also be helpful. Another common strategy is to enrich the population of *R. solanacearum* by culturing the environmental sample on SMSA broth or SMSA plates prior to PCR (Elphinstone et al., 1996; Ozakman & Schaad, 2003; Pradhanang et al., 2000b; Weller, Elphinstone, Smith, & Stead, 2000b). This approach has the added advantages of reducing the detection threshold by providing a biological amplification before the detection step and eliminating the need for DNA purification, because amplifiable DNA is prepared simply by heating the cells at 100^{o} C for several minutes.

Other PCR variations can enhance sensitivity of detecting *R. solanacearum*. Two-stage nested PCR is the simplest method (Elphinstone et al., 1996; Poussier & Luisetti, 2000; Pradhanang et al., 2000b), but the added sample manipulation step increases the risk that contamination will generate a false positive. Co-operational PCR is related to nested PCR, but the reaction is performed in a single tube, so contamination is expected to be less of a problem (Caruso, Bertolini, Cambra, & Lopez, 2003). Real-time PCR using TaqMan fluorescent probes can detect 10 to 30 cfu/ml of potato tuber extract after the *R. solanacearum* population has been enriched by growth on SMSA (Ozakman & Schaad, 2003; Weller et al., 2000a; Weller et al., 2000b). This method also eliminates post-PCR analyses, provides a quantitative estimate of the target sequence in a sample, and demonstrates viability of *R. solanacearum* cells when the amount of target sequence increases in samples removed at intervals while incubating the enrichment culture.

Another approach to detect viable cells is to target RNA, which degrades quickly after death. Nucleic acid sequence based amplification (NASBA), based on isothermal amplification of *R. solanacearum* 16S rRNA, detects only live cells (Bentsink et al., 2002). NASBA was converted to a real-time detection method (called AmpliDet RNA) by incorporating a fluorescent molecular beacon into the reaction (van der Wolf, 2004). Similar to a TaqMan probe, the fluorescence of a molecular beacon increases linearly with the amount of homologous sequence in the reaction mixture. In a 90 minute reaction, AmpliDet RNA detected 10^4 cfu/ml in potato tuber extract and 10 cfu/ml in a water sample previously concentrated 200-fold.

5. THE GENERAL PATHOGEN CYCLE

Before describing diseases caused by *R. solanacearum* on representative hosts, general aspects of the pathogen's life cycle (disease cycle) will be reviewed. Susceptible hosts

suffering from BW contain massive populations of *R. solanacearum* (10^8 to 10^{10} cfu/g tissue) and usually die from the disease. Therefore, the first hurdle for the pathogen is to survive until it can infect another host plant (Coutinho, 2005; Hayward, 1991; Persley, 1986b). The large number of pathogen cells shed from roots of symptomatic and nonsymptomatic plants (Elphinstone, 1996; Swanson et al., 2005) and bacterial ooze on plant surfaces (Buddenhagen & Kelman, 1964; Kelman, 1953) enter the surrounding soil or water, contaminate farming equipment, or may be acquired by insect vectors.

Survival of *R. solanacearum* in water can range from weeks to years depending on the interacting abiotic and biotic factors. The pathogen can survive for >40 years when stored in pure water at 20-25°C, and this is still an acceptable method for long term storage. Temperature extremes (especially storage at 4°C), pH extremes, and the presence of salts or other contaminants directly reduce survival (Caruso et al., 2005; van Elsas, Kastelein, de Vries, & van Overbeek, 2001). These factors can also affect the number, type, and activity of other microorganisms present in non-sterile water that compete with or prey upon *R. solanacearum*. Contaminated irrigation water also is an important route for pathogen dispersal and inoculation (Elphinstone, 1996; Janse, 1996; Swanson et al., 2005).

Survival in soil is less well understood, due to the greater complexity of this substrate and the difficulty in detecting pathogen populations <100 cfu/g soil. In the short term (up to 2 years), survival in soil is largely controlled by moisture (matric potential) and temperature. *R. solanacearum* can survive in moderately dry soil (e.g., -0.3 MPa ≈ 20% moisture), but lower moisture levels drastically reduce survival. Other factors may include the soil type, soil depth, host plant debris, organic matter content, nutrient level, and the microflora and microfauna (Coutinho, 2005; Hayward, 1991; Persley, 1986b). Increased amounts of organic material often correlates with decreased pathogen survival (Gorissen, van Overbeek, & van Elsas, 2004; Hayward, 1991; Schönfeld et al., 2003), but why some soils are 'conducive to disease' while others are 'suppressive' remains unclear. For long term survival (>2 years) in the absence of a true host, the pathogen must either colonize the more nutrient rich soil near roots or latently infect roots of plants that remain asymptomatic (Coutinho, 2005; Elphinstone, 1996; Janse et al., 2004; Pradhanang et al., 2000a). After multiplying in these sheltered sites, the pathogen returns to the bulk soil to repeat the cycle.

Recently, several research groups have claimed that *R. solanacearum* can enter a state where it is viable but not culturable (VBNC) as a result of incubation at 4°C or exposure to copper ions (Caruso et al., 2005; Grey & Steck, 2001; van Elsas, van Overbeek, & Trigalet, 2005b). Cells in the VBNC state do not form colonies on most laboratory media but remain metabolically active, as determined by their enlargement in response to nutrients or their retention of an intact cytoplasmic plasma membrane (van Elsas et al., 2005b). The existence of the VBNC state is controversial, because

(for example) nonculturability may be a laboratory artifact and 'resuscitation' of VBNC cells implies that they were never nonculturable. In practical terms, however, the potential for some or most of a pathogen population to escape detection methods that require *in vitro* multiplication (i.e., plating on SMSA, detection by IFCS, enrichment prior to PCR) means that our understanding of the ecology and epidemiology of *R. solanacearum* may be incomplete and could compromise quarantine practices. It is possible that cold-stressed viable cells are not a threat to agriculture, because in a controlled test strain 1609 (biovar 2 race 3) held at 4°C for 100 to 125 days became avirulent on tomato (van Overbeek, Bergervoet, Jacobs, & van Elsas, 2004). However, this may not always be the case, because *R. solanacearum* strains recovered during the winter from a Spanish river retained virulence on tomato (Caruso et al., 2005).

R. solanacearum also survives in hosts that, due to resistance or cool temperatures, are latently infected and do not develop symptoms. Pathogen populations are often low enough to make detection difficult, but can be as high as 10^8 cfu/g tissue in parts of some latently infected plants (Elphinstone et al., 1996; Swanson et al., 2005). Latently infected propagating tissues also are a major route for long-distance dispersal of *R. solanacearum*. The most important plants in this regard are potato, banana (and other musaceous spp.), ginger, and geranium. It is widely believed that cool-temperature adapted biovar 2 strains pathogenic on potato were repeatedly introduced into Europe via infected seed and ware potatoes (Janse, 1996) and that strains pathogenic to banana were moved from Central America to the Philippines (Buddenhagen, 1986; Fegan & Prior, in press). There is no doubt that biovar 2 strains recently were introduced onto the USA and Europe on latently infected geranium cuttings (Janse et al., 2004; Williamson et al., 2002). There are only a few studies of the survival and dispersal on true seed, and these are rare events (Coutinho, 2005; Martins, Nabizadeh-Ardekani, & Rudolf, 2005).

After survival and/or dispersal, the next step in the *R. solanacearum* life cycle is to invade a potential host. Like most other phytopathogenic bacteria, *R. solanacearum* usually enters via a wound that exposes internal tissues. Unlike many phytopathogenic bacteria, *R. solanacearum* potentially requires only one entry site to establish a systemic infection that results in BW. Normal agricultural practices frequently wound plants, either accidentally or intentionally, and infested tools may simultaneously inoculate the pathogen. Growth in natural soil also exposes roots to wounding by a variety of other agents, foremost of which are nematodes. In the case of Sumatra disease of clove trees caused by *R. syzygii*, xylem-feeding spittlebugs (*Hindola* spp.) both disperse the pathogen and introduce it directly into a suitable infection court (Bennet, Hunt, & Asman, 1985; Roberts et al., 1990).

However, 'natural wounds' also appear during normal plant development. For example, in the case of bananas and plantains, abscission of male flowers creates a

moist site with open xylem vessels that can be inoculated by bees and other insects that inadvertently vector the pathogen from diseased plants that are oozing bacteria (Buddenhagen & Kelman, 1964). A more general example is the genesis of lateral roots, because a meristem growing outward from the central cylinder breaks through the endodermis, root cortex and epidermis. Before it heals, the 'wound' at the lateral root axil provides a route for small molecules to reach the xylem (Peterson, Emanuel, & Humphreys, 1981) and, at least for tomato plants, this site also can be invaded by *R. solanacearum* (Araud-Razou, Vasse, Montrozier, Etchebar, & Trigalet, 1998; Kelman & Sequeira, 1965; Schmit, 1978; Vasse, Frey, & Trigalet, 1995).To access 'wounded' tomato roots, the pathogen benefits from both flagellar-mediated swimming motility (Tans-Kersten, Brown, & Allen, 2004; Tans-Kersten, Huang, & Allen, 2001) and chemotaxic attraction to root exudates (C. Allen, personal communication), but these attributes were not essential at the high soil populations used in controlled tests.

After invading a susceptible host, *R. solanacearum* must multiply and move systemically within the plant before BW symptoms occur. The pathogen's goal is to maximize its population size by using its rare ability to exploit the ecological niche within a plant. Consequently, wilting should be considered as the most visible side affect that usually, but not always, occurs after extensive pathogen colonization. There are only a few histological studies of the systemic colonization of host plants by *R. solanacearum* and most of these examined tomato plants. When plants are grown in liquid nutrient medium, bacteria that enter naturally via a lateral root axil multiply in the root cortex for 2 to 6 days before breaching the endodermis (Schmit, 1978; Vasse, Danoun, & Trigalet, 2005; Vasse et al., 1995). The pathogen then colonizes the intercellular spaces within the central cylinder, invades the xylem vessels, and begins rapidly migrating through the vascular tissue (Vasse et al., 1995). In contrast, Wallis and Truter (1978) reported that, after inoculating the severed end of the tomato taproot, the pathogen first multiplies within cells (maybe tracheids or xylem parenchyma) adjacent to vessels. How bacteria invade these cells was not determined. The pathogen migrates into some of the many tyloses that form within the xylem vessels, and bacteria were observed in vessel lumena 3-4 cm above the root tip beginning 3 days after inoculation.

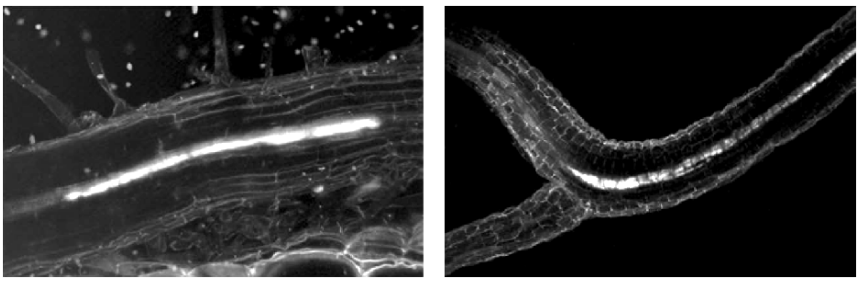

Fig. 4. Confocal microscopy of fluorescent strain AW1-gfp38 colonizing the central cylinder of tomato lateral roots 2 days after soil-drench inoculation.

A somewhat different scenario was observed when unwounded roots of young tomato plants growing in a soil-less potting mixture were inoculated by drenching with strain AW that constitutively expresses green fluorescent protein (GFP) (Denny, unpublished results). The site of pathogen entry was not observed, but within the first day or two the central cylinder of a few lateral roots became brightly fluorescent due to a high density of pathogen cells (Fig. 4). Bacteria also were observed in xylem vessels in the taproot and lower stem 2 or 3 days after inoculation (Fig. 5A) and plants began to wilt after 4 days. Similarly rapid colonization of plants with unwounded roots was observed based on viable cell recovery (Araud-Razou et al., 1998; McGarvey, Denny, & Schell, 1999; Saile, Schell, & Denny, 1997). These results indicate that in soil the pathogen invades a very low percentage of lateral roots almost immediately after inoculation rather than first colonizing the root cortex as observed for roots in liquid culture. Thus, roots of tomato plants in hydroponic culture probably are not well suited for studying natural invasion processes.

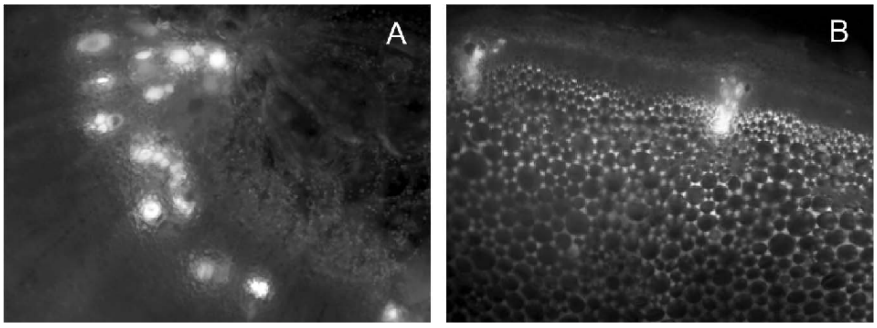

Fig. 5. Epifluorescent microscopy of fluorescent AW1-gfp38 colonizing tomato plants after soil-drench inoculation. A. Bacteria in tap root xylem vessels 3 days post inoculation. B. Bacteria in stem pith intercellular spaces 6 days post inoculation.

Once the pathogen has invaded tomato root xylem vessels there are relatively few physical barriers preventing it from systemically colonizing the remaining plant body. As in most plants, the end walls of functional vessels have completely degenerated, so there is nothing blocking the axial movement of bacteria unless tyloses develop from adjacent parenchyma cells. However, in susceptible tomato plants tylose formation occurs too infrequently or too slowly to prevent pathogen migration, and may instead contribute to vascular dysfunction by obstructing uncolonized vessels (Grimault, Gélie, Lemattre, Prior, & Schmit, 1994; Nakaho, Hibino, & Miyagawa, 2000). Radial movement of bacteria is initially blocked by the pit membranes that separate a colonized vessel from adjacent vessels, xylem parenchyma, or ray parenchyma. The

pathogen tends to congregate near the pits and adjacent parenchyma cells often die. The pit membranes eventually break down and allow bacteria to invade adjacent vessels or the space occupied by dead parenchyma cells (Grimault et al., 1994; Nakaho et al., 2000). Immunofluorescence microscopy and tracking of GFP+ cells revealed that after leaving xylem vessels the pathogen migrates radially through intercellular spaces to colonize primarily the pith, but also the cortex, of roots and stems (Fig. 5B) (McGarvey et al., 1999; Denny, unpublished results).

Wilting begins to appear as pathogen density increases throughout a plant. In a susceptible tomato variety that was root-inoculated, the onset of wilt was correlated with a bacterial density exceeding 4 x 10^7 cfu/g tissue at the midstem (McGarvey et al., 1999) and with one of the major vascular bundles becoming heavily colonized from the taproot to near the shoot tip (Denny, unpublished results). At this time, extracellular polysaccharide (EPS1) content is about 10 μg/g tissue in the taproot, hypocotyl and midstem; EPS1 concentrations later are ≥100 μg/g tissue in fully wilted plants (Denny & Baek, 1991; McGarvey et al., 1999). Wilting is due to vascular dysfunction that prevents sufficient water from reaching the leaves (Buddenhagen & Kelman, 1964; Denny, Carney, & Schell, 1990). There is no evidence for excessive transpiration due to loss of stomatal control as might result from a systemic toxin (Buddenhagen & Kelman, 1964; Van Alfen, 1989). How *R. solanacearum* colonization reduces water flow is not completely clear; the primary factor is probably plugging of pit membranes in the petioles and leaves by the high molecular mass EPS1 (Van Alfen, 1989), but high bacterial cell densities, byproducts of plant cell wall degradation, and plant-produced tyloses and gums may be contributing factors.

6. DISEASES

In 1953 the list of 'true' hosts included plants in 35 families that are naturally infected or highly susceptible when inoculated (Kelman, 1953). Hayward (1994b) updated this host list, and susceptible plants are now known in over 50 families. Some widely cultivated plants, such as sweet potato, strawberry, and groundnut, are only attacked by *R. solanacearum* when they have been moved outside of their normal range (Hayward, 1994b). Many more dicots (Class Magnoliopsida) suffer from BW than do monocots (Class Liliopsida), and five of the nine monocot families that include hosts (*Cannaceae*, *Heliconniaceae*, *Musaceae*, *Strelitziaceae* and *Zingerberfloraceae*) are in the order *Zingiberales*. Why some families have more species susceptible to BW is not known. This section describes disease symptoms on representative hosts and includes some relevant aspects of pathogen diversity and epidemiology.

6.1. BW of Tomato and Tobacco

Tomato is probably the crop most often affected by BW, because it is grown world wide and is susceptible to almost all pathogen strains. The youngest leaves are usually

the first to become flaccid, and in the field this often does not happen until after flowering has begun (McCarter, 1991). Onset of wilt is rapid at warm temperatures (>28°C); it may at first be unilateral, but wilting of the whole plant usually ensues (Fig. 6). The rate of wilt is slower at cooler temperatures, and there may be enough time before the plant dies for adventitious root primordia to appear on the lower and middle stem. Wilting plants are stunted and, if cut open, browning of the vascular system is evident. The stem pith rots during the later stages of the disease either before or after the plant dies. A majority of plants may be affected by BW in fields heavily infested by *R. solanacearum*.

BW of tobacco (also known as Granville Wilt in the USA) occurs in many tobacco-growing countries with moist tropical to warm-temperate climates. Symptoms in tobacco are similar to those on tomato, but unilateral wilt is often more obvious due to the large leaves (Echandi, 1991). During disease onset it is not uncommon for only half of one or two leafs to become flaccid. When the disease progresses slowly leaves on infected plants become light green and then yellow and necrotic areas may appear between veins and at leaf margins (scorch symptoms). Similar to tomato, the vascular system becomes discolored brown and then black, and the pith eventually rots leaving a hollow stem. Lesions may appear on the stem surface.

Fig. 6. Bacterial wilt of tomato. An uninoculated control plant is on the left.

6.2. BW of Potato

BW of potato, often called brown rot, can be caused by strains in biovars 1, 3, 4 and 2-T in warm climates and by biovar 2 (race 3) strains in both warm and cool climates. BW is a limiting factor in production of potato in both lowland and highland tropics (Allen et al., 2001; French, 1994; Lemaga, Kakuhenzire, Kassa, Ewell, & Priou, 2005). In warm climates there may initially be transient wilting only when

transpiration rates are high, but leaves and stems soon wilt permanently (although remaining green) and then die and desiccate. Petiole epinasty may occur. The stem above the soil line may become streaked brown due to discoloration and necrosis of vascular tissues, and extensive secondary rotting may develop. Tubers may not show external symptoms before they begin to rot, but in well established infections vascular discoloration may be visible through the periderm. There may be external signs of the pathogen when bacteria ooze from eyes and the stolon-end attachment site, which may then retain a soil coating (Fig. 7). Tuber internal symptoms usually include distinct grayish brown discoloration of the vascular tissue and necrosis of the vascular ring and adjacent tissues that can be confused with ring rot caused by *Clavibacter michiganesis* subsp. *sepidonicus*.

Unlike most *R. solanacearum* strains, some biovar 2 strains are virulent on potato even when temperatures are $\leq 24^{o}$C (French, 1986; Swanson et al., 2005). These conditions are common at higher elevations in the tropics and at higher latitudes. Thurston (1963) reported epidemics of potato brown rot in Columbian highlands when temperatures averaged ~13^{o}C (23^{o}C maximum). He also demonstrated that potato plants inoculated by stem wounding slowly develop wilt symptoms when incubated constantly at 18^{o}C and more rapidly at 24 and 30^{o}C. Other researchers reported that following inoculation of wounded stems or roots some biovar 2 strains latently infect and cause at least limited wilt symptoms at 16^{o}C and are highly virulent at $\geq 20^{o}$C (Ciampi & Sequeira, 1980; Swanepoel, 1990). In contrast, biovar 1 and 3 strains cause no wilt at <20^{o}C and are highly virulent only at $\geq 24^{o}$C. Recently, Swanson et al. (2005) inoculated unwounded roots of potato and tomato and found that a biovar 2 strain from geranium is more virulent than a biovar 1 strain from tomato at 24^{o}C, but the reverse is true at 28^{o}C. Nothing is known about the genetics or physiology responsible for the ability of biovar 2 strains to cause disease at cool temperatures, except that it is not strongly correlated with the ability to multiply *in vitro* at 16^{o}C (Ciampi & Sequeira, 1980; French, 1986). It is also unclear if all biovar 2 strains exhibit this trait, because relatively few strains have been tested and in one case only 2 of 13 strains from potato were highly virulent at cool temperatures (Ciampi & Sequeira, 1980).

Fig. 7. Bacterial wilt of potato. Tubers with dirt adhering to bacterial ooze emerging from the eyes. Courtesy of Dr. Joe Thurston, Cornell University.

In cooler climates yellowing of the foliage and stunting may appear before wilt or there may be no foliar symptoms. Apparently healthy plants can produce tubers that are latently infected by biovar 2 strains. Processing of infected ware potatoes can release the pathogen into streams and rivers where alternate hosts like *S. dulcamara* can become infected and further infest the watercourse. Irrigating with contaminated water will often result in fields becoming infested by *R. solanacearum* biovar 2 strains (Elphinstone et al., 1996; Janse, 1996).

6.3. BW of Geranium

During the last decade it became apparent that in addition to biovar 1, biovar 2 strains from potato also affect cultivated zonal geranium (*Pelargonium* x *hortorum*) (Janse, 1996; Williamson et al., 2002). Several companies produce geranium cuttings in regions of Central America and Africa were biovar 2 strains are endemic. Irrigation with infested water or other phytosanitary failure results in plants latently infected by *R. solanacearum* and infected cuttings have been shipped to the US and Europe were they are rooted and grown for resale. If the plants are maintained at cool temperatures they may show no disease symptoms, but unknown stresses or incubation at warmer temperatures (>25^{o}C) are conducive to disease. Early symptoms are upward curling and then wilting of lower leaves and subsequent sectorial chlorosis and necrosis. Stems may show external brown to black discoloration and internal discolored vascular tissue. The disease sometimes progresses and the whole plant desiccates and dies, but other times plants 'out grow' the disease (but not the infection). BW of geranium would not be a serious problem for horticulturalists except that detection of biovar 2 infection triggers quarantine restrictions that usually result in destruction of many thousands of plants.

6.4. BW of Banana and Related Plants

Cultivated *Musa* species are hybrids between the diploid species *Musa acuminata* (genome A) and *Musa balbisiana* (genome B). Sterile triploid plants with different proportions of the A and B genomes determine the sweetness or starchiness of the fruit: dessert bananas (commonly Cavendish type) are AAA, whereas cooking bananas may be either AAB (e.g. Plantain type) or ABB (e.g. Bluggoe type) (Jones, 2000). These and other triploid cultivars are propagated vegetatively throughout the humid tropics and provide the main carbohydrate consumed by many of the world's poor. A few varieties are grown on large plantations for export, and they are a major cash crop in some countries (Jones, 2000; Sequeira, 1998). *R. solanacearum* and the BD bacterium cause wilt diseases of *Musa* and *Heliconia* species in Latin America and the Caribbean, the Philippines, and Indonesia (Thwaites et al., 2000). Relevant *R.*

solanacearum strains are in phylotype II, whereas BD bacteria are in phylotype IV (Table 3). Colonies of the BD bacterium are smaller than those of *R. solanacearum* and are non-fluidal. When first isolated they do not use glucose, but readily metabolize galactose and glycerol, and do not reduce nitrate to nitrite (Eden-Green, 1994).

BW of bananas in Latin America and the Caribbean is usually referred to as Moko disease, the name having originated in the early 1900's during an epidemic in Trinidad on the local variety called Moko (a Bluggoe type) that is very susceptible (Thwaites et al., 2000). Serious epidemics of Moko disease in Central and South America during the 1950's and 1960's in commercial dessert banana plantations and in Bluggoe bananas planted in gardens and smallholdings attracted substantial scientific attention. The original literature described two subgroups of *R. solanacearum* stains causing Moko disease based on bacterial cultural characteristics and whether the pathogen is vectored by insects (French, 1986; Sequeira, 1998). Colonies of strains in the 'B' subgroup were described as elliptical with lace-like EPS1 slime and a light pink center on medium containing triphenyl tetrazolium chloride (TZC). This subgroup is synonymous with sequevar 3 (RFLP MLG 24) (Fegan, 2005; Prior & Fegan, 2005). These strains are mainly soil-borne and are transmitted by root-to-root contact, cultivation or pruning. Infected dessert bananas exhibit rapid yellowing and wilting of leaves and suckers, vascular discoloration in the pseudostem, premature fruit ripening and blackening, and dry rot of fruit pulp (Fig. 8). 'B' strains may be transmitted by insects, but this is infrequent because infected plants exude relatively little bacterial ooze.

Colonies of strains in the 'SFR' (small, fluidal, round) and 'A' subgroups were described as being nearly round with little to plentiful faintly lace-like EPS1 slime and a light pink center (in a faint spiral pattern for A types). More importantly, strains in these subgroups are readily insect-transmitted and enter plants via natural wounds on male flowers (Buddenhagen & Kelman, 1964). Bluggoe type cultivars are particularly susceptible, because male flowers abscise every day for several months, whereas flower infection is uncommon in cultivars with persistent bracts. Infected flower buds and peduncles (flower stalks) become blackened and shriveled and there is internal blackening and rotting of fruit of the affected bunch. Copious bacterial ooze exudes from the bases of bracts or abscission sites on diseased peduncles, and some of the many insects that visit these sites become infested. During the 1961 epidemic in Costa Rica, the pathogen was frequently transmitted from diseased inflorescences to uninfected plants nearby and infrequently to plants one mile or more away. Typical Moko disease wilt symptoms may appear months later after the pathogen has systemically colonized the pseudostem and rhizome. Nearby plants not susceptible to flower infection by insects may then exhibit typical Moko disease symptoms after being infected via soil, flood water, root contact, or pruning tools. Genetic analyses show that SFR strains are found in sequevars 4 and 6 (MLGs 25 and 28) (Fegan, 2005; Prior & Fegan, 2005; Raymundo, Aves-Ilagan, & Denny, 1998; Thwaites et al., 1999).

Surprisingly, sequevar 4 is closely related to B strains that cause typical Moko disease (sequevar 3) in the phylotype IIa subcluster, whereas sequevar 6 is in the phylotype IIb subcluster. Therefore, although SFR strains share unusual pathogenic traits, they are not a clonal.

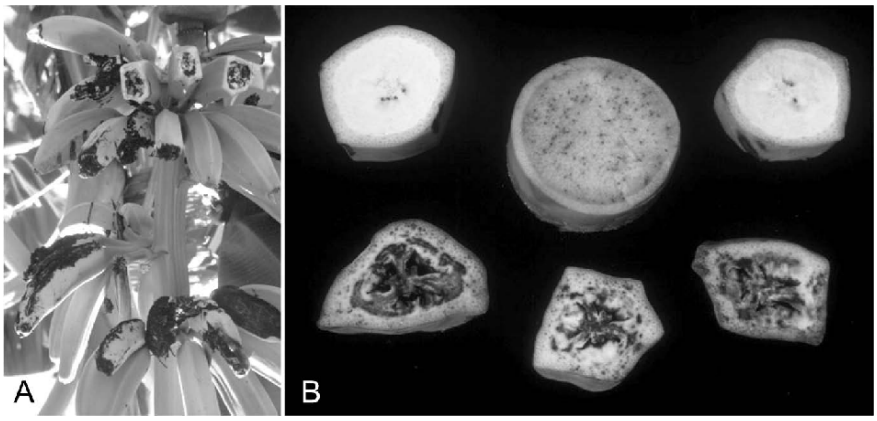

Fig. 8. Moko disease of banana. A. Diseased raceme with severe symptoms on fruit (some have been cut open). B. Cross sections of pseudostem (top middle) and fruit (lower three sections) from a diseased plant. Fruit in upper corners are from a healthy plant. Courtesy of Dr. Joe Thurston, Cornell University.

In the Philippines the local cooking banana varieties Saba (ABB or BBB), Cardaba (ABB or BBB) and Latundan (AAB) suffer from a disease known as Bugtok (or Tapurok), which was already widespread in 1965 when the first description was published (Soguilon, Magnaye, & Natural, 1994; Thwaites et al., 2000). Symptoms are largely confined to the inflorescence, with the peduncle becoming blackened, dry and distorted, and fruit pulp becoming discolored grayish black to yellowish red and later becoming hard. There may be an associated reddish brown discoloration of the vascular tissue of the peduncle and pseudostem, but rarely does discoloration extend into the rhizome. Because the pathogen is never fully systemic there are no wilt symptoms and the plant appears relatively normal to the untrained eye. Bugtok remains a common disease in bananas cultivated by smallholders in the Philippines. Unexpectedly, numerical taxonomy and genetic analyses determined that the *R. solanacearum* strains responsible for Bugtok are indistinguishable from those causing Moko disease on Cavendish type dessert bananas in the Philippines and are very similar to the 'B' subgroup of Moko strains from Honduras (Eden-Green, 1994; Fegan & Prior, in press; Ilagan, Lavina, Natural, & Raymundo, 2003; Raymundo et al., 2005; Thwaites et al., 1999). In addition, the very low genetic diversity among *R. solanacearum* strains isolated from *Musa* spp. in the Philippines (Fegan & Prior, in press; Raymundo et al., 2005) suggests that a single genotype of the pathogen was

introduced from Central America. There probably are several reasons why the 'same' strains can cause two apparently different diseases in the Philippines. First, Bugtok starts with infection of male flowers by insects (similar to infection by SFR strains) rather than via stems or roots like in Moko disease. Only Moko disease is observed in the commercial banana plantations, because the standard control practices of removing male flowers and protecting fruit in insect-proof bags prevent infection of the inflorescence. Second, the cooking banana varieties cultivated in the Philippines may resist systemic infection better than the Bluggoe type (ABB) varieties popular in the Americas. Third, unique environmental factors in the Philippines may make cooking bananas more resistant to systemic infection than they are in the Americas.

In Indonesia cooking and dessert bananas and native *Heliconia* plants suffer from another wilting disease known as blood disease (BD). The pathogen may have originated on Salayar Island near Sulawesi, and BD was first reported in the early 1900's after the introduction of dessert bananas (Eden-Green, 1994; Thwaites et al., 2000). Genetic analyses indicate that there is little diversity among strains of the BD bacterium (Fegan & Prior, in press; Thwaites et al., 1999). BD was 'rediscovered' in Java in the late 1980's (Thwaites et al., 2000) and was common on local cooking banana cultivars in Sulawesi (Stover & Espinoza, 1992). Unfortunately, the pathogen has spread to most of the larger Indonesian islands and average yield losses exceed 35% (Supriadi, 2005). It has also been reported on the island of New Guinea (see (Fegan & Prior, in press)). Symptoms of BD are quite similar to Moko disease caused by insect-transmitted strains in the SFR/A subgroup, namely discoloration and shriveling of the male flower bud and peduncle, reddish dry rot of the fruit pulp, and reddish discoloration of vascular tissue throughout the plant, which emits reddish-brown bacterial ooze when cut. Older leaves become yellow, followed by wilting, necrosis and collapse; younger leaves turn bright yellow before becoming necrotic and dry. The pathogen rapidly colonizes the entire plant, and suckers will also wilt and die. Insect transmission of the BD bacterium is strongly implicated by the sequence of symptoms and the rapid dispersal of the pathogen. However, poor hygienic practices of banana traders may also be a contributing factor. Unlike *R. solanacearum* strains causing Moko and Bugtok, the BD bacterium is not pathogenic on tomato and eggplant seedlings (Eden-Green, 1994; Supriadi, 2005).

6.5. BW of Ginger

Edible ginger is a perennial herb cultivated in the warm, humid tropics. BW of ginger was first reported in Australia and Hawaii during the 1960's (Hayward, 1994b), and it is now a serious problem in Hawaii, Japan, China, India, and several southeast Asian countries (Alvarez et al., 2005; Elphinstone, 2005). In some countries BW is the most important disease of ginger. Typical BW symptoms start with the yellowing and wilting of lower leaves, but the whole plant soon becomes golden brown and wilted. The base of the pseudostem may become watersoaked and will easily break away from

the rhizome (the edible root). Diseased rhizomes are grayish brown with transparent patches covering milky white tissue below. Copious white, milky exudate oozes out after pseudostems or rhizomes are cut.

In Oceania, some biovar 4 strains (race 4) are responsible for BW of ginger (Hayward, 1994b), and genetic analysis showed that they cluster as a relatively homogenous group separate from selected strains from tomato, banana, and potato (Alvarez et al., 2005; Cook et al., 1994). Until recently, the few biovar 3 strains found that attack ginger were weakly aggressive (Alvarez et al., 2005; Hayward, 1994b), but in India a clonal group of biovar 3 strains are more aggressive than biovar 4 strains from the some localities (Kumar et al., 2004). These contrasting findings indicate the necessity for a comprehensive genetic survey of ginger strains from many countries.

6.6. Sumatra Disease of Clove

On the Indonesian islands of Sumatra and Java *R. syzygii* causes a lethal wilting disease of clove trees (*Syzygium aromaticum*), a high value crop grown for manufacture of kretek cigarettes (Bennet et al., 1985; Eden-Green, 1994; Roberts et al., 1990). *R. solanacearum* can colonize the roots and lower trunk, but only *R. syzygii* systemically colonizes and kills clove trees. Observed since the 1920's, Sumatra disease is a recurring problem in the western coastal region of Sumatra, especially on trees planted near forests 400 to 1000 meters above sea level. Seedlings and saplings <2 years old are unaffected, but susceptibility increases with age and most of the trees >10 years old are killed. The first symptom is unseasonal yellowing and leaf-drop from tips of branches high in the crown. Leaves may also wilt suddenly and turn brown, but stay attached (fire blight symptoms). Affected twigs turn reddish brown and progressively die back. Internally, there is a pale grayish-brown discoloration of the newly-formed wood adjacent to the cambium (in an arc or complete ring) that is diagnostic for Sumatra disease. Infected branches often release a milky white to pale brown bacterial ooze from cut surfaces. The discolored xylem can be traced down the trunk into one or more major roots. Symptoms typically progress to lower branches until the whole crown is affected, and the tree dies within 6 to 18 months. Insect transmission of the pathogen by xylem-feeding spittlebugs (*Hindola* spp.) combined with the greater susceptibility of older trees often results in death of all the mature trees in a region. The disease then disappears for years until young trees mature and the cycle repeats. The host range of *R. syzygii* has not been determined. It has been experimentally transmitted to some other myrtaceous species, but if it follows the pattern of some other xylem-limited bacteria, then its host range (including symptomless hosts) might be substantially larger (Purcell & Hopkins, 1996).

7. DISEASE CONTROL

No single strategy can reduce the incidence and/or severity of BW in regions where the pathogen is endemic (Hartman & Elphinstone, 1994; Saddler, 2005). Nevertheless, losses due to BW can be greatly reduced by following a holistic approach, often referred to as Integrated Disease Management (IDM), which employs multiple disease control strategies. For BW, all successful IDM packages include use of pathogen-free planting material, planting less susceptible host varieties, and rotation of susceptible crops with those resistant or immune to BW (Akiew & Trevorrow, 1994; French, 1994; Lemaga et al., 2005; Saddler, 2005).

7.1. Host Resistance

Planting disease resistant cultivars is almost always the most successful, most economical and most environmentally benign disease control strategy (Boshou, 2005). Unfortunately, for most crops susceptible to BW, there are almost no sources of high level, gene-for-gene type resistance encoded by single dominant genes. Instead, available sources of resistance are usually polygenic and it has been difficult or impossible to transfer all the identified quantitative trait loci (QTL) into desirable cultivars due to their number or linkage to undesirable traits. The only exception is groundnut, where dominant resistance genes were identified long ago and introduced into all four botanical types of *Arachis hypogaea* to produce cultivars with high-level resistance. Remarkably, Schwarz 21, a groundnut cultivar developed over 80 years ago, is still resistant in different regions of the world (Boshou, 2005).

The best that normal breeding has achieved for most solanaceous crops is tolerance of BW (i.e., satisfactory yield despite infection) on a regional level when conditions are not excessively hot or wet. Some potato cultivars are less susceptible to BW at least on a regional scale, and they are useful as part on an IDM package (French, 1994; Lemaga et al., 2005). Due to potato's importance as a subsistence crop, there are still active resistance breeding programs, some of which are focusing on resistance to latent infection (Priou et al., in press). Three distinctly different cultivars of eggplant very tolerant of BW have been released for cultivation in India, and might perform well elsewhere (Gopalakrishnan et al., 2005). A small number of tolerant tobacco cultivars with multiple recessive genes have been developed and some have been widely planted (Akiew et al., 1994). However, even the most tolerant tobacco cultivars bred in the USA must be used as part of an IDM package, because they still suffer serious losses when disease pressure is high (Fortnum & Kluepfel, 2005). In contrast, despite extensive international research that has produced some highly resistant tomato breeding lines, such as Hawaii 7996, there are still no acceptable large-fruited cultivars generally available (Prior, Grimault, & Schmit, 1994; Wang, Hanson, & Barnes, 1998).

There are several reports where genetic engineering has increased BW tolerance of tomato and tobacco. One approach is to constitutively activate defense responses (e.g.,

pathogenesis-related (PR genes (Van Loon & Van Strien, 1999)) and other defense-response genes) that are normally induced only after pathogen infection. For example, transgenic tomato lines constitutively expressing the *Arabidopsis NPR1* gene (nonexpressor of *PR* genes) are outwardly normal looking but overexpress a subset of PR genes (Lin et al., 2004). NPR1 protein is a conserved and essential intermediate regulator in salicylic acid and jasmonic acid/ethylene-dependent activation of PR genes and acquired resistance. The *NPR1* overexpressing lines are almost as resistant to BW after soil drench inoculation as the control resistant line Hawaii 7997, and also suppress *R. solanacearum* multiplication *in planta*. Constitutive expression of the tomato stress response factor gene *TSRF1* also enhances BW tolerance in transgenic tobacco and tomato (Zhang et al., 2004). TSRF1 protein is a member of the family of ethylene-responsive factors (ERFs), which are transcription factors that help regulate plant pathogen resistance, abiotic stress tolerance and plant development. Like other ERFs, over production of TSRF1 activates expression PR genes that have promoters containing a GCC box, probably by binding to the GCC motif. It is not known in either example whether resistance is due to over production of PR proteins or to activation of uncharacterized responses.

The second approach for engineering tolerance has been to constitutively express foreign proteins or peptides with antimicrobial activity. *R. solanacearum* is sensitive to some peptides *in vitro* (e.g., potato pseudothionin-St1, lactoferrin, and bovine lactoferricin), but is unusually tolerant of others (e.g. Cecropin B, a cationic lytic peptide, and Shiva-1, a synthetic analog of Cecropin B) (Alan & Earle, 2002; Jaynes et al., 1993; Segura, Moreno, Madueno, Molina, & García-Olmedo, 1999; Zhang, Coyne, Vidaver, & Mitra, 1998). Tobacco expressing Shiva-1 from a wound-inducible promoter are moderately more tolerant to BW than normal, especially when inoculated via a stem wound (Jaynes et al., 1993). Some transgenic potato plants expressing a different Cecropin B analog are similarly more BW tolerant (Montanelli, Stefanini, Chiari, Chiari, & Nascari, 1995). Tobacco and tomato plants that constitutively produce iron-binding human lactoferrin protein are more tolerant to BW than controls and inhibited multiplication of the pathogen *in planta* (Lee, Coyne, Clemente, & Mitra, 2002; Zhang et al., 1998). Tolerance could be due to bacteriostatic sequestration of iron by lactoferrin, the bactericidal lactoferricin peptide released from lactoferrin by proteolysis, or to unknown side effects of the transgenes. However, regardless of the approach used to genetically engineer enhanced BW resistant crops, there are major scientific and societal hurdles that must be cleared before they are ready for field production (Denny, 2005).

7.2. Cultural Control

A variety of cultural practices help to reduce losses due to bacterial wilt. In regions or fields where *R. solanacearum* is not present, the fist line of defense is to avoid introducing the pathogen by using pathogen-free propagative tissue (e.g., tubers and

rhizomes) and good sanitation. In some developed countries, regulatory agencies have promulgated quarantine regulations for biovar 2 (race 3) strains. These may include testing all lots of seed potatoes for latent infection, surveying ware and starch potatoes and destroying loads containing any infected tubers, monitoring surface water and prohibiting use of contaminated waterways for irrigation, and requiring that affected farms disinfest machinery, storage facilities, etc. and plant grasses in infested fields for 4 or 5 years (Janse, Araluppan, Schans, Wenneker, & Westerhuis, 1998). Planting clean seed potatoes is helpful even in regions where the pathogen is endemic, but in developing countries there may not be enough certified seed tubers or they are too expensive for subsistence farmers. An innovative on-farm seed-plot technique pioneered in eastern Africa has the potential to help satisfy farmer's needs for high quality, pathogen-free seed potatoes (Kinyua et al., 2005).

Where *R. solanacearum* is already endemic, the best cultural control is crop rotation. Several grasses are especially effective in reducing BW incidence (Akiew et al., 1994; French, 1994; Hartman et al., 1994; Saddler, 2005). Other crops that are locally desirable can also be effective, with sweet potato in Africa being a good example (Lemaga et al., 2005). Elimination of volunteer plants and weeds that promote survival of *R. solanacearum* is an important component of successful rotation. Rotation may provide an additional advantage if it reduces nematode populations that attack the susceptible crop. The number of years that a rotation crop must be grown depends on the level of infestation, the survival capacity of the pathogen in local soils and climate, and other factors. In some cases a single year of rotation can significantly reduce BW (at least in the short term), but the usual recommendation is for a two or three year rotation schedule. Bare fallowing and flooding can serve the same purpose (Hartman et al., 1994), but generally are not feasible. Unfortunately, farmers often do not adopt effective rotations because of limited land availability and pressures to produce a subsistence crop or one with high cash value.

A variety of other cultural controls are available, some of which are crop or region specific. One of the simplest is to shift cropping dates to avoid the peak periods of warm and/or moist conditions conducive to disease. Soil amendments, such as organic matter (compost, bagasse, rice husk powder), inorganic fertilizers, or other material like oyster shell powder, may modify native soil microbial communities to suppress the size or activity of the *R. solanacearum* population, but have not been widely studied or generally applied (Lemaga et al., 2005; Saddler, 2005; Schönfeld et al., 2003; van Elsas, van Overbeek, Bailey, Schönfeld, & Smalla, 2005a). Solarization appears to be marginally effective at best, and in one case the *R. solanacearum* population increased in treated beds (Saddler, 2005; Sharma, Rajesh, Dohroo, & Rajesh, 2004; van Elsas et al., 2005a). Grafting susceptible, horticulturally desirable scions of eggplant and tomato onto BW-resistant rootstocks reduces disease and increases yield (Grimault & Prior, 1994a; Nakaho et al., 2000), and has been used

successfully in Japan, India and southeast Asia. For banana and plantain, a combination of sanitation, roguing of diseased plants and those nearby (and soil disinfestation), removing the male flower and bagging fruit clusters all help to control BW.

7.3. Chemical Control

Commercial chemicals and antibiotics generally are ineffective in controlling BW (Hartman et al., 1994; Saddler, 2005). One exception may be fumigation with chloropicrin either alone or combined with other fumigants (Enfinger, McCarter, & Jaworski, 1979; Fortnum & Martin, 1998) or with solarization. However, for tobacco in the southeastern USA, fumigation was not as effective as rotation or use of tolerant cultivars. Moreover, fumigation is environmentally destructive and the cost effectiveness of fumigation has not been examined, so this strategy is unlikely to be part of an IDM package in developing countries.

7.4. Biological Control

There has been a great deal of interest in finding bacteria that can be coated on propagating tissues or added to soil to reduce infection of susceptible crops or reduce *R. solanacearum* populations, respectively (Prior, Allen, & Elphinstone, 1998; Akiew et al., 1994; Hartman et al., 1994; Saddler, 2005; Trigalet, Frey, & Trigalet-Demery, 1994). Theoretically, biological control agents (BCAs) may work directly by competing with the pathogen for limited resources in the soil, the rhizosphere, or within the plant, or by producing antibiotics, bacteriocins, or bacteriophage. BCAs may also work indirectly by stimulating plant defense capabilities. BCAs often are nonpathogenic bacteria, but the greatest effort on developing a BCA for *R. solanacearum* has focused on non-pathogenic *hrpO* mutants of the pathogen. Unfortunately, although showing promise when tested in controlled conditions, none of the potential BCAs have effectively or consistently reduced BW in field conditions. Development of a useful, affordable BCA is unlikely in near term, but progress is being made and there is still hope for success in the future.

8. MECHANISMS OF PATHOGENESIS

The biochemical and genetic mechanisms underlying *R. solanacearum* pathogenesis have been studied for over 50 years (Denny, 2005). In this literature, virulence factors are defined as those which contribute to the incidence, rate or severity of wilt symptoms, whereas pathogenicity factors are essential for disease. Most virulence factors enhance the pathogen's ability to colonize host tissues systemically and to reach populations $>10^8$ cfu/g tissue; disease symptoms appear only after, and largely because of, this extensive colonization. The expectation is that basic research into *R. solanacearum*'s pathogenic mechanisms will result in new or improved approaches

for disease control. This topic has been recently reviewed (Denny, 2005; Hayward, 1995; Schell, 2000), so work published in the last five years will be emphasized.

Compared to most other aspects of *R. solanacearum* pathobiology, the mechanisms of pathogenesis have been studied by relatively few research groups using only a few pathogen strains. Strain K60 (the type strain; phylotype II, biovar 1), isolated from a wilted tomato in North Carolina, was first studied by A. Kelman, then by L. Sequeira, and most recently by C. Allen. Strain AW, which was isolated from tomato in Alabama and is genetically and biologically similar to K60, has been studied primarily by T. Denny and M. Schell. Strain GMI1000 (phylotype I, biovar 3) was isolated from tomato in French Guyana and used primarily to investigate pathogenicity factors by a French consortium lead by C. Boucher. The complete genomic sequence of GMI1000 is available (Salanoubat et al., 2002). All three strains are highly virulent on tomato, the primary host for pathogenesis research, but only K60 causes BW of tobacco.

8.1. Virulence Factors

8.1.1. Extracellular Polysaccharides

A major virulence factor of *R. solanacearum* is its acidic extracellular polysaccharide EPS1, a long (>10^6 Da) polymer with a repeating unit of three unusual *N*-acetylated monosaccharides (Orgambide et al., 1991). EPS1 is produced in massive amounts by *R. solanacearum* on various laboratory media and in plants (Araud-Razou et al., 1998; McGarvey, Bell, Denny, & Schell, 1998). Tests with EPS1-specific antibodies show that diverse *R. solanacearum* strains produce EPS1 (or a very similar polymer) and that 85% of the EPS1 is released as a cell-free slime (McGarvey et al., 1998). Many proteins comprising the EPS1 biosynthetic pathway are encoded by the 16-kb *eps* operon. Mutants of *R. solanacearum* unable to produce EPS1 rarely wilt or kill plants, even when bacteria are injected directly into the stem (Araud-Razou et al., 1998; Saile et al., 1997). Potted plants inoculated by soil-drenching, the most natural laboratory method available, showed that EPS1 promotes rapid systemic colonization of tomato plants, because EPS1$^-$ mutants generally colonize only the roots and lower stems (Saile et al., 1997). In contrast, EPS1$^-$ mutants do not move into the xylem vessels of hydroponically-grown plants, but instead multiply in the intercellular spaces of the root cortex (Araud-Razou et al., 1998).

Lipopolysaccharide (LPS), consisting of lipid A, core polysaccharide, and O-antigen polysaccharide, is a major component of the outer leaflet of the outer membrane of Gram-negative bacteria, making it the primary environmental interface. Most *R. solan-acearum* strains characterized have an O-antigen repeating unit that contains three rhamnose and one acetylglucosamine (Kocharova et al., 1993; Varbanets, Kocharova, Knirel, & Moskalenko, 1996). Studying the role of LPS has been difficult, because LPS mutants are usually pleiotropic. For example, mutations that interfere with synthesis of the core polysaccharide in K60 reduce virulence (Kao

& Sequeira, 1991; Titarenko, López-Solanilla, García-Olmedo, & Rodríguez-Palenzuela, 1997), but they also reduce production of EPS1 and increase sensitivity to plant antimicrobial peptides. Many years of research in Sequeira's lab suggested that the LPS could play several roles in host-pathogen interactions (Sequeira, 1985), but without well-characterized LPS-minus mutants the overall results were inconclusive. Recently, Kang et al. (2004) specifically blocked synthesis of the O-antigen side-chain without affecting production of EPS1 by inactivating the gene (*waaL*; RSc2204) in GMI1000 that appears to encode the lipid A core:O-antigen ligase, an enzyme that couples the completed O-antigen oligosaccharide subunits to the core polysaccharide (Whitfield, 1995). The mutant is resistant to an LPS-specific phage and analysis of purified LPS indicated that it lacks the O-antigen moiety. Although the mutant is normal for multiple other traits, it is dramatically less virulent than the wild type. These results suggest that the O-antigen contributes to *R. solanacearum* virulence, but the mechanism remains undetermined.

8.1.2. Protein Appendages: Flagella and Pili (Fimbriae)

R. solanacearum can produce two or three lophotrichous flagella, but in a rich broth culture cells are motile only at cell densities between 10^7 and 10^9 cells ml^{-1} (Clough, Flavier, Schell, & Denny, 1997). Similarly, bacteria recovered from within tomato plants are overwhelmingly nonmotile (Tans-Kersten et al., 2001) and microscopic observations of GFP+ *R. solanacearum* in tomato plants confirms this observation (Denny, unpublished results). K60 Mutants that are nonflagellated due to inactivation of the flagellin structural gene (*fliC*) are reduced in virulence when applied to potted tomato plants in a soil drench, but exhibit normal virulence when inoculated via a severed petiole (Tans-Kersten et al., 2001). This suggests that flagella are not important for pathogenesis once bacteria are inside a tomato plant. Non-chemotactic K60 mutants are similarly reduced in virulence when inoculated by soil drench (Allen, personal communication). Unlike the flagellin protein from some bacteria (Asai et al., 2002), *R. solanacearum* FliC does not trigger an innate defense response by *Arabidopsis thaliana* or tobacco (Pfund et al., 2004).

Polar, retractable, type 4 pili (Tfp) are produced by diverse bacteria, and give them the ability to migrate over solid surfaces, a process called twitching motility (Strom & Lory, 1993). *R. solanacearum* strains make Tfp composed of PilA protein, and Tfp are essential for twitching motility and virulence on tomato when plants were inoculated either by a soil drench or via severed petioles (Kang, Liu, Genin, Schell, & Denny, 2002). A Tfp-minus strain also is reduced in autoaggregation and biofilm formation in broth culture, and does not exhibit polar attachment to cultured tobacco cells or to tomato roots. The Hrp pili made by *R. solanacearum* will be discussed below.

8.1.3. Plant Cell Wall Degrading Enzymes (CWDEs)

The involvement of *R. solanacearum* CWDEs in virulence has been examined in all three strains, usually with tomato as the host (González & Allen, 2003; Liu, Zhang, Schell, & Denny, in press; Schell, 2000). Six extracellular enzymes have been identified: a β-1,4-endoglucanase (Egl), an exoglucanase (ChbA), an endopolygalacturonase (PehA or PglA), two exopolygalacturonases (PehB and PehC), and a pectin methylesterase (Pme). Inactivation of single genes has shown that none of these exoenzymes is essential and that their relative contribution to disease may vary with the strain. In K60, PehA and PehB, but not PehC or Pme, contribute to pathogen colonization and wilt severity (González & Allen, 2003; Huang & Allen, 2000; Tans-Kersten, Guan, & Allen, 1998), but the cellulolytic enzymes have not been studied. The PehA-PehB double mutant was less virulent than either single mutant (Huang & Allen, 2000), but eliminating PehC in addition partially restored virulence (González & Allen, 2003). This counterintuitive result was attributed to the absence of pectic breakdown products that stimulate plant defenses (see also Jha, Rajeshwari, & Sonti, 2005). In GMI1000, however, where mutants lacking one to all six enzymes were recently studied, no combination of the four pectolytic enzymes contribute significantly to disease when using a soil drench inoculation (Liu et al., in press). Both Egl and CbhA contribute to virulence of GMI1000 regardless of the inoculation method, and the Egl-CbhA double mutant is the least virulent CWDE mutant strain. Similar to the results for K60, simultaneous elimination of Peh enzymes consistently enhances virulence of GMI1000. Why the CWDEs do not contribute equally to virulence of K60 and GMI1000 is not known.

8.1.4. Other Potential Virulence Factors

In *R. solanacearum* most of the CWDEs and many other extracellular proteins transit the inner membrane via the Sec-dependent general export pathway and the outer membrane via the type II secretion system (T2SS) (Preston, Studholme, & Caldelari, 2005; Schell, 2000). The one known exception is PehC, which is exported by the twin-arginine translocation (Tat) system (González and Allen, personal communication) and secreted by the T2SS. Inactivation of the T2SS results in proteins that normally are secreted accumulating in the periplasm or cytoplasm. A T2SS mutant of strain AW does not secrete CWDEs, multiplies less than the wild type in tomato stems, and does not cause wilt symptoms (Kang, Huang, Mao, He, & Schell, 1994). A more thorough examination of a GMI1000 T2SS mutant lacking its outer membrane secretin (SdpD), found that it does not secrete any of the six CWDEs and is much less virulent than either the Egl-CbhA double mutant or a mutant lacking all six CWDEs (Liu et al., in press). The T2SS mutant also colonized the lower stem of about 50% fewer plants than the wild type. These results indicate that extracellular proteins in addition to Egl and CbhA contribute to the ability of GMI1000 to systemically colonize tomato plants.

It has long been suspected that phytohormones produced by *R. solanacearum* might contribute to virulence (Buddenhagen & Kelman, 1964; Hayward, 1995).

Infected plants may exhibit leaf epinasty and over production of adventitious root primordia, suggesting an imbalance in indoleacetic acid (IAA) or auxin. K60 produces relatively little IAA in culture and increased IAA concentrations in plants may be due the pathogen reducing its degradation. *R. solanacearum* can make cytokinin and ethylene. Inactivation of the *tzs* gene responsible for production of cytokinin (Akiyoshi, Regier, & Gordon, 1989) reduces the virulence of strain AW, but inactivation of the gene encoding the putative ethylene-forming enzyme (RSp1529), which makes ethylene and succinate from oxoglutarate, does not affect virulence (Ratnayake, 2002).

Many bacteria produce siderophores (low-molecular mass extracellular iron-scavenging compounds) to help them acquire essential iron, which is often in forms that are biologically unavailable to aerobes (Andrews, Robinson, & Rodriguez-Quinones, 2003). Some phytopathogenic bacteria need their iron acquisition system for full virulence whereas others do not. *R. solanacearum* strain K60 had been reported to produce the dihydroxamate siderophore called schizokinen, but more recent work showed that this strain (and probably all seven others tested, including AW and GMI1000) produce the polycarboxylate siderophore staphyloferrin B (Bhatt & Denny, 2004). However, tomato xylem fluid may have sufficient iron to repress expression of the pathogen's iron-acquisition system, and a mutant that does not make staphyloferrin B is fully virulent on tomato.

8.1.5. Regulated Production of Virulence Factors

Spontaneous loss of virulence, EPS1 and other traits by *R. solanacearum* in culture was until recently a vexing and perplexing problem for scientists studying BW (Denny, 2005; Kelman, 1953; Sequeira, 1985). Although there are ways to cope with this propensity (Buddenhagen & Kelman, 1964), the genetics of phenotype conversion (PC) remained unclear until the discovery of PhcA, a LysR-type transcriptional regulator that controls expression of many virulence genes (Fig. 9) (Brumbley, Carney, & Denny, 1993; Schell, 2000). There is no evidence that *phcA* spontaneously mutates at a high frequency; instead, *phcA* mutants (PC-types) accumulate because they are selected for during some stressful conditions (e.g., prolonged stationary phase in culture or *in planta*, high salt or low oxygen concentrations) (Denny, Brumbley, Carney, Clough, & Schell, 1994). DNA replication errors and transposition of IS elements can inactivate *phcA* (Brumbley et al., 1993; Jeong & Timmis, 2000; Poussier et al., 2003). Traits in strain AW currently known to be positively regulated directly or indirectly by PhcA are: (i) production of EPS1, (ii) production of Egl, (iii) production of Pme, (iv) competence for natural transformation by DNA, and (v) an acyl-homoserine lactone quorum sensing system (Kang et al., 2002; Schell, 2000). Negatively regulated traits (i.e., those expressed better in PC-types) are: (i) production of PehA, (ii) production of staphyloferrin B siderophore, (iii) production of type 4 pili and thereby twitching motility, autoaggregation and biofilm formation, (iv) flagellar motility at high cell density, (v) salt tolerance, and (vi) activity of the HrpG transcriptional regulator (Bhatt & Denny, 2004; Genin, Brito, Denny, & Boucher,

2005; Schell, 2000). There are likely to be additional genes controlled by PhcA. The acyl-homoserine lactone quorum sensing system does not regulate virulence, but it likely controls more than just *aidA* (whose function is unknown). Although some PC-types are stimulated to revert to wild type by plant compounds (Poussier et al., 2003), spontaneous mutation of *phcA* is usually just a genetic mistake and a pathological dead end for *R. solanacearum*. However, wild type PhcA plays a critical role as part of a complex regulatory network that enables *R. solanacearum* to cycle between two very different phenotypic states (i.e., reversible PC) in response to nutrient availability and cell density (Denny, 2005; Schell, 2000). Levels of functional PhcA are controlled by a unique confinement-sensing system encoded by the *phcBSR* operon. PhcB appears to be a small-molecule methyltransferase that synthesizes 3-OH palmitic acid methyl ester (3-OH PAME), which accumulates in the extracellular environment when bacteria are growing rapidly within a confined space (Fig. 9). PhcS and PhcR comprise a two-component regulatory system that senses and responds to threshold concentrations of 3-OH PAME by elevating the level of functional PhcA. It is likely that 3-OH PAME stimulates PhcS to phosphorylate PhcR, and that PhcR ~ P lacks the ability to post-transcriptionally inhibit production of functional PhcA. In other words, cells at low-density have little functional PhcA and, like *phcA* mutants, exhibit a low virulence phenotype. These cells are hypothesized to be optimized for survival and invasion of plant tissues, because low densities of *R. solanacearum* routinely occur in soil and in plants at the leading edge of infection. In contrast, cells at high densities, like those in well colonized xylem vessels, have abundant functional PhcA and so produce multiple virulence factors (while suppressing production of survival/invasion factors) that promote multiplication and further colonization of tissues. Functional PhcA activates expression of some genes, like *egl* and *xpsR*, directly by binding to their promoter, while traits like EPS1 are controlled indirectly (Fig. 9) (Schell, 2000). Most research on confinement-sensing was done in culture, but the *eps* operon is similarly regulated in tomato plants during pathogenesis (Kang, Saile, Schell, & Denny, 1999; McGarvey et al., 1998).

PhcA has a central role in regulating virulence, but there are additional regulatory proteins in the supporting cast (Fig. 9) (Schell, 2000). Transcription of the *eps* operon is the best example, because downstream of PhcA is a complex cascade that may respond to two additional signals. Functional PhcA first activates transcription of *xpsR*, an intermediate regulator whose expression is also enhanced by the VsrA/VsrD two-component system. XpsR, a unique and very basic protein, then works in conjunction with VsrC, a response regulator paired with the VsrB sensor, to activate *eps* transcription (Garg, Huang, Yindeeyoungyeon, Denny, & Schell, 2000). The signals, if any, sensed by VsrA and VsrB are not known. VsrC binds to a 20 nucleotide region of the *eps* promoter that is also essential for activation by XpsR. The biochemical role of XpsR remains unknown due to poor solubility of the purified protein. EpsR, an atypical response regulator-type protein (Chapman & Kao, 1998), inhibits EPS1 production when borne on a multicopy plasmid, and binds to the same 20 nucleotide region. However, since inactivation of *epsR* in the genome has no major effect on *eps* expression or EPS1 production, the physiological role of EpsR remains obscure.

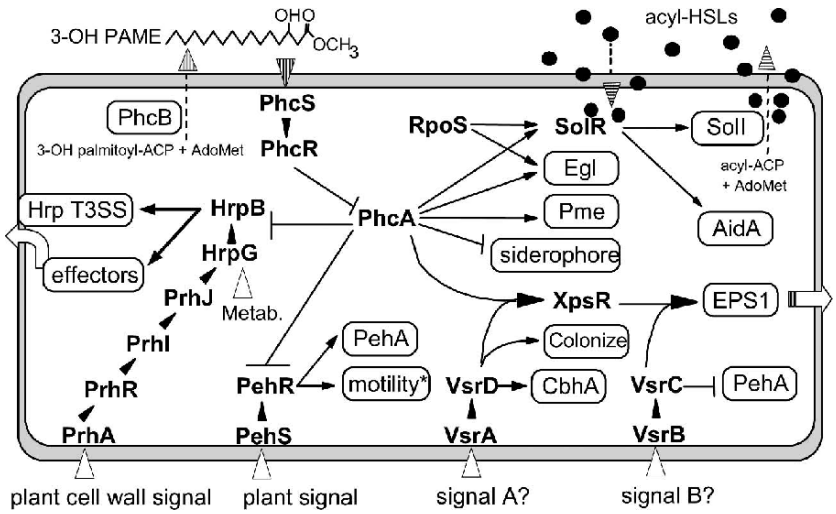

Fig. 9. Model of the regulatory networks in *R. solanacearum*. The major circuits are shown, but there is cross talk between many of these pathways (Brown and Allen, 2004). Within this network the four known two-component regulatory systems are PhcS-PhcR, PehS-PehR, VsrA-VsrD, and VsrB-VsrC. Except for 3-OH PAME, the novel autoinducer sensed by PhcS-PhcR, the signals for the two-component systems are not known. Other transcriptional regulators are PhcA, SolR (that responds to acyl-homoserine lactones (AHLs)), and PrhI, PrhJ, HrpG, and HrpB in the Prh/Hrp signal cascade. Also involved with regulating transcription are RpoS (an alternative sigma factor) and XpsR (a signal integrator). Proteins essential for biosynthesis of extracellular signal molecules are PhcB (predicted to be an S-adenosylmethionine-dependent methyltransferase) and SolI (an AHL synthase). Predicted substrates for these enzymes are 3-OH palmitoyl-acyl carrier protein (3-OH palmitoyl-ACP), C6- and C8-acylated-acyl carrier proteins (acyl-ACPs), and S-adenosyl-methionine (AdoMet). AidA is a protein of unknown function; its structural gene is regulated by SolR in response to sufficient concentrations of acyl-HSLs. Dashed lines with hatched arrowheads represent diffusion of signal compounds into and out of the cell. The metabolic/nutritional signal affecting hrp gene expression is not known; also unknown is whether this signal affects HrpG activity, the step between HrpG and HrpB, or HrpB directly. Motility refers to both flagellar swimming and type 4 pili-mediated twitching. Open arrowheads represent perception of extracellular signals by two-component sensors; filled arrowheads represent presumed transfer of phosphate from sensor proteins to response regulators. Lines with filled arrowheads or bars represent positive or negative control, respectively.

Both the VsrBC and VsrAD two-component systems regulate other traits. Inactivation of either *vsrB* or *vsrC* increases PehA production about seven fold by an

undetermined mechanism. Inactivation of *vsrA* or *vsrD* strongly reduces production of CbhA and largely eliminates the ability of *R. solanacearum* to cause disease symptoms. The poor ability of a *vsrAD* mutant to colonize stems and multiply *in planta* is not due to the reduced EPS1 production that accompanies loss of this regulator (McGarvey, 1999), so it is likely that VsrAD activates expression of some genes that promote rapid *in planta* growth and colonization.

Unexpectedly, pectinolytic CWDEs are not coordinately controlled, since functional PhcA activates production of Pme, represses production of PehA, and has little effect on PehB and PehC (Schell, 2000). PhcA similarly controls both flagellar and twitching motility, because unlike in the wild type, *phcA* mutants express both swimming and twitching motility at high cell density. Functional PhcA indirectly controls PehA production and motility by reducing the function of the PehSR two-component system that positively controls their expression (Fig. 9). Expression of *pehR* is activated by an uncharacterized plant signal (Tans-Kersten et al., 2004), and its inactivation results in loss of PehA and flagellar and twitching motility. In culture, PehR controls flagellar motility by activating expression of *flhDC*, which encode a tetrameric regulatory protein, but *in planta* there appear to be other activators of *flhDC* expression and repressors of motility (Tans-Kersten et al., 2004). PehR controls twitching motility by activating expression of *pilA*, which encodes the major pilin protein (Kang et al., 2002).

8.2. Hypersensitive Response and Pathogenicity (Hrp) System

Most bacteria that kill plant cells during pathogenesis have a type III secretion system (T3SS) similar to that in some animal pathogens (He, Nomura, & Whittam, 2004; Preston et al., 2005). *R. solanacearum* was among the first phytopathogenic bacteria found to harbor a T3SS, and mutants lacking this system have the typical *hrp* phenotype: HR-negative on nonhosts (usually tobacco) and nonpathogenic on hosts (usually tomato) (Schell, 2000; van Gijsegem, Vasse, De Rycke, Castello, & Boucher, 2002). Similar to other phytopathogenic bacteria, *R. solanacearum* T3SS mutants multiply very little after being infiltrated into a host's leaves. They retain the ability to invade unwounded roots of tomato and to colonize the tap root and lower stem as rapidly as the wild type, but they poorly colonize the upper half of infected tomatoes. The population size of T3SS mutants in infected tissues is typically 100 to 1000-fold lower than for the wild type (Etchebar, Trigalet-Demery, van Gijsegem, Vasse, & Trigalet, 1998; Frey et al., 1994; Vasse, Genin, Frey, Boucher, & Brito, 2000), but in young plants grown in conducive conditions the mutant population can equal that of the wild type and cause transient wilt (Denny, unpublished results).

The primary purpose of the T3SS probably is to secrete 'effector' proteins into the cytoplasm of plant cells, where they facilitate nutrient release and/or suppress basal defense responses (Alfano & Collmer, 2004; Büttner & Bonas, 2002a; He et al.,

2004). Individual effectors usually have subtle and nonessential biochemical functions, so determining their role in pathogenicity has been difficult. However, some plants have evolved (or been bred for) proteins that recognize an effector and trigger a rapid, vigorous defense response, often manifested as a HR (Keen, 1990; Nimchuk, Eulgem, Holt, & Dangl, 2003; White, Yang, & Johnson, 2000). In such cases, the effector is called an avirulence protein because it restricts the pathogen's host range by making it nonpathogenic (incompatible) on a resistant host. Thus, a single effector protein can be a 'double agent' (Alfano & Collmer, 2004) by promoting pathogenesis in a susceptible cultivar, but triggering HR in a resistant cultivar.

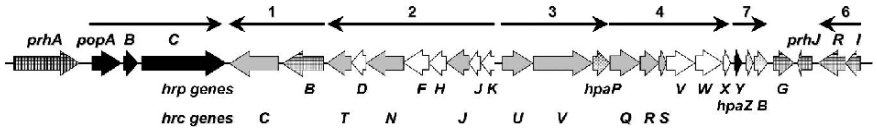

Fig. 10. Genetic organization of the *R. solanacearum* hrp/hrc gene cluster. Thin arrows indicate operons; thick arrows indicate genes. Conserved hrc genes are represented by filled grey arrows, genes encoding T3SS secreted proteins by filled black arrows, hpa genes by stippled arrows, and regulatory genes by hatched arrows. Modified from Van Gijsegem et al., 2002 (with permission).

8.2.1. The hrp/hrc *Gene Cluster and the T3SS*

In *R. solanacearum* the T3SS is encoded by a gene cluster containing five transcriptional units located in a 23-kb region of the megaplasmid (Fig. 10) (van Gijsegem et al., 1995; van Gijsegem et al., 2002). Most genes in the GMI1000 T3SS are very similar to and nearly syntenic with those in several *Xanthomonas* species (the hrp2 group (He et al., 2004)), but the gene cluster is arranged quite differently in *P. syringae* and *Erwinia* spp. (the hrp1 group). Available evidence indicates that the GMI1000 T3SS co-evolved with the rest of the genome rather than being recently acquired by horizontal gene transfer (Salanoubat et al., 2002). The nine *hrc* (*hrp* conserved) genes encode proteins essential for the core secretion apparatus (the secreton) of all T3SS. Most Hrc proteins localize to the inner membrane of Gram-negative bacteria, with the exceptions being the HrcN ATPase, which is cytoplasmic, and the HrcC secretin, which creates a pore in the outer membrane. In contrast, the *hrp* genes encode proteins with more species-specific roles in translocating effector proteins into the plant cell cytoplasm. In GMI1000, *hrpB*, *hrpF*, *hrpK*, *hrpW*, *hrpX* and *hrpY* are essential for the HR in tobacco, virulence on tomato, production of a Hrp pilus, and secretion of the PopA protein (see below), but *hrpD*, *hrpH*, *hpaP* (*hrp* associated), *hrpV*, and *hrpJ* are variably impaired in these traits. (Schell, 2000; van Gijsegem, Vasse, Camus, Marenda, & Boucher, 2000; van Gijsegem et al., 2002). Mutants of *hpaZ*, which encodes a HrpY-like protein, and *hpaB* have not been studied in GMI1000. However, in *R. solanacearum* strain RS1000 (biovar 4, from Japan), *hpaZ* mutants exhibit wild-type virulence and HR induction, but *hpaB* mutants are

negative for both traits despite production of Hrp pili (Mukaihara, Tamura, Murata, & Iwabuchi, 2004). *hrpY*, *hpaZ* and *hpaB* are all regulated by HrpB (see below) and constitute operon 7 in RS1000 (Fig. 10) (Cunnac, Occhialini, Barberis, Boucher, & Genin, 2004b; Mukaihara et al., 2004).

Only a few Hrp proteins have well characterized functions. The best example is HrpB, which is an AraC-type transcriptional regulator that activates expression of the other transcriptional units in the *hrp/hrc* gene cluster as well as a majority of the known and putative effector proteins (see below) (Cunnac, Boucher, & Genin, 2004a; Cunnac et al., 2004b; Mukaihara et al., 2004). It is also clear that HrpY is the major subunit of the *R. solanacearum* Hrp pilus (Kang et al., 2002; van Gijsegem et al., 2000), and probably forms a hollow conduit through which these proteins move (He et al., 2004). However, HrpY pili are not required for the polar adherence of *R. solanacearum* bacteria to cultured plant cells (van Gijsegem et al., 2000). HrpX is essential for assembly of Hrp pili (but not for secretion of HrpY pilin), but how is unknown (van Gijsegem et al., 2002). HrpJ is suggested to function in translocation of a subset of effectors, because a *hrpJ* mutant has a very low HR-inducing potential but is almost normally virulent on tomato. HrpV and HpaB may have a similar functions in effector 'exit control', because they are orthologous to the better characterized HpaA and HpaB proteins in *X. campestris* pv. vesicatoria (Büttner, Gurlebeck, Noel, & Bonas, 2004; Huguet, Hahn, Wengelnik, & Bonas, 1998) and *hpaB* mutants of RS1000 are nonpathogenic and HR-negative despite making Hrp pili (Mukaihara et al., 2004). HrpV is also needed for efficient display of Hrp pili (van Gijsegem et al., 2002).

The *hrp/hrc* gene cluster in *X. campestris* pv. vesicatoria also has *hrpF*, which is not present in the 23-kb *hrp/hrc* region of GMI1000. *hrpF* is not required for secretion of effectors into the culture supernatant, but it is required for translocation of a the AvrBs3 avirulence protein into the plant cytosol (Rossier, Van den, & Bonas, 2000). HrpF also exhibits *in vitro* lipid-binding activity and pore-formation potential (Büttner, Nennstiel, Klusener, & Bonas, 2002), so it is proposed to be part of an oligomeric protein channel (the translocon) in the plant cell membrane (Büttner & Bonas, 2002b). GMI1000 has two genes, *popF1* and *popF2* (*Pseudomonas* outer protein), that encode proteins about 50% identical to HrpF. These genes are located on the megaplasmid >30 kb away from the *hrp/hrc* cluster. Similar to HrpF, PopF1 and PopF2 are both secreted by the T3SS and, although essential for translocation of the AvrA avirulence protein into plant cells, they are not required for assembly of Hrp pili or secretion of proteins to the supernatant (M. Arlat, personal communication). If additional work proves that PopF1 and PopF2 are part of the translocon, then they are members of the *hrp* subset of T3SS genes and probably should be renamed to reflect this status.

8.2.2. Secreted Proteins and Effectors

The definition of an effector is in flux now, but I use it to refer only to proteins that are translocated via the T3SS directly into the host cell (Alfano & Collmer, 2004). Although ten GMI1000 proteins are known to transit the T3SS, only six have been proven to be effectors. Nevertheless, bioinformatics and transcriptomic analyses suggest that GMI1000 may produce 60-80 effectors (Genin & Boucher, 2004).

Avirulence Proteins. The best known effectors are those which trigger a HR in plants that have a effector-specific recognition system, commonly called a gene-for-gene interaction (Keen, 1990). There are only a few gene-for-gene type interactions between *R. solanacearum* and a host, so unlike phytopathogenic pseudomonads and xanthomonads, there are few known avirulence effectors. The first avirulence gene (*avrA*) was found in AW and encodes a protein necessary for this strain to elicit a HR on 27 cultivars of tobacco (*Nicotiana tabacum*) (Carney & Denny, 1990). Thus, unlike most avirulence proteins that determine cultivar-level specificity, AvrA determines host specificity of AW on *N. tabacum* at the species level. GMI1000 has an AvrA ortholog that is 60% identical to AvrA in AW, and despite lacking 33 amino acids (from four locations within the protein) it is responsible for this strain eliciting a HR on *N. tabacum* cultivars (Carney & Denny, 1990; S. Genin, personal communication). Furthermore, GMI1000 translocates AvrA fused in frame to the *Bordetella pertussis* calmodulin-dependent adenylate cyclase reporter (CyaA=) into plant cells (S. Genin, personal communication), and transient expression of *avrA* from GMI1000 in *N. tabacum* cells elicits a HR and. In K60, which is virulent on tobacco, *avrA* has been inactivated by insertion of a miniature inverted transposable element (Robertson et al., 2004). Inactivation of *avrA* is at least partly responsible for the virulence on tobacco of K60 and most of the *R. solanacearum* strains isolated from tobacco in North and South Carolina, USA. AvrA is not homologous to any protein in the GenBank nonredundant database, so bioinformatics has provided no clues as to its potential biochemical function.

PopP1 and PopP2 in GMI1000 are encoded by genes in chromosomal regions with atypical codon usage and both are secreted by the T3SS into the culture medium (Deslandes et al., 2003; Lavie, Shillington, Equiluz, Grimsley, & Boucher, 2002). GMI1000 also carries an inactivated *popP3* gene (Lavie, Seunes, Prior, & Boucher, 2004). Most of the strains in phylotypes I and III have one or more of the *popP1*, *popP2* or *popP3* genes, but they are all absent in K60 and most other phylotype II strains with the exception of strains in MLG 25 (sequevar 4; mostly from banana) (Lavie et al., 2004). Although PopP1 and PopP2 are only slightly related, amino acid sequence analysis shows that they both are members of the YopJ/AvrBsT family of ubiquitin-like cysteine proteases (Alfano & Collmer, 2004; Deslandes et al., 2003; Lavie et al., 2004; Lavie et al., 2002). Neither protein has been demonstrated to have the predicted enzymatic activity *in vitro* or *in planta*. *popP1* and *popP2* behave like avirulence genes, because mutants are pathogenic on *Petunia* cultivars and *Arabidopsis* ecotypes, respectively, that normally resist BW caused by GMI1000. In

addition, a PopP2-CyA' fusion protein is translocated from bacteria into plant cells (Cunnac et al., 2004b). PopP2 appears to interact with the RRS1-R resistance protein in *Arabidopsis* (Deslandes et al., 2003) and, due to its functional nuclear localization signal, it directs the movement of RRS1-R into the plant nucleus. Like most effector genes in *R. solanacearum* and other phytopathogenic bacteria, *popP2* is positively regulated along with the *hrp/hrc* operons (by HrpB in this case); however, *popP1* is expressed constitutively.

Other Proteins. Seven other genes activated by HrpB in GMI1000 encode proteins that transit the T3SS in culture. Four *rip* genes (for *Ralstonia* effector injected into plant cells) encode effectors, because their adenylate-cyclase fusion proteins are translocated from bacteria into plant cells (Cunnac et al., 2004b). RipA is a member of the *R. solanacearum* AWR family (five genes in GMI1000 that have a conserved Alg-Trp-Arg motif). RipB is homologous to the *P. syringae* effector HopPtoQ and has a putative nucleoside N-ribohydrolase domain. RipG has 18 leucine-rich repeats (LRRs) and is a member of the LRR-GALA subfamily in GMI1000. RipT is related to the YopT family of cysteine proteases. The functions of these proteins have not been demonstrated.

Relatively large amounts of PopA1 protein are secreted into culture medium, where it was identified by its ability to elicit a HR when an unnaturally high protein concentration is infiltrated into tobacco and some BW-resistant Petunia cultivars (Arlat, van Gijsegem, Huet, Pernollet, & Boucher, 1994). Tomato cultivars are unaffected by PopA proteins. This HR-inducing activity is similar to the 'harpins' that *P. syringae* and *Erwinia* spp. secrete when cultured in *hrp*-inducing minimal media (MM) (Alfano & Collmer, 2004; He et al., 2004). Like other harpins, PopA is glycine rich and heat stable. In GMI1000, *popA* is the first gene in the HrpB-activated *popABC* operon that is adjacent to the *hrp/hrc* gene cluster (Fig. 10). Removal of 93 amino acids from the N-terminus of PopA1, which occurs in culture and *in planta*, creates PopA3 that retains HR-inducing activity. PopA is not required by GMI1000 either for pathogenicity on susceptible tomato and *Petunia* cultivars or for eliciting a HR on tobacco (Arlat et al., 1994). Interestingly, a mutant of strain OE1-1 constitutively expressing *popA* is not virulent on tobacco, tomato, eggplant and pepper (Kanda et al., 2003); however, since PopA1 is inactive in tomato, the reason for this mutant's reduced virulence is unclear. No biochemical data or sequence motif indicates that PopA is translocated into plant cells, and it may normally be targeted to the plant apoplast.

In contrast, PopB and PopC have amino acid sequence motifs that suggest they have evolved to function inside eukaryotic cells (Gueneron, Timmers, Boucher, & Arlat, 2000). PopB is a basic protein with a functional bipartite nuclear localization signal and PopC carries 22 tandem LRR repeats that match the predicted eukaryotic cytoplasmic LRR consensus present in some resistance gene products. These proteins

are detected in culture supernatants only when Congo Red is added to the *hrp*-inducing growth medium, because this dye appears to stabilize some extracellular proteins. PopB or PopC are not required for pathogenicity or HR induction, and whether they are translocated into plant cells has not been reported.

8.2.3. Regulated Production of the T3SS

Expression of genes for the T3SS and most effectors is environmentally regulated. Like in all other phytopathogenic bacteria, transcription of these genes in *R. solanacearum* is coordinately increased *in planta* and in minimal media (MM), which mimics some conditions in the apoplast (Genin & Boucher, 2004; He et al., 2004; Schell, 2000). Addition of a complex nitrogen source (e.g., peptone) to MM strongly represses gene expression. In parallel with the two *hrp/hrc* evolutionary lineages in phytopathogenic bacteria, *R. solanacearum* and *X. campestris* pv. vesicatoria (hrp2 group) have a regulatory cascade completely different from *P. syringae* and *Erwinia* spp. (hrp1 group).

In GMI1000, *hrpB* and *hrpG* are essential for activating *hrp/hrc* operons in minimal medium (Schell, 2000), but how they sense nitrogen status is unknown. HrpB mostly controls genes with promoters containing a hrp_{II} box (TTCGn16TTCG) (Cunnac et al., 2004a). This motif is very similar to the putative PIP box motif identified by sequence analysis of *X. campestris* pv. vesicatoria promoters regulated by HrpX (the ortholog of HrpB) (Büttner & Bonas, 2002a). Deletion and mutation analyses of promoters showed that the direct repeat sequence, the distance (but not the sequence) between the repeats, and the distance of the hrp_{II} box from the transcriptional start site are all important for activation by HrpB (Cunnac et al., 2004a). However, HrpB/HrpX have not been shown to interact physically with the hrp_{II} box (e.g., by footprinting), and other aspects of HrpB/HrpX function are not understood. For example, in RS1000 a few HrpB-regulated *hpx (hrpB-dependent expression)* genes lack a hrp_{II} box, have an imperfect hrp_{II} box, or have a hrp_{II} box abnormally far upstream (Mukaihara et al., 2004). Similarly, in *X. campestris* pv. vesicatoria, HrpX activates some promoters that lack a PIP box, and also does not activate some promoters with a PIP box (Büttner & Bonas, 2002a). Furthermore, in *R. solanacearum* strain OE1-1, HrpB activation of *hrpY* occurs more quickly than activation of *popABC* after bacteria are infiltrated into tobacco leaves, which suggests that factors in addition to HrpB regulate the *popABC* operon (Kanda et al., 2003).

The HrpG transcriptional regulator is a member of the OmpR subclass of two-component response regulators (and is orthologous to HrpG in *X. campestris* pv. vesicatoria) (Brito, Marenda, Barberis, Boucher, & Genin, 1999). A cognate two-component sensor kinase has not been identified. Transcription of *hrpG* does not increase in MM, but is stimulated >15-fold by cocultivation with *Arabidopsis* or tomato cells. Nevertheless, HrpG is required for activating expression of *hrpB* and

downstream *hrp/hrc* operons both in MM and in cocultivation with *Arabidopsis* and tomato cells, so it acts as a convergence point for separate metabolic/nutritional and plant signals that affect expression of genes in the T3SS. Contrary to the original report, *hrpG* mutants are nonpathogenic on tomato and HR-negative on tobacco. HrpG also appears to regulate additional genes important for pathogenesis, because a *hrpG* mutant colonizes tomato plants less aggressively than a *hrpB* mutant (Vasse et al., 2000).

Contact with plant cell walls is another environmental signal that induces expression of *hrp/hrc* and coregulated genes in GMI1000. Four additional genes (*prhA*, *prhR*, *prhI*, and *prhJ* (plant regulator of *hrp* genes)) in a linear pathway upstream of *hrpG* sense and transduce the plant signal (Fig. 9) (Aldon, Brito, Boucher, & Genin, 2000; Brito, Aldon, Barberis, Boucher, & Genin, 2002; Brito et al., 1999; Marenda et al., 1998), but they are not required for induction of *hrpB* in MM. PrhA is similar to TonB-dependent outer membrane siderophore receptor proteins, PrhR is similar to signal transducer proteins that span the inner membrane, and PrhI is similar to members of the ECF subset of σ^{70} factors that induce genes in response to extracytoplasmic stimuli. These three proteins may comprise a signal transduction module analogous to the FecA-FecR-FecI system that controls expression of genes for ferric citrate transport in *E. coli*, except that the *R. solanacearum* module does not respond to low iron concentrations. Thus, PrhA (a putative outer membrane protein) may respond to wall contact by stimulating PrhR, which spans the inner membrane. PrhR in turn stimulates the PrhI σ factor to activate expression of *prhJ*, which encodes a member of the LuxR/UhpA family of transcriptional activators that then activates expression of *hrpG*. Mutation of *prh* genes results in varying degrees of reduction in HR elicitation on tobacco and virulence on tomato or *Arabidopsis*, with mutations toward the beginning of the signal cascade having progressively less impact on T3SS function.

A third environmental signal controlling *hrp/hrc* gene expression is the concentration of 3-OH PAME autoinducer (see above). As culture density increases in a confined space, the PhcSR sensing system reacts to increasing autoinducer concentration by enhancing PhcA activity. Genin et al. (2005) found that inactivation of *phcA* in GMI1000 increased *hrpB* expression up to 60-fold in normally repressive rich culture medium and up to 6-fold in normally inductive MM. That a *phcA* mutant of AW overproduces Hrp pili in rich medium (Kang and Denny, unpublished results), and a GMI1000 strain constitutively expressing *phcA* elicits partial and delayed HR on tobacco both support the conclusion that active PhcA depresses functionality of the T3SS. Genetic evidence indicates that PhcA represses *hrpB* transcription by modifying the activity of the HrpG response regulator post-transcriptionally, possibly by affecting its phosphorylation state (Genin et al., 2005). Thus, in the wild type, HrpG integrates signals from two environmental pathways and uses quorum sensing to further modulate the transduced signal (Fig. 9). During pathogenesis, *R. solanacearum* is

envisioned as maximally producing its T3SS and effectors when at low cell density in the apoplast but fully repressing them in highly colonized tissue of a wilted plant, presumably to conserve resources and to prepare for surviving after the host dies.

9. PLANT RESPONSES TO INFECTION

In comparison to the research on pathogenic mechanisms, there have been relatively few reports describing the genetics, physiology, or histology of host responses to infection by *R. solanacearum*. Most of the genetic research has emphasized aspects related to breeding for resistance (Hartman & Hayward, 1993; Prior, Allen, & Elphinstone, 1998; Allen, Prior, & Hayward, 2005), and will not be reviewed. I will instead focus on basic research using tomato and *Arabidopsis* as models for understanding the nature of resistance loci and subsequent plant responses. Additionally, only studies in which roots were inoculated (with or without wounding) will be considered, because responses to other inoculation methods, especially leaf infiltration, may be artifactual.

9.1. Genetics of Susceptibility

Susceptibility has been studied much less than resistance, but it is logical to consider this aspect first, because it is the most common outcome of *R. solanacearum* infection of host plants. Feng et al. (2004) isolated a single fast-neutron-induced *nws1* (no wilt symptoms) mutant of *Arabidopsis* that, unlike the wild type glabrous Col-0 parent, does not develop wilt symptoms after inoculation with GMI1000 or other strains. Pathogen multiplication in *nws1* plants is reduced, similar to that in a well-characterized resistant ecotype. Reduced susceptibility is only expressed under high light intensity, which also makes the plants smaller and have shorter roots than normal. Unlike many resistant mutants, *nws1* plants do not spontaneously develop leaf lesions (due to abnormal constitutive defense responses), abnormally express pathogenesis-related (PR) genes associated with defense responses (Van Loon & Van Strien, 1999) prior to or after inoculation, or become more resistant to other pathogens. Since *nws1* is recessive, this suggests that *NWS1* encodes a 'susceptibility factor' required for normal disease development. *Arabidopsis* genes also have been found that are required for susceptibility to powdery mildew (Vogel, Raab, Somerville, & Somerville, 2004) and downy mildew (Van Damme et al., 2005). Therefore, BW disease does not occur just due to an absence of resistance, but requires active plant metabolism before or after inoculation. *NWS1* has not been characterized and its function is unknown, but theoretical functions include production of a cell wall binding site necessary for stimulating expression of *hrpB* or a metabolite that stimulates pathogen genes required for multiplication or virulence (see IVET research below).

The ethylene signal transduction pathway may also contribute to symptom development in *Arabidopsis*, because *ein2-1* mutant plants (ethylene insensitivity;

EIN2 encodes a signal transducer downstream of the ethylene receptors (Chang & Shockey, 1999)) wilt more slowly than susceptible Col-0 plants inoculated with GMI1000 (Hirsch, Deslandes, Feng, Balague, & Marco, 2002). Bacterial colonization is less than normal in the *ein2-1* plants, but greater than in a resistant ecotype. The ethylene responsive genes *PR-3* and *PR-4* that are strongly induced by infection of Col-0 are not expressed in the *ein2-1* mutant. In contrast, wilt symptoms develop normally in Col-0 plants lacking either of two ethylene receptors or a transcriptional factor downstream of *EIN2.* Therefore, it is not clear if ethylene itself is required for expression of wilt symptoms. It is worth noting that Col-0 plants expressing *NahG* (which do not accumulate salicylic acid required for resistance), and Col-0 mutants constitutively expressing PR genes or insensitive to jasmonic acid are normally susceptible to *R. solanacearum* (Hirsch et al., 2002).

9.2. Genetics of Resistance in Tomato

Despite decades of work, researchers in public and private institutions have had only limited success in developing a tomato cultivar with large fruit and high level resistance to BW in diverse geographical locations. Useful levels of resistance exist in *L. esculentum* var. cerasiforme (e.g. L285) and in *L. pimpinellifolium* (especially PI 127805 A), and a number of breeding lines and a few named cultivars have been created by introgressing one or more loci into *L. esculentum*. When 35 tomato lines were assessed for BW resistance at 11 locations world wide, L285 exhibited intermediate resistance while Hawaii 7997 (and related lines with PI 127805 A as a parent) performed well at all sites and had the highest average survival rate (97%) (Wang et al., 1998). Crosses between L285 and H7997 (or three additional resistant lines) did not produce any F1 progeny that were significantly more resistant to BW than the parents (Hanson, Licardo, Hanudin, Wang, & Chen, 1998). Unfortunately, H7997 is susceptible to some *R. solanacearum* strains (e.g. two isolated in Taiwan (Jaunet & Wang, 1999)), so even its resistance is not 'universal'.

Modern marker-assisted genetics methods revealed that BW resistance in both L285 and H7997 is polygenic and carried on two or more chromosomes. Danesh et al. (1994) crossed L285 and susceptible line C286 and developed an RFLP linkage map with 79 markers that cover about 75% of the genome. Root inoculation of F_2 progeny with strain UW364 (biovar 4 from China) revealed a quantitative trait locus (QTL) on chromosome 6 centered on markers CT184 and TG240 that accounts for 77% of the variation in disease response. This locus may be strain specific, because it is not associated with resistance to strain Pss4 (biovar 3 from Taiwan) (see Wang et al. (2000)). A less effective locus on chromosome 10 between RFLP markers CT225b and TG230 accounts for 24% of the variation. Both QTLs are also found when F_3 plants are inoculated with UW364, and they are partially dominant. The presence of a major QTL on chromosome 6 is interesting, because some genes for resistance to other plant pests are also on this chromosome.

A series of studies examining the progeny of a cross between the highly resistant line H7997 and the very susceptible *L. pimpinellifolium* line WVa700 identified multiple QTLs using a linkage map covering about 75% of the genome. Growth chamber tests of plants inoculated with strain GMI8217 (biovar 1 from Guadeloupe) revealed a major locus on chromosome 6 spanning a large region 10-20 cM distal to the QTL observed in L285 (Thoquet et al., 1996a). This QTL and weaker ones on chromosomes 10 and 11 account for up to 56% of the variation in resistance. Two QTLs originally identified on chromosome 4 were later determined to be statistical artifacts (Wang et al., 2000). Field tests of the F_2 clones and additional F_3 families inoculated with GMI8217 in Guadeloupe identified four QTLs (Thoquet et al., 1996b). Besides the previously identified loci on chromosomes 6 and 11, two new QTLs were found on chromosomes 3 and 8. Temporal analysis of BW development in a field test of an F_3 population inoculated with GMI8217 revealed that the large QTL on chromosome 6 contains two linked loci about 30 cM apart on the upper arm of chromosome 6 (Mangin, Thoquet, Olivier, & Grimsley, 1999). One locus is near the end of the chromosome close to the *Cf-2* gene for *Cladosporium fulvum* resistance and the *Mi* gene for nematode resistance. The close proximity of the BW QTL and *Mi* makes recombination between these loci very difficult and explains why introgression of the *Mi* gene from *L. peruvianum* into *L. esculentum* results in progeny that are more susceptible to BW (Deberdt, Olivier, Thoquet, Queneherve, & Prior, 1999). The second locus on chromosome 6 is in the same region as the QTL in L285 (near marker TG240), but likely is a separate locus or different allele. In contrast, a greenhouse test of the F_3 population root-inoculated with strain Pss4 (biovar 3) identified a new major QTL on chromosome 12 that appears to be active specifically against this strain (Wang et al., 2000). In this case, the QTL on chromosome 6 near marker TG240 contributes a little to resistance to Pss4 and the QTL near *Mi* is inactive. Wang et al. (2000) speculated that BW QTLs may act like single resistance genes that determine race-cultivar specificity, but that they provide partial resistance only to selected strains. Tomato lines like H7997 may have a collection of such loci on chromosome 6 that make it resistant to almost all *R. solanacearum* strains (Jaunet & Wang, 1999; Wang et al., 1998).

9.3. Genetics of Resistance in Arabidopsis thaliana

Arabidopsis has multiple genes for BW resistance, and the facile genetics of this model organism has resulted in some of them being well characterized. A single dominant locus for resistance is present in ecotype S96, which develops a HR-like response when leaves are infiltrated with *R. solanacearum* strain Ps95, but the gene's identity has not been reported (Ho & Yang, 1999). Similarly, resistance is dominant in F_1 progeny from a cross between resistant accession Col-0 and highly susceptible L*er* when inoculated with *R. solanacearum* strain 14.25 (Godiard et al., 2003). Analysis of 100 F_9 recombinant inbred lines revealed that resistance involves the major QTL loci *QRS1* (quantitative resistance to *R. solanacearum*) and *QRS2* on chromosome 2 and

the weaker *QRS3* on chromosome 5, which together explain about 90% of the variation in resistance. Unexpectedly, analysis of both an L*ER* line and two L*er* lines transformed with the wild-type *ERECTA* gene, which is located near to QRS1 and normally affects development of aerial organs, showed that *ERECTA* also contributes quantitatively to BW resistance.

In contrast, BW resistance to GMI1000 in accession Nd-1 is inherited as a single recessive gene in crosses with the susceptible Col-5 accession (Deslandes et al., 1998). The DNA sequences of the dominant *RRS1-S* and recessive *RRS1-R* alleles are 98% identical up to where a stop codon in the former results in a protein lacking the C-terminal 90 amino acids present in the latter (Deslandes et al., 2002). Normally susceptible Col-5 plants carrying the cloned *RRS1-R* allele are resistant and prevent normal pathogen multiplication *in planta*, so this allele encodes a resistance *R* gene. The biochemically nonfunctional but genetically dominant *RRS1-S* is proposed to encode a protein that interferes with the function of RRS1-R (a dominant negative effect). Analysis of the putative *RRS1* alleles show that they are members of the large TIR-NBS-LRR subclass of *R* genes (Lahaye, 2002), but are unique in having a group III conserved C-terminal WRKY transcriptional activation domain. WRKY transcriptional regulators are known to interact with the W box in many pathogen-responsive plant promoters (Genin & Boucher, 2004). Another unusual feature of RRS1-R is that it physically interacts with the PopP2 avirulence effector secreted by GMI1000, which is responsible for triggering resistance in accession Nd-1 (Deslandes et al., 2003). PopP2 has a functional nuclear localization signal and co-expression of fluorescent PopP2 and RRS1-R fusion proteins in *Arabidopsis* cells results in their colocalizing in the nucleus. It therefore seems likely that BW resistance in Nd-1 plants is manifested when PopP2 is injected into the plant cytoplasm by the T3SS and carries RRS1-R 'piggyback' into the nucleus where it activates defense-response genes. A separate study found that a homozygous *ein2-1* mutant of Nd-1 is normally resistant, so ethylene sensing is not required for resistance (Hirsch et al., 2002).

9.4. Manifestation of Resistance

The mechanisms of BW resistance are even less well understood than its genetics. Most of our knowledge has come from spatial-temporal analyses of pathogen colonization and microscopic comparisons of susceptible and resistant tomato lines (especially Hawaii 7996, 7997 or 7998) infected with *R. solanacearum*. The pathogen multiplies equally well in the xylem sap collected from H7996 and a susceptible cultivar, so there appear to be no pre-existing inhibitory compounds in the xylem (McGarvey et al., 1999). Instead, resistance is probably due to rapid development of physical or chemical 'barriers' in response to pathogen infection.

Several papers by Grimault and associates showed that, after wounded roots are inoculated, resistance in H7996 is most clearly manifested by a low percentage of

plants being colonized at the lower and mid-stem levels (Grimault, Anais, & Prior, 1994; Grimault & Prior, 1993; Grimault & Prior, 1994b). Despite most H7996 plants having 10^5 to 10^6 cfu/gram fresh tissue in their taproot and collar tissue, all the plants remained healthy looking. Grafting experiments showed that resistance is associated with limited *R. solanacearum* invasion of the lower stem, because resistant scions (CRA66 or Caraïbo) grafted 10 cm above the collar of a susceptible root stock (Floradel) wilted, but the susceptible scion on a resistant root stock did not (Grimault & Prior, 1994a). However, in plants inoculated by soil drench without intentionally wounding the roots, the pathogen also multiplies much less well in the taproot of H7996 than in a susceptible cultivar (McGarvey et al., 1999). *R. solanacearum* also inefficiently invades the unwounded roots of H7996 within 5 hours after inoculation. Microscopic examination of H7997 growing in hydroponic culture similarly found that invasion of unwounded roots of this resistant line is less efficient than in a susceptible cultivar, and that tissue browning near infection sites and deposition of polyphenolic-like material on vessel walls and in the lumen of some vessels was only observed in H7997 (Vasse et al., 2005).

The resistance response was examined using light and electron microscopy of vascular tissue from the lower stem of two cultivars. Unlike in the susceptible cultivar Floradel, many tyloses occluded colonized xylem vessels and adjacent vessels in resistant Caraïbo plants 15 days after inoculation (Grimault et al., 1994). Deposits of electron-dense material (gums) were also common only in Caraïbo. In contrast, no tyloses were seen in vessels from the lower stem of either susceptible cultivar Ponderosa or resistant line LS-89 (a selection from H7998) 14 days after inoculation (Nakaho et al., 2000). Nevertheless, pathogen movement between vessels is restricted in LS-89, which correlates with its pit membranes being thicker and more electron dense than in Ponderosa and the development of a more conspicuous electron-dense layer near the pits and along vessel walls. In both experiments, however, plants were examined only once late in pathogenesis, so it is unknown whether the responses observed occurred before or after resistance was manifested. The timing of defense responses is crucial, because it is often the rapidity and not that nature of the response that differentiates resistant and susceptible cultivars. Because the impaired colonization of resistant tomato lines by wild-type *R. solanacearum* is very similar to that of susceptible cultivars by *hrp* mutants (Etchebar et al., 1998; Frey et al., 1994; Vasse et al., 2000), it may be that effector proteins secreted by the T3SS delay induction of defense responses in susceptible cultivars but not in resistant plants (Alfano & Collmer, 2004).

10. GENOME ANALYSIS

GMI1000 was one of the first phytopathogenic bacteria to have its complete genome sequenced (Salanoubat et al., 2002), and this accomplishment ushered in a new phase of research on *R. solanacearum* (Denny, 2005). In addition, by the time this chapter is

published, draft sequences of three additional strains should also be available (Gabriel et al., in press) (S. Genin and C. Boucher, personal communication). With these resources, anyone with an Internet connection can access in minutes knowledge about a specific locus that previously would have required months of laboratory work. It then is easy to create mutants necessary to test the gene's function experimentally. However, rather than just providing shortcuts in standard experimental strategies, the genomic sequence also makes possible novel approaches to study gene content, function, and expression on a large scale.

10.1. Genome Structure and Predicted Gene Functions

At 5.81 megabases (Mb), GMI1000 has a genome about the same size as other phytopathogenic proteobacteria. There are two circular replicons of 3.716 and 2.094 Mb that have almost the same G + C content. The larger replicon, which has an origin of replication typical of bacterial chromosomes and carries genes for all essential life functions, is referred to as the chromosome. The smaller replicon has an origin of replication characteristic of plasmids and it is well known in the literature as a megaplasmid. However, three observations indicate that the megaplasmid is an essential part of the genome. First, no derivative of GMI1000 has been found that completely lacks the megaplasmid, and most other strains have a megaplasmid (Genin & Boucher, 2004). Second, it carries the *hrp* gene cluster, many virulence-associated genes (e.g., CWDEs, flagellar motility, EPS1 biosynthesis), and genes that allow the pathogen to exploit diverse environments. Third, the megaplasmid probably co-evolved with the chromosome rather than being acquired recently by horizontal gene transfer (Coenye & Vandamme, 2003). *R. solanacearum* is, therefore, considered to have a bipartite genome. Unlike many phytopathogenic bacteria, only a few *R. solanacearum* strains are known to carry small plasmids (<100 kb).

The most significant information on GMI1000 gene content and genome structure that can be gleaned from DNA sequence analysis was recently reviewed (Genin & Boucher, 2004). The chromosome and megaplasmid together are estimated to have approximately 5,120 protein-coding open reading frames (ORFs), about half of which have a functional assignment based on homology with genes in other organisms. Among these are multiple genes and protein secretion systems that may contribute to virulence, but other than those already described above, they have not been studied experimentally. Some of the conserved genes explain the metabolic versatility of GMI1000 (typical of biovar 3), because they should encode for utilization of a wide range of amino acids, carbohydrates, and fatty acids and some phenolic compounds. Less versatile biovar 1 and 2 strains presumably lack these genes. Unexpectedly, GMI1000 also has multiple genes possibly involved with detoxification of noxious compounds. For example, eight gene clusters are related to those for heavy metal tolerance exhibited by *Cupriavidus metallidurans* strain CH34, which was isolated from a waste tank at a zinc factory (Mergeay et al., 2003). If functional, such

detoxification loci may improve the fitness of GMI1000 in soil. These and many other genes probably were acquired by horizontal gene transfer, which may be facilitated by the natural ability of *R. solanacearum* to take up DNA from its environment (Bertolla, van Gijsegem, Nesme, & Simonet, 1997). Many of these genes are present in 93 ACURs (alternative codon usage regions) that also have significantly different G + C content from the rest of the genome. In addition, there are an unusually large number of mobile genetic elements (e.g., insertion sequences, defective prophage, and a potential conjugative transposon).

I want to emphasize that only a predicted function is available for the vast majority of genes in GMI1000 and other sequenced genomes. Prediction of a new gene's function is most reliable when deduced from homology with a gene whose function has been demonstrated experimentally. Unfortunately, non-curated databases (e.g., GenBank) contain many genes whose functions are, for one or more reasons, incorrectly predicted (Valencia, 2005). Furthermore, even when orthologous proteins in two organisms appear to have a similar biochemical function (e.g., enzyme activity), their biological function may be different. Therefore, it is important to know when an inference is based only on sequence analysis so as to avoid over-interpreting or misinterpreting the data.

10.2. Utilization of Genomic Sequence Data

Promoters of genes encoding proteins in the T3SS and many of its co-regulated secreted effectors in phytopathogenic bacteria have one of several conserved motifs (Preston et al., 2005). After analysis of the *hrpY* promoter in GMI1000 demonstrated that the hrp_{II} box is essential for induction by the HrpB transcriptional regulator (see above), computer-assisted analysis of the GMI1000 genomic sequence identified 95 transcriptional units (estimated to comprise 110 genes and 4 pseudogenes) with a properly positioned hrp_{II} box (Cunnac et al., 2004a). Nineteen of these genes encode proteins that may transit the T3SS; among these are the putative translocon proteins PopF1 and PopF2, and 14 proteins previously identified as having domains suggestive of their functioning within eukaryotic cells. Cunnac et al. (2004b) subsequently created transcriptional *lacZ*-reporter fusions to 71 of these candidate promoters and found 48 genes that are positively regulated by HrpB. Among these *brg* (HrpB-regulated) genes are those encoding the avirulence proteins AvrA and PopP2. Six *brg* genes appear to encode accessory components of the T3SS, eight genes encode proteins homologous to known harpins or effectors, and the remaining genes encode hypothetical proteins. A complementary study by Mukaihara et al. (2004) examined random transposon-induced *lacZ*-reporter fusions in strain RS1000 and found 25 of the 48 *brg* genes (which they called *hpx* genes) and 3 genes predicted to be HrpB-regulated (Cunnac et al., 2004a). Four other *hpx* genes are unusual, because two don't have a hrp_{II} box, one has an imperfect hrp_{II} box, and one has a hrp_{II} box far upstream

from its normal position. Therefore, strategies in addition to sequence analysis are necessary to identify all of the genes regulated by HrpB.

Fortunately, the genomic sequence also facilitates production of microarrays and other methods that make it possible to evaluate transcription of all genes simultaneously (the transcriptome) in different strains or in one strain subjected to different conditions (Rhodius, Van Dyk, Gross, & LaRossa, 2002). A microarray with one oligonucleotide for each of 5047 predicted ORFs in GMI1000 was generated and used to determine the HrpB transcriptome (Occhialini, Cunnac, Reymond, Genin, & Boucher, 2005). Microarray slides were hybridized to cDNAs prepared from pairs of strains cultured under *hrp*-inducing conditions: GMI1000 and the *hrpB* mutant GMI1525, or GMI1000 overexpressing a plasmid-borne *hrpB* and GMI1525 with an empty vector. A stringent threshold of differential gene expression detected 143 *brg* genes positively controlled by HrpB and 50 genes that are negatively controlled by HrpB; regulated expression was confirmed for 91.4% of the representative genes tested. However, 81 genes may be within operons, so only 112 HrpB-regulated promoters were identified. Among the *brg* genes, 64 (or 92%) had been previously identified as being HrpB-regulated (i.e., known *hrp*, *brg*, and *pop* genes), and a disproportionate number are on the megaplasmid. Four known and 36 new *brg* genes do not have a hrp_{II} box within 500 bases of the start codon of the cognate gene or the first gene of the operon and none of the negatively regulated promoters have a hrp_{II} box. Promoters lacking a hrp_{II} box may be controlled indirectly by other transcriptional regulators, some of which may be directly controlled by HrpB. Many of the new HrpB-regulated genes with annotated function appear to be involved in chemotaxy or intermediate metabolism. Thus, HrpB appears to be the master regulator in a developmental program to up and down regulate many functions associated with the shift from saprophytic to parasitic life.

Analysis of the 50 N-terminal amino acids in five secreted Pop proteins, four Rip proteins, and many Brg proteins revealed that they are rich in serine and proline, low in leucine, and most lack acidic residues in the first 12 positions. Similar sequence features have been identified previously in *P. syringae* T3SS effectors (Preston et al., 2005), although what role they play during protein secretion is not known. The microarray results identified 26 new *brg* genes that also have these characteristics, so GMI1000 may have a total of 70-80 effectors or T3SS-accessory proteins (Occhialini et al., 2005). It is unlikely that all of these *R. solanacearum* proteins will be true effectors, because recent results showed that a minority of the *P. syringae* proteins predicted to be T3SS substrates are translocated into plant cells (Chang et al., 2005).

In vivo expression technology (IVET) provides an experimental approach to identify promoters that are induced by the environment within an animal or plant host, which acts as a selective 'medium'. Brown and Allen (2004) used IVET to screen a library of potential promoter-containing DNA fragments from *R. solanacearum* K60

for induction in tomato plants. They cloned the DNA fragments into a custom-made vector upstream of promoterless, tandem copies of the K60 *trpEG* locus and a β-glucuronidase gene, introduced the library into the genome of strain K909 (Δ*trpEG*), and inoculated pools of exconjugants into tomato plants. Cells with promoters active *in planta* produced tryptophan, multiplied, and were recovered from wilting plants three days later. Assaying for β-glucuronidase activity *in vitro* during growth on a rich medium differentiated uninteresting strains containing constitutively expressed promoters from strains containing inducible promoters, and the cloned DNA in 307 of the latter was sequenced. Homology searches in the GMI1000 genomic DNA database revealed 153 unique *in planta*-expressed (*ipx*) genes. Based on the GMI1000 genome annotation, the *ipx* genes can be organized in eight categories: phage and transposases (5 genes), transport/ secretion systems (8), signal peptide motif (12), stress response (15), regulators (19), novel with no motif (21), transmembrane motif (26), and metabolism (31). Fifteen have the identifiable promoter aligned backwards with respect to the *trpEG* locus. *In planta* expression was confirmed for 44 *ipx* promoters and ranged from 2-fold to over 35-fold. Nineteen of 32 fusions are not expressed in minimal medium, suggesting that their promoters are specifically triggered by *in planta* conditions or signals. Seven known virulence-associated genes are among the *ipx* genes (*pehR*, *vsrB*, *vsrD*, *rpoS*, *hrcC*, *pme*, and *gspK*). The diverse functions of *ipx* genes indicate that *R. solanacearum* significantly modifies its physiology to adapt to a stressful, low-nutrient environment *in planta*. Surprisingly, only three *ipx* genes are also positively regulated by HrpB (Occhialini et al., 2005), suggesting that, because bacteria were recovered from plants after extensive pathogen colonization, this IVET screen may have identified mostly genes expressed late in pathogenesis.

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12. AFFILIATION

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