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JURY

Monique ROYER, Chercheur CIRAD Montpellier, Rapporteur
Joël RENAUDIN, Directeur de Recherche INRA, Bordeaux, Rapporteur
Valerie VERDIER, Directrice de recherche, IRD, Montpellier, Rapporteur
Silvia RESTREPO, Professeur et Directeur de Recherche de La Universidad de Los Andes, Bogotá, Colombie, Examinateur
Mathhieu ARLAT, Professeur de l'Université Paul Sabatier, Toulouse, Examinateur
Stéphane GENIN, Directeur de Recherche, CNRS, Toulouse, Directeur de Thèse

Ecole doctorale : *SEVAB*
Unité de recherche : *Laboratoire des Interactions Plantes Micro-organismes*
Directeur(s) de Thèse : *Stéphane GENIN*
Rapporteurs : *Monique ROYER et Joël RENAUDIN*

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ABREVIATIONS

Acr	Acridine Orange
ACUR	Alternative codon usage region
acyl-HSL	Acyl homoserine lactone
AdoMet	S-adenosylmethionine
ATP	Adenosine Triphosphate
Avr	Avirulence
BDB	Blood Disease Bacterium
CbhA	β-1,4-exocellobiohydrolase
CFU	Colony formit unit
CGH	comparative genomic hybridization
OD	Optical Density
DNA	Deoxyribonucleic acid
Dpi	Days post infection
Egl	*R. solanacearum* endoglucanase
EPS	Exopolysaccharide
ET3	Effector type III
ETI	Effector triggered immunity
ETS	Effector triggered susceptibility
Hdf	3-hydroxy-oxindole
HR	Hypersensitive response
Hrc	*Hrp* conserved
Hrp	Hypersensitive response and pathogenicity
IAA	indole acetic acid
IFC	immunofluorescence colony staining
ITS	Internal Transcription Sequence
Kb	Kilobase
kDa	Kilodalton
Km	kanamycin
LPS	Lypopolysaccharide
MAMP	Microbial associated molecular patterns
Met	Methionine

MIMP	Microbe induced molecular patterns
PAMP	Pathogen associated molecular patterns
Pb	Pair of bases
PCR	Polymerase chain reaction
PGs	polygalacturonases
Pme	pectin methylesterase
PR	Resistance Protein
PRR	Pathogen recognition receptors
PTI	PAMP triggered immunity
pv.	pathovar
R3bv2	Race 3 Biovar2
Rip	Ralstonia injected protein
rpm	Revolutions per minute
Sp	Spectinomycin
Tc	Tetracycline
T3SS	type III Secretion System
T6SS	Type VI Secretion System
tad	tight adherence
Tfp	Type IV pili
VBNC	Viable but non culturable

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CHAPTER I

GENERAL INTRODUCTION

CHAPTER I

1 *Ralstonia solanacearum:* the phytopathogen and the bacterial wilt disease

Ralstonia solanacearum is a gram negative, aerobic rod classified in the β-subdivision of the proteobacteria class, order Burkholderiales and family Burkholderiaceae. It is a soil-borne plant pathogen responsible for the development of bacterial wilt, one of the most important and destructive bacterial plant disease worldwide (Hayward, 1991). This bacterium infects more than 200 vegetal species in 50 different families (Hayward, 1991, and 1994) and it is responsible of economic damage in several crops which are critical for developing countries due to their strategic importance as cash crops or subsistence foods, like potato (*Solanum tuberosum*), tomato (*S. lycopersicum*), eggplant (*S. melongena*), cooking banana (*Musa ssp.*) and peanut (*Arachis hypogea*) (Remenant et al., 2010). *R. solanacearum* is pathogenic also on model plants such as *Arabidopsis thaliana* and *Medicago truncatula* therefore facilitating analysis of basic molecular mechanisms governing pathogenicity. The origin and subsequent dissemination of the disease remain undetermined. The earliest reports were published about the same time, towards the end of the 19th century, in diverse parts of Asia, South America, USA and Australia, where it was already apparently well-established (Kelman, 1953). Within this period, intensification of the culture of the more susceptible solanaceous hosts such as tomato, potato and tobacco in subtropical or warm-temperate zones made the disease easier to be fully recognized (Kelman, 1953).

1.1 Taxonomy

This wilt disease was first described by E.F. Smith from potato, tomato and eggplant in 1896 and the pathogen was called as *Bacillus solanacearum* since it seemed to have peritrichous flagella (Smith, 1896). Later, E.F. Smith suggested referring to the pathogen as *Pseudomonas solanacearum* (Smith, 1914) provided it was found to have a single polar flagellum (Smith, 1914; Kelman, 1953). For nearly 80 years *R. solanacearum* was then called *P. solanacearum* and was a member of the *Pseudomonas* rRNA homology group II that included many other non-fluorescent pseudomonads such as *P. mallei*, *P. caryophylli*, *P. cepacia*, *P. pickettii*, and *P.*
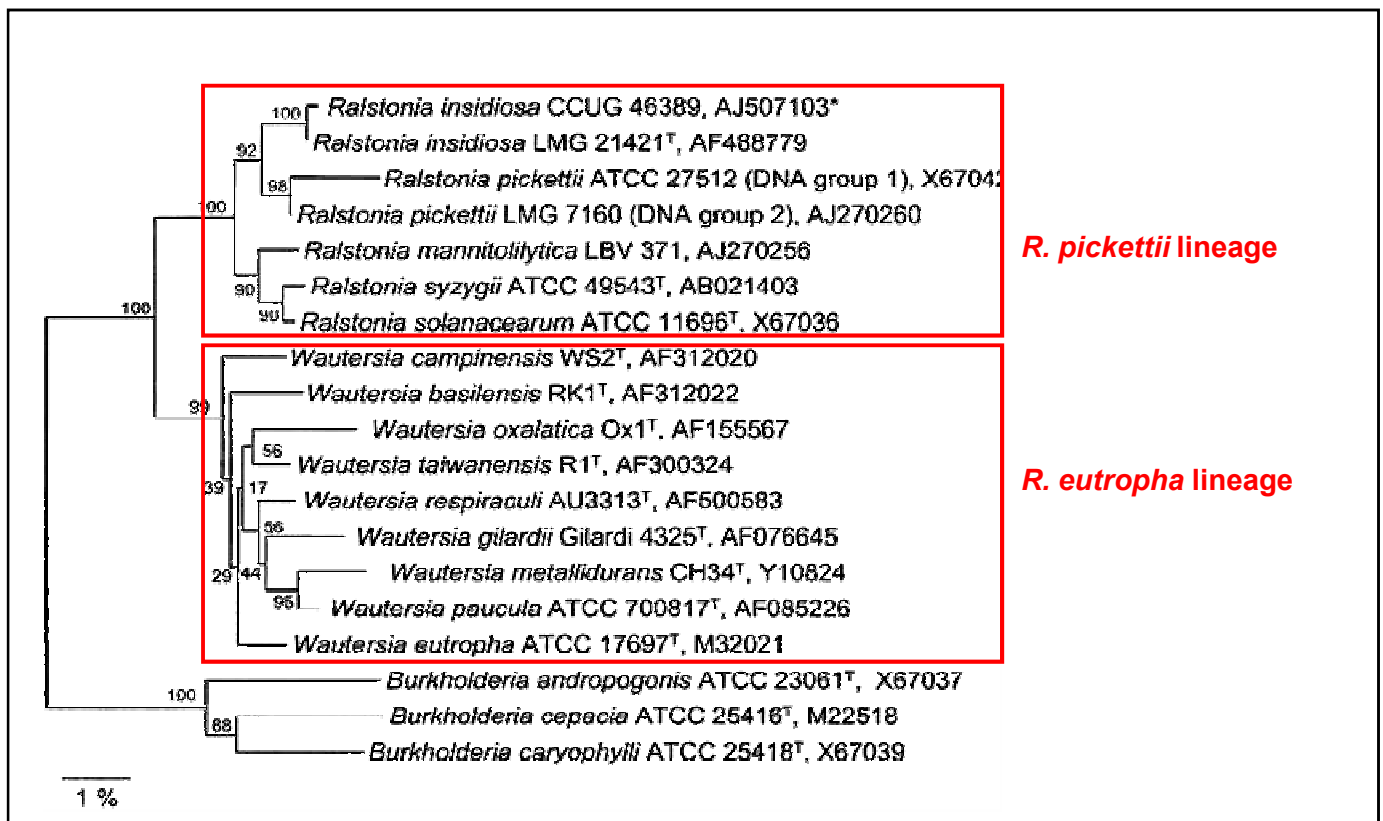
Figure 1. Phylogenetic tree based on 16S rRNA gene sequence homology for species of the genera *Ralstonia* and *Wautersia* and of some species of Gram-negative non-fermenters.

Cluster analysis was based on the neighbour-joining method, with *Burkholderia cepacia* ATCC 25416T (M22518) as the outgroup. Numbers at branch-points are the proportion of 100 bootstrap resamplings that support the tree topology. Bar, 1 % indicates estimated sequence divergence. (Adapted of Vaneechoutte et al., 2004).

Figure 2. *R. solanacearum* colony morphology on TZC medium [from P. Champoiseau, University of Florida]

gladioli (Palleroni & Doudoroff, 1971). In 1992 most of this group was proposed to be placed in the new genus *Burkholderia* on the grounds of 16S rRNA sequences, DNA-DNA homologies, fatty acid analyses, and other phenotypic characteristics (Yabuuchi et al., 1992). Finally phylogenetic and polyphasic phenotypic analyses allowed to accommodate the bacterium in a new established genus of *Ralstonia*, in 1995 (Yabuuchi et al., 1995). Since then, the bacterium is named *Ralstonia solanacearum.* Each time new species have been described in the genus *Ralstonia* and thanks to comparative 16S rDNA sequence analysis two distinct groups have been established within the genus, the first one is the *R. eutropha* lineage, comprising *R. basilensis, R. campinensis, R. eutropha, R. gilardii, R. metallidurans, R. oxalatica, R. paucula, R. respiraculi R. taiwanensis*; and the second correspond to *R. pickettii* lineage, comprising *R. insidiosa, R. mannitolilytica, R. pickettii, R. solanacearum* and *R. syzygii* previously *P. syzygii*, renamed based on new DNA–DNA hybridization data (Vaneechoutte et al., 2004). This phylogenetic discrimination was supported by phenotypic differences: members of the *R. eutropha* lineage have peritrichous flagella, do not produce acids from glucose and are susceptible to colistin, whereas members of the *R. pickettii* lineage have one or more polar flagella, produce acid from several carbohydrates and are colistin resistant. The *R. eutropha* lineage was re-classified in the new genus *Wautersia* in 2004 (Vaneechoutte et al., 2004). (Figure 1)

1.2 Morphological and physiological characteristics

R. solanacearum is a rod-shaped bacterium with an average size varying from 0.5 to 0.7 by 1.5 to 2.5 μm and it is considered as an organism strictly aerobic (Denny & Hayward, 2001). The principal biochemical characteristics are catalase positive, oxidase positive, and nitrate reduction. The pathogen does not hydrolyze starch and does not readily degrade gelatin. In broth culture, the organism is inhibited by concentrations of sodium chloride (NaCl) greater than 2%. Liquid and solid (agar) growth media are commonly used for culture of the bacterium. On solid agar medium, individual colonies are usually visible after 36 to 48 hours of growth at 28°C and Kelman's tetrazolium chloride (TZC) agar is regularly used for its isolation (Kelman 1954). After two days on TZC medium, virulent wild-type colonies are large, elevated, fluidal, and either entirely white or with a pale red center (Figure 2). For

most strains, the optimal growth temperature is 28-32ºC; however some strains that are pathogenic on potato have a lower optimal growth temperature of 27ºC.

1.3 Host range

The high economic and social impact of this organism results from its wide geographical distribution around the world. Over 200 plant species, especially tropical and subtropical crops, are susceptible to one or other of the races of *R. solanacearum*. These 'races' were defined more than 50 years ago (Buddenhagen et al 1962) and mainly correspond to pathovars (*i.e* groups of strains adapted to a single or a group of hosts). Aspects on the biodiversity of the *R. solanacearum* species and strain classification will be detailed below.

Worldwide, the most important plants susceptible to *R. solanacearum* are: tomatoes, Musa spp., tobacco (*Nicotiana tabacum*) and potatoes. Some minor host crops are: *Anthurium* spp., artichoke, groundnuts (*Arachis hypogaea*), *Capsicum annuum*, cotton (*Gossypium hirsutum*), rubber (*Hevea brasiliensis*), cassava (*Manihot esculenta*), castor beans (*Ricinus communis*), egglant (*Solanum melongena*), ginger (*Zingiber officinalis*), *Eucalyptus, Pelargonium, Piper hispidinervium, Pogostemon patchouli*, pumpkin, sesame, turmeric. In addition, many weeds are also hosts of the pathogen and therefore increase the potential of *R. solanacearum* to build up inoculum (Kelman 1953; Bradbury 1986; Hayward 1994a and Elphinstone, 2005). From this list, it is apparent that the *R. solanacearum*'s host range is not restricted to solanaceous plants but encompasses many other botanical families among Dicots and Monocots.

1.4 Infectious cycle of *R. solanacearum*

R. solanacearum is primarily a soilborne and waterborne pathogen and is disseminated by soil, contaminated irrigation water, surface water as well as infected plant material as geranium cuttings and seed potatoes (Janse, 1996). In greenhouses, it may also be spread by transplanting infected plants, taking cuttings without disinfecting grafting knives between plants, pinching buds of plants, and especially by irrigating with sub-irrigation or ebb-and-flow systems (Champoiseau et al., 2010). The pathogen does not readily spread from plant-to-plant through the splashing of water, casual contact, or aerially (Swanson et al., 2005). The early

3. Intracellular progression via root cortex and colonization of xylem vessels

2. Entry into root tissues through wounds, root tips and secondary root emerging points

4. Systemic spread and wilting symptoms appearance

5. Plant death. Return to soil-survival and dissemination in aqueous environments

1. Saprophytic life stage/survival soil
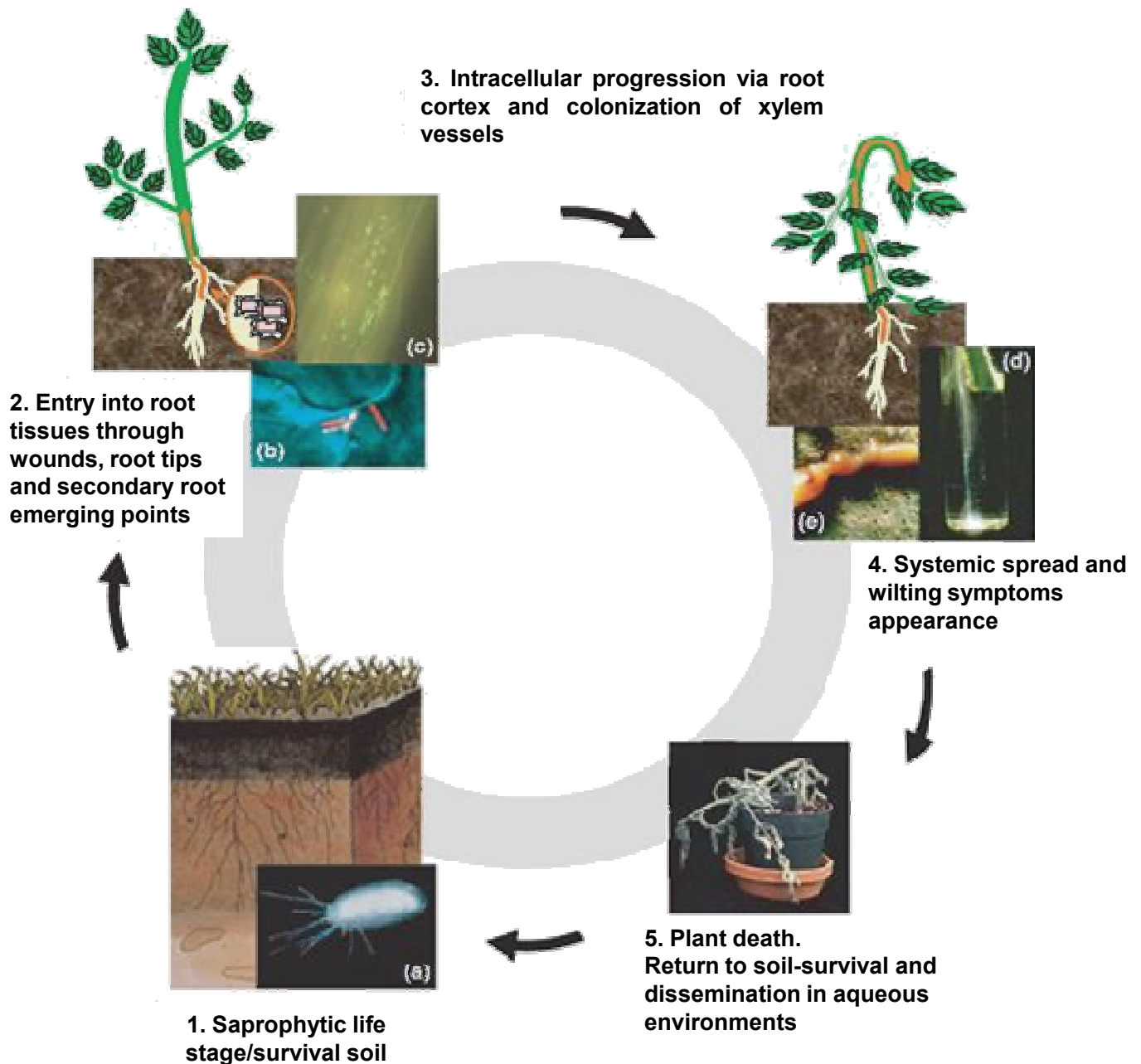
Figure 3. The *Ralstonia solanacearum* infectious cycle. [From Genin, 2010]
(a) Transmission electron microscopy observation of wild-type strain GMI1000.
(b) Confocal observation of bacteria (in red) attached to plant cell surfaces. (c) Green fluorescent protein-expressing bacteria visualized on the surface of a tomato root. (d) Bacteria oozing from an infected tomato stem in water. (e) Bacterial exopolysaccharide matrix oozing out of stem vessels after transversal section. Photographs courtesy of J. Vasse (a), D. Aldon (b & c) and A. Guidot (d).

infection of the plant by this pathogen involves root entry, mostly via lateral root emergence sites or by root damage caused by handling or soilborne organisms (e.g. the root-knot nematode). The bacterium can also enter plants by way of stem injuries from insects, handling, or tools.

Inside the plant, the bacterium are rapidly develops within intercellular spaces of the inner cortex; then, it crosses the natural barrier of the endodermis and penetrates into the vascular cylinder where it multiplies within vascular parenchyma to finally invade protoxylem vessels via cell wall degradation (Vasse et al., 1995). Bacterial proliferation (until 10^{10} cells by centimeter of tomato stem) synchronizes with a massive exopolysaccharide synthesis that obstructs the vessels and blocks the circulation of raw sap (water and and salts) from roots to aerial parts of the plant. This induces the typical wilting symptoms and eventually plant death. (Figure 3) The functional analysis of pathogenicity genes indicates that several hydrolytic enzymes might be necessary to promote the intercellular progression of the bacterium within the inner cortex and during translation towards the xylem vessels (Denny 2006; Genin 2010).

1.5 Symptomatology

Bacterial wilt most characteristic external symptoms are wilting, stunting and yellowing of the foliage (Smith, 1920; Kelman, 1953), however expression of the symptoms and rate of disease development may vary according to the susceptibility and growth conditions of the host plants, and would also be influenced by environmental conditions (Smith, 1914; Kelman, 1953).

On potatoes

At the early stages of diseases, the first visible symptoms usually appear on foliage of plants. These symptoms consist of wilting of the youngest leaves at the ends of the branches during the heat of the day with recovery at night when temperatures are cooler, but soon wilting becomes irreversible, and death of the plants follows (Champoiseau, 2010). The stems of young plants may collapse and/or have narrow dark streaks. Vascular discoloration of the stem appears to be grey or brown and bacterial ooze is present (Champoiseau, 2010). A white, slime mass of bacteria exudes from vascular bundles which are broken or cut. This slime oozes
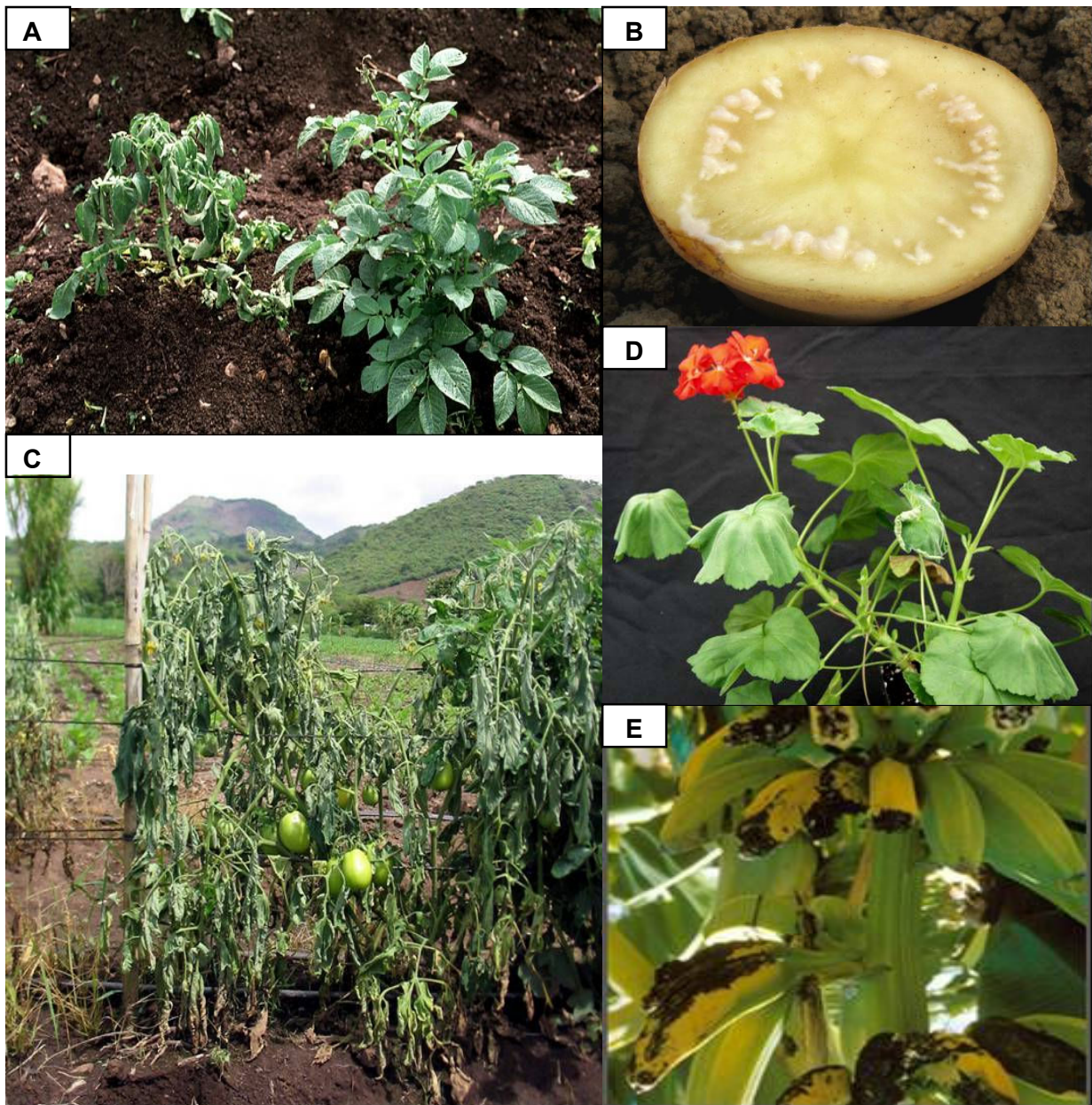
Figure 4. Symptoms caused by R*alstonia solanacearum*. (A) Wilting and stunting of potato plants. **(B)** Bacterial ooze from vascular tissues (vascular ring) in potato tuber. **(C)** Wilting of foliage and stunting of tomato plants. **(D)** Wilting and characteristic upward curling of geranium leaves. **(E)** Splitting and rotting bananas on plant infected with Moko disease. Photographs courtesy of The International Potato Center, Lima, Peru (A), P. Champoiseau, University of Florida (B), C. Allen, University of Wisconsin (C), D. Norman, Mid-Florida Research and Education Center, University of Florida (D) and Cooperative Research Centre for Tropical Plant Protection, The University of Queensland, Brisbane, Australia (E).

spontaneously from the cut surface of a potato stem in the form of threads, when kept in a beaker with water. Such threads are not formed by other bacterial pathogens of potato. This test is of presumptive diagnostic value in the field (EPPO). On tubers, external symptoms may or may not be visible, depending on the state of development of the disease; furthermore, symptoms may be confused with those of ring rot due to other pathogens as *Clavibacter michiganensis* subsp. *sepedonicus* (EPPO/CABI, 1996). *R. solanacearum* can be distinguished by the bacterial ooze that often emerges from the eyes and stem-end attachment of infected tubers. When this bacterial exudate dries, a mass of soil adheres to the tubers at the eyes. Cutting the diseased tuber will reveal a browning and necrosis of the vascular ring and immediately surrounding tissues up to 0.5 cm each side of the ring. A creamy fluid exudate usually appears spontaneously on the vascular ring of the cut surface a few minutes after cutting. In the case of ring rot the tuber has to be squeezed in order to press out a mass of yellowish dissolved vascular tissue and bacterial slime. Initially the vascular ring appears yellow to light brown, but as the infection progresses the ring will become browner (Champoiseau et al., 2009) (Figure 4B). It is important to note that plants with foliar symptoms caused by *R. solanacearum* may bear healthy and diseased tubers, while plants that show no signs of the disease may sometimes produce diseased tubers (EPPO)

On tomatoes

The youngest leaves are the first to be affected and have a flabby appearance, usually at the warmest time of day. Wilting of the whole plant may follow rapidly if environmental conditions are favourable for the pathogen, between 4-7 days after appearance of the first wilt symptoms (Figure 4C). The vascular tissues of the stem show a brown discoloration and, if the stem is cut crosswise, drops of milky-white exudate indicates the presence of dense masses of bacterial cells in infected vascular bundles. (McCarter, 1991; Champoiseau et al., 2010).

On geranium

On geranium, the earliest symptom of bacterial wilt can be very subtle and easily overlooked, developing as a leaf scorch in sectors and abnormal yellowing of lower leaves. A characteristic symptom of Southern wilt of geranium is the upward curling

of leaf margins (Champoiseau et al., 2009). Soon after these initial symptoms appear, geranium plants begin to express wilt symptoms (Figure 4D). Wilting symptoms in geranium caused by *Ralstonia* species are similar to wilting symptoms caused by other pathogens such as *Xanthomonas campestris* pv. *pelargonii*, the causal agent of bacterial blight. Unlike bacterial wilt, bacterial blight produces leaf spots and symptom expression is favored by high temperatures (29-35°C) (Champoiseau et al., 2010).

On bananas

The Musa strains of *R. solanacearum* can cause a range of symptoms which differ according to specificity from their host (plantain, banana Cavendish, *Heliconia* spp.). The various facets of this bacterial vascular disease comprise dwarfism, plant bodies' distortion and wilt and plant destruction (Prior, 2003). It is possible to distinguish two features from this disease according to dissemination nature, by the soil or the machete or by the insects which visit the male flowers or their wounds after abscission. Ascending bacterial colonization results initially in yellowing and wilting of the youngest leaves. Then the oldest leaves wilt and the banana tree dies. In the case of Moko strains, disseminated by the insects, the primary symptoms appear on the flowers and fruits before the leaves wilt. The male flower (cooking) blackens; the inflorescences remain attached and start the production of bacterial oozes from the buds. The fruits yellow in an abnormal way, they burst or chap themselves and a transverse section let appear an intense vascular tanning. A dry and greyish rot develops in pulp fruit. (Figure 4E)

In weed host

In weed hosts, wilt symptoms are rarely observed under natural conditions unless soil temperatures exceed 25ºC or inoculum levels are extremely high. When wilting does occur, the symptoms are as described for tomato. *Solanum dulcamara* (woody nightshade or bittersweet) plants growing in water may show internal discoloration of vascular tissues on the stem base without any obvious wilting. *Solanum dulcamara* has been described as an important weed host of *R. solanacearum* in England (Champoiseau et al., 2010).

Table 1. Races of the *R. solanacearum* species complex. [From Denny and Hayward, 2001].

Race	Host Range	Geographical Distribution
1	Wide	Asia, Australia, Americas
2	Banana, other Musa spp	Caribbean, Brazil, Philippines
3	Potato	Wordlwide
4	Ginger	Asia
5	Mulberry	China

Table 2. Biovars of the *R. solanacearum* species complex. Adapted from Daughtrey 2003 [From Denny and Hayward, 2001].

TEST	BIOVAR				
	1	2	3	4	5
Dextrose	+	+	+	+	+
Mannitol	-	-	+	+	+
Sorbitol	-	-	+	+	-
Dulcitol	-	-	+	+	-
Trehalose	+	-	+	+	+
Lactose	-	+	+	-	+
Maltose	-	+	+	-	+
D(+) cellobiose	-	+	+	-	+
Nitrite from nitrate	+	+	+	+	+
Gas from nitrate	-	-	+	+	+

2   The *R. solanacearum* species complex and its geographical distribution

R. solanacearum is a heterogeneous species showing diversity among strains from different hosts and geographical origins (Alvarez, 2005). Several independent criteria have been used for the classification of *R. solanacearum* strains.

2.1 The 'race' classification scheme

Historically, strains of *R. solanacearum* were classified into five races based loosely on host range (Buddenhagen et al 1962), although this classification does not, with few exceptions, correlate with the taxonomic relationship of the strains (Denny, 2006). The five races of *R. solanacearum* have different host ranges and geographic distributions. Race 1 is a poorly-defined group with a very wide host range (attacks tobacco, many other solanaceous crops and many hosts in other plant families) and is endemic to the southern United States as well as Asia, Africa, and South America. Race 2 principally attacks bananas, and is found mainly in Central America and Southeast Asia. Race 3 is distributed worldwide and has primarily been associated with potato, causing the brown rot disease, which ranks among the most destructive diseases of potato in Africa, Asia and Central and South America (CABI 2003). Race 4 affects ginger in much of Asia and Hawaii, and race 5 affects mulberries in China (Denny, 2006; Champoiseau et al., 2010). (Table 1)

2.2 The 'biovar' classification scheme

The ability of the pathogen to utilize and/or oxidize several hexose alcohols and disaccharides also allowed to classify *R. solanacearum* strains into six biovars (Hayward, 1964, 1991) (Table 2). Both classification schemes constitute informal groupings at the infrasubspecific level (Hayward, 1991). The relationship between them is only evident with race 3 strains, which generally correlate with biovar 2 phenotype (Buddenhagen and Kelman, 1964; Hayward, 1991). Race 1 strains have a wide host range contrary to race 2 and 3 strains which infect a number of plants more restricted; therefore, these classification systems show a little correlation.
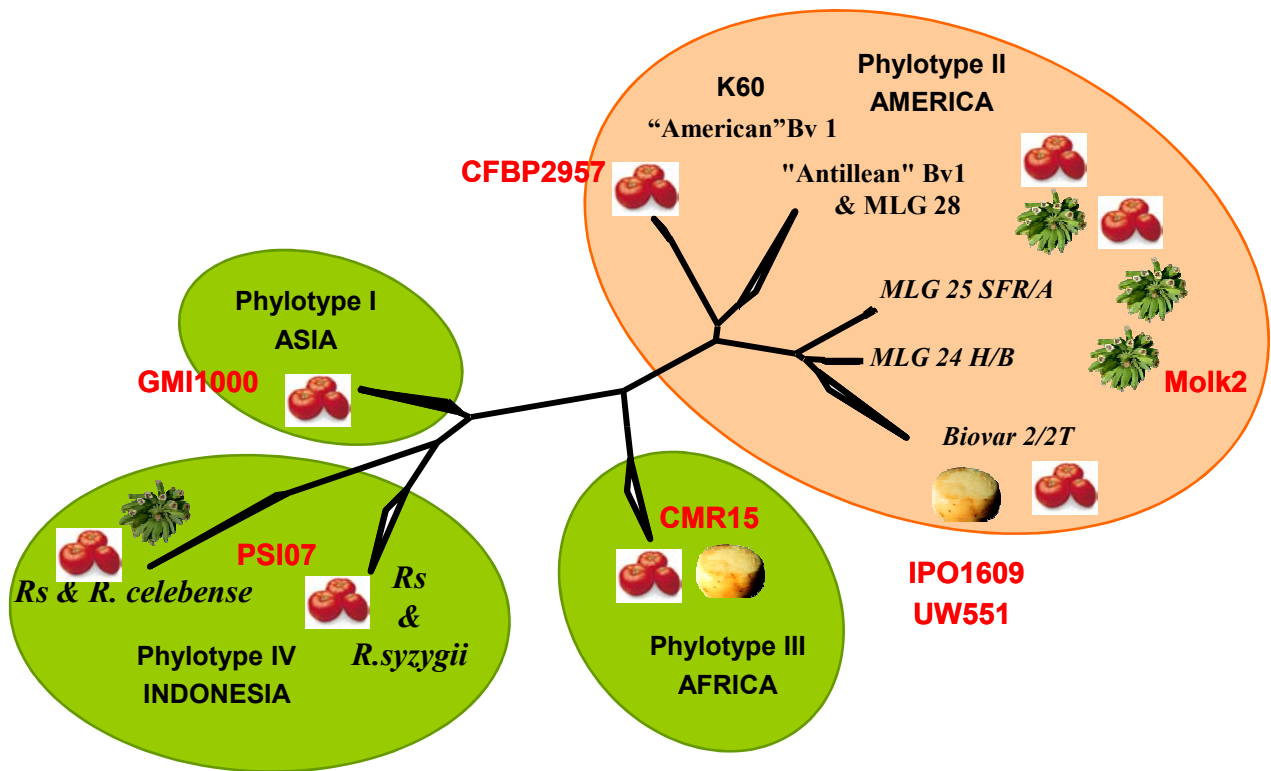
Figure 5. Hierarchical classification scheme showing relationships between *Ralstonia solanacearum* strains

Genomic sequences from strains available are indicated in red. [Adpated from Fegan and Prior, 2005].

2.3 Multi-locus genotyping classification scheme

To better study the diversity of *R. solanacearum* strains, molecular analyses based on the nucleotide sequence comparisons of the 16S-23S internal transcribed spacer (ITS) region, and the *egl* (endoglucanase) and *hrpB* (Type III Secretion System central regulator) genes (Fegan and Prior, 2005; Poussier et al., 2000a; Poussier et al., 2000b) allowed a new hierarchical classification scheme to describe more accurately the intraspecific variation among the strains (Figure 5). This approach resulted in the grouping of *R. solanacearum* strains in four monophyletic groups which reflect their origins as follows: Asia (phylotype I), the Americas (phylotype II), Africa (phylotype III) or Indonesia/Japan/Australia (phylotype IV, which also includes the related pathogens *R. syzygii* and the blood disease bacterium BDB) (Prior and Fegan, 2005). *R. zysygii* is a xylem limited-bacterium responsible of Sumatra disease of cloves (*Syzygium aromaticum*) and some related species in Indonesia and it is spread by the insect *Hindola* spp (Roberts et al., 1990). BDB is the causal organism of blood disease of banana, one of the most important and devastating disease in Indonesia and it is transmitted through the soil and running water and by insect vectors and nematodes (Subandiyah et al., 2005). BDB strains are genotypically closely related to *R. solanacearum* Phylotype IV strains however phenotypically, they differ greatly from each another, metabolic characteristics such as nitrate reductase, starch hydrolysis, citrate utilization, and acid production from sugar, they allowed to differentiate BDB strains form *R. solanacearum* Phylotype IV strains (Safni et al., 2010).

Studies on genetic diversity of a *R. solanacearum* strains and the new comparisons of the genome organization and gene repertoires by comparative genomic hybridization (CGH) have confirmed the differentiation of *R. solanacearum* species complex strains into the same four phylotypes (Remenant et al., 2010; Castillo and Greenberg, 2007; Guidot et al., 2007). Genetic variation level within the strains which has led to consider *R. solanacearum* as a complex species (Gillings et al., 1993) defined as "a cluster of closely related isolates whose individual members may represent more than one species" (Fegan and Prior, 2005), thus explaining the significant genetic and phenotypic differences observed between the strains. New sequences of *R. solanacearum* strains have shown the constant evolution of their genomes, thus the differentiation of this species complex into four phylotypes would probably change in the future.

3 Epidemiology and life in environments other than hosts

R. solanacearum uses different strategies for dissemination and survival in the environment, depending on the bacterial strain, its host, environmental conditions, agricultural practices and other factors that are not yet clearly identified (Boucher and Genin, 2004).

3.1 Survival in plant material

Association of the bacterium with either reservoir plants or plant debris has been frequently suggested to promote survival of the pathogen in soil and water, and favour overwintering in temperate regions (Hayward, 1991; Elphinstone, 1996; Wenneker et al., 1999; van Elsas et al., 2000, Norman et al., 2009). Dispersal of *R. solanacearum* on vegetatively propagation material has been known for many years and it has been one of the distribution ways used by this bacterium to enter into other countries, for example the introduction of *R. solanacearum* race 3 (R3) into Europe and North America by geranium cuttings imported from Kenya (Janse *et al*., 2005; Swanson *et a*l., 2005). The discovery that both solanaceous and non-solanaceous plants can be infected by *R. solanacearum* without showing any symptoms has had a great impact on quarantine and breeding strategies (Coutinho, 2005). The fact that several weeds growing either during the winter or summer seasons can be potential hosts of *R. solanacearum* (Pradhanang et al., 2000) revealed the possibility for persistence of the pathogen in the field during intercropping periods. In Europe, the most frequently reported carrier is bittersweet nightshade (*Solanum dulcamara*, a common perennial semi-aquatic weed inhabiting river banks), and in a lesser extent also black nightshade (*Solanum nigrum*) and stinging nettle (*Urtica dioica*) (Olsson, 1976; Hayward, 1991; Wenneker et al., 1999).

The occurrence of *R. solanacearum* as latent infections in plants not exhibiting wilting symptoms, and thus, regarded as resistant, has highlighted the importance of selecting planting stocks under conditions conducive for disease expression. If plants are selected at low temperatures and the material is then planted at temperatures favorable for disease, the consequences can be devastating (Coutinho, 2005). Seed transmission has been demonstrated in groundnut (Machmund and Middleton, 1991) and artificially contaminated seeds of tomato and capsicum were also reported to transmit the disease (Moffett et al., 1981).

3.2 Survival in water

Irrigation water contaminated with *R. solanacearum* has been responsible for outbreaks of bacterial wilt on a number of crops. In the case of potato, water effluents resulting from domestic or industrial processing of this material led to contamination of water streams (Elphinstone, 1996; Janse, 1996). As mentioned above, in *R. solanacearum*-contaminated waterways, bittersweet nightshade is continuously exposed to the pathogen, which may overcome harsh winter conditions relatively shielded within the roots and stems of this plant, and from there it could be eventually released into the water (Olsson, 1976; Hayward, 1991; Elphinstone et al., 1998; Janse et al., 1998; van Elsas et al., 2001). *R. solanacearum* can survive for considerable periods of time in water. Prolonged survival and even growth has been shown to take place in sterile water under appropriated environmental conditions, Kelman (1956) reported cells keeping culturability for more than 220 days, with higher numbers at 21°C than at 5°C. Temperature, pH, salt level, surfaces provided by particulate matter, and the presence of competing, antagonistic or parasitic organisms are the key factors influencing the bacterium's ability to survive in aquatic habitats (van Elsas et al., 2001). For example levels of seawater salts similar to those of water in coastal areas, inhibited the surviving of the bacterium due to osmotic tensions (van Elsas et al., 2001, 2005). In surface water layers, incident light in a light-dark regime had a detrimental effect on *R. solanacearum* survival, presumably due to either a direct effect on cells caused by photo-oxidative damage or an indirect effect by stimulating growth of algae which might be competitors or antagonists of the pathogen (van Elsas et al., 2001, 2005).

In addition, it has been proposed that the viable but non culturable state (VBNC) of *R. solanacearum* may be also involved in the long term survival of this bacterium in water and soil and at different stages in plant infection (Grey and Steck, 2001).

3.3 Survival in soil

There are several ways *R. solanacearum* can enter soil habitats. First, the organism exudes from infected plant tissue, entering the soil inside a matrix of protective polysaccharide (Shekawat and Perombelon, 1991). Second, infestation of soil sites by the organism may be brought about via irrigation or surface runoff water. Finally, infested machines used in agriculture may spread the organism over fields (van

Elsas et al., 2000). In any case, *R. solanacearum* can survive in the soil; however the survival time of this pathogen in this habitat has been a point of differentiation between different researches (Coutinho, 2005). There are evidences that this pathogen does not survive in bulk soil away from plant material except for short periods of time (Graham and Lloyd, 1979). For example, Van Elsas et al. (2000), reported a decrease in bacterial population in the three different fields evaluated over the time. In the other hand, other evidences support that long-term survival of *R. solanacearum* in the soil could be associated to the ability of the organism to infect the roots of susceptible or latent hosts, or to colonize the rizosphere of non host (Graham and Lloyd, 1979; Granada and Sequeira, 1983). For example, the presence of weeds and plant debris in the soil contributed to *R. solanacearum* survival (van Elsas et al., 2000). However, it is important to note that soil moisture content, temperature and soil type can all play a critical role in the survival of *R. solanacearum* in this habitat. The extent to which these factors affect survival can vary and the ultimate effect depends on the physiology and physical requirements of this bacterium as well as the interactions between these factor (Coutinho, 2005). Van Elsas et al. (2000), in a work of soil survival of *R. solanacearum* (race 3) in temperate soils reported a poor persistence of the bacterium in topsoil in the three experimental field sites tested, where IFC counts as well as PCR signals generated by directly extracted soil DNA, declined progressively to levels below the respective limits of detection. Nevertheless, Graham and Lloyd (1979), reported that survival of *R. solanacearum* (race 3) was enhanced in deeper soil layers, presumably due to less temperature fluctuation, to a lower degree of predation by protozoa or of competition or antagonism by the indigenous microbiota.

The viable but non culturable state (VBNC) is considering as a survival mechanism which some non-sporulating bacteria exhibit when environmental conditions become adverse (Roszak and Colwell, 1987; Grey and Steck, 2001; Oliver, 2005). Bacterial cells in this state, although metabolically active (viable), are unable to grow on the media usually used for their culture (Oliver, 1993) and form dormant, non-proliferating cells. The VBNC of *R. solanacearum* may be involved in the long term survival of this bacterium in water and soil and at different stages in plant infection (Grey and Steck, 2001). A range of factors that affect cellular growth, such as nutrient starvation, incubation outside the normal temperature, range of growth,

elevated or lowered osmotic concentrations, oxygen concentrations, heavy metals and even exposure to white light have been reported to trigger the VBNC state (Van Elsas et al., 2005; Oliver, 2010).

4 Disease Control

Bacterial wilt caused by *R. solanacearum* is a disease difficult to control in field production on crops, since pathogen's properties as a soilborne bacterium, their broad host range and the genetic variation level within the strains; they have not allowed implementing a universal control measure for this disease (Saddler, 2005 and Denny, 2006). Resistance breeding has been effective with tobacco and groundnut, but success with solanaceous crops appears to be limited or linked to climate conditions (Hayward, 1991; French, 1994). In other cases, the resistance or more accurately tolerance as asymptomatic infections are commonplace with solanaceous hosts can be neutralized by the actions of other pests as nematodes (Saddler, 2005; Yen et al., 1997). However, effective and long term control is possible by using a combination of diverse control methods including the use of resistant/tolerance varieties, chemical and biological control and cultural practices as parts of an integrated pest management strategy.

4.1 Host resistance

The best that normal breeding has achieved for most solanaceous crops is moderate level of host resistance to bacterial wilt on a regional level when conditions are not excessively hot or wet (Champoiseau et al., 2010). Some potato cultivars are less susceptible to bacterial wilt at least in some regions. There are active potato resistance breeding programs, some of which are focusing on resistance to latent infection. Seven genotypes from two wild Andean potato species were found to have high levels of resistance to wilt and tuber infection, which provide new resistance sources for developing commercial resistant potato cultivars (CIP, 2004). Additionally, a pentaploid sexual hybrid between *Solanum commersonii* and the cultivated species *S. tuberosum* was showen to be resistant to *R. solanacearum*, latent bacterial colonizations were detected in roots, whereas no bacterial populations were detected within stems. Today, these hybrids display a good fertility

Table 3. **Antagonist bacteria isolated from different host and used towards _R. solanacearum_** [from Saddler, 2005]

Bacteria	Host
Pseudomonas aeruginosa, _P. fluorescens_, _Burkolderia glumae_, _Bacillus_ spp., _B. polymyxa_, _B. subtilis_, _Streptomyces mutabilis_, _actinomycetes_	Tomato
P. fluorescens, _Bacillus_ spp., _B. subtilis_, B. cereus, _actinomycetes_	Potato
Corynebacterium sp., _Bacillus_ spp., _Escherichia_ sp., _Serratia_ sp., _Pseudomonas_ sp.	Pepper
P. fluorescens, _Bacillus_ spp.	Banana, Eggplant, Tomato
Bacillus spp	Tobacco
Ectomycorrhizal fungi	Eucalyptus

and are being used for further breeding efforts (Carputo et al., 2009). In the case of eggplant crops, three cultivars of eggplant resistant to bacterial wilt have been used in India (Gopalakrishnan et al., 2005). Extensive international research has produced some highly resistant tomato breeding lines, such as Hawaii 7996, but the resistance is usually linked with undesirable traits like small fruit size. Some large-fruited resistant tomato cultivars (e.g. FL7514 and BHN 466) have become commercially available in recent years, and provide moderate resistance against bacterial wilt (Champoiseau et al., 2010). Grafting susceptible tomato cultivars onto resistant tomato or other solanaceous rootstocks is effective against Asian strains of *R. solanacearum* and is used on the commercial scale in different locations worldwide (Saddler, 2005).

4.2 Chemical control

Direct control of bacterial wilt diseases caused by *R. solanacearum* in the field has showed to be difficult due to the fact that the bacterium localizes inside the plant xylem and is able to survive at depth in soil (Mariano et al., 1998). Chemical controls with Actigard (e.g., acibenzolar-S-methyl) and phosphorous acid for bacterial wilt have been show to have efficacy both greenhouse and to a lesser extent field (Anith et al., 2004, Ji et al., 2007, Norman et al., 2006, Pradhanang et al., 2005). Other control ways include the treatment of irrigation or effluent water with low doses of chlorine or peracetic acid and the soil fumigation with vapam, methyl bromide, or chloropicrin, but it is of limited efficacy and utility. The plant-derived volatile compound thymol was found to effectively reduce bacterial wilt incidence on tomato when used as pre-plant soil fumigation (Ji et al., 2005); however, its utility as an eradicant has not been evaluated.

4.3 Biological control

The biological control is one of the control means most investigated today, however the promising results generated under controlled conditions have failed to be replicated in the field. A number of soil bacteria and plant growth promoting rhizobacteria (PGPR) are currently being investigated for their role in the control of *R. solanacearum*. A list of saprophytic bacteria that have been studied as biological control agents against wild disease is presented in (Table 3). Other studies have

focused on the use of derivates from the *R. solanacearum* wild type itself, for example spontaneous avirulent mutants deficient in exopolysaccharide production capable of colonize the host but survive asymptomatically. Other example is the use of *hrp*O mutants which showed a significant reduction in the onset of disease and disease severity when plants where subsequently challenged with a pathogenic strain (Saddler, 2005). However the use of all these biological organism are currently being investigated in small scale experiments and none are currently available commercially.

4.4 Cultural control

In regions where the disease is endemic cultural control methods and phytosanitation have the potential to ameliorate its worst excesses (Saddler, 2005). In regions where bacterial wilt of potato is endemic or in locations where *R. solanacearum* is present but not yet established, these methods can be effective under some conditions (Champoiseau et al., 2010). A number of cultural practices can reduce disease, including crop rotation which dependent on factors such as the ability of the local *R. solanacearum* strains to survive and maintain a remaining infective population in the absence of a host (Akiew & Trevorrow, 1994; Mariano et al., 1998). Thus, only partial control of the disease can usually be achieved with short-term rotations. Intercropping of different time length with several plant species such as bean, cabbage, cowpea, onion, pea, or several cereals like maize or wheat has proved variable efficiency in bacterial wilt control (Devaux et al., 1987; Hayward, 1991; Akiew & Trevorrow, 1994; French, 1994; Dhital et al., 1996; Terblanche, 2002; Katafiire et al., 2005; Lemaga et al., 2005). Other methods include, planting healthy (tested) seed, using of cover crops and other measures to reduce the impact of weed hosts as herbicides, since there are many considered as asymptomatic to *R. solanacearum* infection (López & Biosca, 2005), and avoidance or testing and treatment of surface water for irrigation (Champoiseau et al., 2010). An important aspect for successful control of *R. solanacearum* dissemination and bacterial wilt eradication is the realization of systematic surveillance on both, imported and home-produced plant material susceptible to the disease (Elphinstone, 2005).

5   Economical Impact

The high economic and social impact of this organism results from its wide geographical distribution in all warm and tropical countries of the globe but also in more temperate countries from Europe and North America as the result of the dissemination of strains adapted to cooler climates. Losses greatly differ in the distinct areas in dependance on (i) local climates, soil types and cropping practices, (ii) the choice of crop and plant cultivar, and (iii) the virulent characteristics of the *R. solanacearum* strains present (Elphinstone, 2005). In areas where the organisms has quarantine status, considerable economic losses can result from the destruction of entire infected crops, additional eradication measures and restriction on further production on contaminated land (Elphinstone, 2005). Disease severity mostly increases if *R. solanacearum* is found in association with root nematodes. In tobacco, nematode infestation changes the physiology of the plants, causing susceptibility to bacterial wilt (Chen, 1984). Experiments in India showed that the combined pathogenic effects of *R. solanacearum* and *Meloidogyne javanica* were greater than the independent effects of either (Sitaramaiah & Sinha, 1984).

In the case of *R. solanacearum* R3, one of the most damaging pathogens on potato worldwide (Hayward, 1991; Janse, 1996), it has been estimated to affect 3.75 million acres in approximately 80 countries with global damage estimates exceeding $950 million per year (Floyd, 2007). The potential economic impact of potato brown rot caused by R3 is unknown in temperate climates such as where much of US potato production occurs (Champoiseau et al., 2010). The actual potential of this race to cause losses in temperate climates is uncertain because data on yield reductions are limited due to quarantine and eradication efforts in potato fields in the UK, the Netherlands and Sweden. Despite widespread detection of the pathogen in northern Europe, direct losses of potato to the disease have been limited to a few outbreaks during unusually hot summers (Priou et al., 2006).

In the case of banana, moko disease has affected susceptible bananas and plantains over thousands of square miles in central and South America (Sequeira, 1998). This disease is one of the most important phytopathological problems of the banana agribusiness in tropical countries. In Colombia, the region of Uraba and Magdalena which represent the main exporting region of banana in this country, this disease causes a destruction estimated in 16.5ha/year (Castañeda and Espinosa, 2005).

Additional, reported losses in plantain crops over the period 1998-2000 exceed $570,000 in only six municipalities of the Quindio region (Buitrago, 2001). In the Philippines, a survey of 163 farmer fields on Negros Oriental showed a 60-92% incidence of *R. solanacearum* race 2 causing bugtok disease on cooking bananas (Molina, 1996).

6 The R. solanacearum genome

6.1 The GMI1000 genome

The first *R. solanacearum* strain to be completely sequenced was the strain GMI1000 (Salanoubat et al., 2002), isolated by Bernard Digat from a wilted tomato plant in Guyana, and classified as Phylotype I, race 1, biovar 3. The sequence analyses established that the genome of this organism is organized in two large circular replicons of 3.7 and 2.1 megabases, and designated as chromosome and megaplasmid, respectively. Both replicons have a high G+C content (average value of 67%) and the genome has a total coding potential for approximately 5120 proteins (Genin and Boucher, 2004). *R. solanacearum* GMI1000 genome was originally described as a "mosaic structure" since it contains regions in which the codon usage considerably differs from the average *R. solanacearum* codon usage. These regions account for approximately 7% of the genome and were designated ACURs for Alternative Codon Usage Regions (Salanoubat et al 2002). ACURs vary in size from 2 to up to 50 kilobases and are most often found in the vicinity of phages, insertion sequences and mobile genetic elements, indicating that ACURS might have been acquired relatively recently through horizontal gene transfers (Genin and Boucher 2004).

6.2 Genome plasticity and horizontal gene transfers

Gene's comparison among a collection of 18 strains of *R. solanacearum* from different phylotypes, that represented the biodiversity of strains, was performed by comparative genomic hybridization using a DNA microarray which encompasses 5,074 oligonucleotides representative of the 5,120 predicted genes from the GMI1000 strain and it was composed of 70- or 65-mer oligonucleotides (Guidot et al., 2007). This comparison showed that a large proportion of variable genes are

Table 4 General features of *R. solanacearum* genomes [adapted from Remenant et al., 2010]

Strains	Geographical origin	Source	Phylotype	Sequence lenght	GC%	#CDS	rRNA operons	tRNA	Genome Organization
CMR15	Cameroon	Tomato	III	5,593,041	66,9	5149	3	59	CHR/ MPL/SPL
PSI07	Indonesia	Tomato	IV	5,606,288	66,3	5247	1	49	CHR/ MPL/SPL
CFBP2957	French West Indies	Tomato	IIA	5,683,402	66,9	5310	1	56	CHR/ MPL
GMI1000	French Guyane	Tomato	I	5,810,922	67	5630	4	57	CHR/ MPL
IPO1609	Netherlands	Potato	IIB3	5,523,292	66,7	5203	1	Na	CHR/ MPL
Molk2	Indonesia	Banana	IIB1	5,862,101	66,7	5438	1	Na	CHR/ MPL

Data for chromosomes and megaplasmids are combined. (#CDS = number of coding sequences, tRNA=number of tRNA, Na= Not available, CHR= Chromosome, MPL= Megaplasmid, SPL= Small plasmids

organized in genomic islands which are dispersed over two replicons. Most genomic islands are included in regions with an alternative codon usage, suggesting that they originate from acquisition of foreign genes through lateral gene transfers (Guidot et al., 2007). It has been hypothesized that the fact that this bacterium exhibits a strong adaptative potential could be related to this horizontal gene transfer potential. This is supported by results demonstrating competence development and natural transformation-mediated gene transfer during plant colonization (Bertolla et al., 1999). This ability was also confirmed *in vitro* by Coupat et al. (2008) who showed that *R. solanacearum* was able to exchange large DNA fragments ranging from 30 to 90kb by DNA replacement indicating that the natural transformation mechanism could be the main driving force of genetic diversification of the *R. solanacearum* species complex. However, Guidot et al. (2007) showed that other genomic islands present in the *R.solanacearum* mosaic genome do not correspond to ACURs and contain genes that have the same base composition as 'core' genes, suggesting that these genes either might be ancestral ones lost by deletion in certain strains or might originate from rather 'ancient' horizontal gene transfers.

6.3 Comparative Genomics

Actually (September 2010), the genome sequence of seven *R. solanacearum* strains is publicly available. These strains belong to different main taxonomic groups (phylotypes) and also differ in terms of host range and from the geographical areas they were isolated, thus providing a better illustration of the biodiversity of the species at the genomic level (Table 4).

Beside GMI1000, there are three draft genome sequences (Molk2, IPO1609 and UW551) and three assembled genomes (CMR15, CFBP2957 and PSI07). Both Molk 2 and IPO 1609, belong to phylotype IIB. Molk 2 is a strain of race 2 isolated from a banana tree and strictly limited to this host, whereas IPO1609 is a race 3 strain isolated from potato in The Netherlands (van Elsas et al., 2001). Another draft genome from race 2 strain was obtained and annotated in The United States by Gabriel et al. (2006) which corresponds to *R. solanacearum* strain UW551 isolated in Wisconsin from a wilted geranium grown from a cutting originating in Kenya, but highly pathogenic on potato and tomato (Swanson et al., 2005). The draft nature of three genome sequences actually limits the accuracy of global comparisons but a

comparative analysis made between UW551 (Phylotype II) and GMI 1000 (Phylotype I) *R. solanacearum* genomes revealed a 71% synteny at the gene level. Most genes encoding pathogenicity determinants appeared to be conserved in both strains, while one 22-kb region specifically present in GMI1000 and apparently acquired by horizontal gene transfer was absent from UW551 is likely to encode enzymes essential for utilization of the three sugar alcohols that distinguish biovars 3 and 4 from biovars 1 and 2 (Gabriel et al., 2006).

Very recently the genomes of three other tomato *R. solanacearum* strains, collected from different geographic locations were sequenced and manually annotated (Remenant et al., 2010): CFBP2957 (Phylotype IIA) was isolated in the French West Indies (Prior and Steva, 1990), CMR15 (Phylotype III) in Cameroon (Mahbou Somo Toukam et al., 2009) and PSI07 (Phylotype IV) in Indonesia (Fegan and Prior, 2005). The genome comparisons between all the seven available genome sequences confirmed that more than 60% of the genes of the megaplasmid and 70% of those on the chromosome are syntenic, thus clearly showing that all these diversified strains have a common ancestor. The three new genomic sequences also revealed the presence of some previously unknown traits in the species such as for example the presence of small plasmids in strains CMR15 and PSI07 (Remenant et al., 2010). Analyses of these genomes has permitted the identification of pathogenicity determinants globally conserved between all the strains whatever their host range or origin which include those related to biosynthesis of lytic enzymes and EPS, (*hrp*) genes, T3 Effector proteins, and others. However, the basis for host range or other specificities might rely on 'discrete' characters that are apparently not easy to identify from genomic sequences.

7   Pathogenicity determinants

Over the last three decades, genetic and molecular studies led by several international groups unraveled important mechanisms underlying *R. solanacearum* pathogenicity. Several important adaptation and pathogenicity factors were identified and characterized (for general reviews see Schell 2000; Denny, 2006; Genin 2010). Current knowledge about the most important factors has been summarized below.

7.1 Attachment and Motility of *R. solanacearum* to plant cell surfaces

7.1.1 Chemotaxis

Bacterial chemotaxis is the movement towards regions that contain higher concentrations of beneficial or lower concentrations of toxic chemicals and is required along with the motility for many pathogenic species to colonize and invade a host (Wadhams and Armitage, 2004). *R. solanacearum* has a complex and effective chemotaxis system, which it apparently uses to move toward more favourable conditions to effectively locate and infect plant hosts in its natural niches, increasing its ecological fitness (Yao and Allen, 2006; Genin 2010). The bacterium is attracted to diverse amino acids and organic acids; however the attraction response differs between the strains raising the possibility that chemotactic responses may be differentially selected traits that confer adaptation to various hosts or ecological conditions. For example, it has been demonstrated that *R. solanacearum* is more attracted by root exudates from the host plant tomato but it less attracted by rice (a non host plant) exudates (Yao and Allen, 2006). Two nonchemotactic *R. solanacearum* mutants, lacking either core chemotaxis signal transduction proteins CheA or CheW, but with their normal swimming motility, showed significantly reduced virulence by a soil soak pathogenicity assay on tomato plants, indistinguishable from that of a nonmotile mutant. However, the demonstration that nontactic strains could be as virulent as the wild-type strain, when they were introduced directly into the stem through a cut petiole, indicated that taxis is an important factor in the early stages for successful invasion of host tissues The wild-type strain out-competed the nontactic mutants by 100 folds when co-inoculated. The results revealed that chemotaxis is an essential trait required for virulence and pathogenic fitness in *R. solanacearum* (Yao and Allen, 2006).

7.1.2 Swimming motility

R. solanacearum can produce one to four polar flagella which provide it with swimming motility (Tans-Kersten et al., 2001). This ability is related with cell density and it was confirmed by Clough et al. (1997) who demonstrated that a high numbers of motile cells were obtained in exponential phase, whereas in stationary phase there would be a majority of non-motile. A soil soak pathogenicity assay on tomato plants with two non-motile mutants constructed by disrupting the *fliC* (encoding the subunit

of the flagellar filament) and *fliM* (encoding the flagellar motor switch protein) genes, showed a reduction in virulence of mutants compared to the wild-type strain, but this difference cannot be observed after wounded petiole inoculations, suggesting that swimming motility makes its most important contribution to virulence during early stages of host plant invasion (Tans-Kersten et al., 2001).

7.2 Type IV pili and fimbrial structures

R. solanacearum produces Type IV pili (Tfp) required for twitching motility (Liu et al., 2001), a form of flagella-independent translocation of bacteria over solid surfaces. In GMI1000, there are >20 chromosomal genes organized in four unlinked operons that are candidates for the synthesis and function of Tfp (Genin & Boucher, 2004). *R. solanacearum* Tfp is composed primarily of a single pilin protein, PilA, assembled to a flexuous polar filament. In fact, Tfp play multiple roles in the biology of *R. solanacearum* differently to twitching motility, as adherence to various surfaces (attachment to tobacco or tomato cells) and natural transformation (i.e., the natural ability to take up DNA from its milieu) (Kang et al., 2002). It has been reported that *R. solanacearum* variants having mutations in type IV pili were markedly less virulent on host plants, and consequently motility, adherence and/or type IV pili appear to have a significant role in *R. solanacearum* pathogenesis (Liu et al., 2001; Kang et al., 2002). Taken together, these results demonstrate the pilus formation probably contributes to pathogenesis during both invasion of roots and inside the plant by promoting adherence to host cell surfaces, colonization of root surfaces, migration to wound sites and biofilm development.

Analysis of the *R. solanacearum* GMI1000 genome provided strong presumptions that this bacterium produces multiple pili/fimbriae structures. For example, GMI1000 possesses two distinct tad-related gene clusters (RSc0649-0661 and RSp1082-1092) that belong to genomic islands widespread in many pathogenic or soil Proteobacteria (Genin & Boucher 2004). *tad* (for t̲ight a̲dherence) genes in *Actinobacillus actinomycetemcomitans* were shown to produce a type of bundled pili that mediate adherence and are required for tenacious biofilm formation (Planet et al., 2003). There is indeed suspicion that *R. solanacearum* is able to form biofilms in the plant on host xylem vessel walls; these specialized aggregates may protect the pathogen from host defenses and help bacterial survival during latent infections and

saprophytic life (Genin and Boucher, 2004). In addition to pili structures, more than 15 ORFs in GMI1000 code for large proteins containing variable internal repeats typically found in many bacterial nonfimbrial adhesin/hemagglutinin molecules; one of them, encodes a protein similar to various bacterial adhesins that promote tight adhesion to mammalian cells (Genin and Boucher, 2004). In plant pathogenic bacteria, similar adhesins were also shown to be involved in aggregation and attachment to host surfaces (Rojas et al., 2002) but their contribution to virulence was minor (Ray et al., 2002) or detectable only on specific plant species using in vitro pathogenicity assays (Rojas et al., 2002). Because adhesin-like molecules appear to be widely distributed among various non pathogenic soil Proteobacteria, it seems plausible that most of these cell surface-associated determinants are general attachment factors useful in the soil environment or for plant/rhizosphere interactions (Genin and Boucher 2004).

7.3 Exopolysaccharide production

An important pathogenicity determinant produced by *R. solanacearum* is the exopolysaccharide (EPS I), a high-molecular-mass acidic extracellular polysaccharide. It is a heterogeneous polymer containing a trimeric repeat unit of N-acetyl galactosamine, 2-N-acetyl-2-deoxy-L-galacturonic acid, and 2- N-acetyl-4-N-(3-hydroxybutanoyl)-2-4-6-trideoxy-D-glucose (Orgambide et al., 1991). Genes coding for the biosynthetic pathway for EPSI are encoded by the 20kb *eps* operon, which is subjected to a complex genetic control (Schell, 2000). In *R. solanacearum*, it has been reported that all virulent wild-type strains (mucoid colonies) produce EPS (Kelman, 1954; Husain & Kelman, 1958; Buddenhagen and Kelman, 1964; Boucher et al., 1992; Poussier et al., 2003), while EPS-deficient mutants (non-mucoid colonies) are avirulent. EPS I is the most important virulence factor of *R. solanacearum*, since EPS mutants do not cause wilt symptoms even when introduced directly into stem wounds although they remain slightly pathogenic (Denny & Baek, 1991). Therefore, it has been generally assumed that EPS plays a more important role in the last stages of host colonization, probably act by occluding xylem vessels, and thus interfering directly with normal fluid movement of the plant (Husain & Kelman, 1958; Denny, 1995) or by breaking the vessels due to hydrostatic overpressure (van Alfen, 1989). However, it has been reported that EPS mutants can

Table 5. Protein secretion pathways in *R. solanacearum* GMI1000 [from Poueymiro and Genin, 2009]

Secretion pathways	Genes	Substrates	Impact on pathogenicity
Type I	Several predicted	Unknown/not characterized	Unknown
Type II	RSc3105–RSc3116 gene cluster	Several plant cell wall degrading enzymes and other uncharacterized proteins	Essential for pathogenicity
Type III	hrp gene cluster (RSp0849–RSp0874)	74 predicted Type III effectors	Essential for pathogenicity
Type IV	RSc2574–RSc2622 gene cluster	Conjugative transposon export machinery	Unknown
Type V	Two predicted autotransporter adhesin-like proteins (RSc0115 and RSc3162)		Unknown
Type VI	RSp0739–RSp0753 gene cluster	Unknown	Unknown
Two-partner secretion	Eight predicted systems	Adhesin-like proteins	Unknown
Tat export pathway	RSc2940–RSc2942	70 putative Tat-exported proteins	Reduced virulence
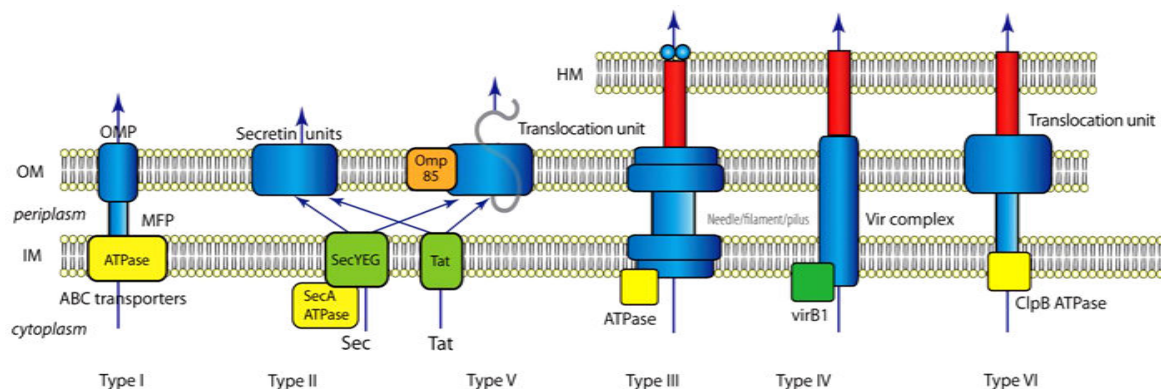
Figure 6. Summary of know bacterial secretion systems
In this simplified view only the basics of each secretion system are sketched.
HM: Host membrane; **OM**: outer membrane; **IM**: inner membrane; **MM**: mycomembrane; **OMP**: outer membrane protein; **MFP**: membrane fusion protein. ATPases and chaperones are shown in yellow.[From Tseng *et al.,* 2009]

multiply more slowly, and colonized poorly the stem of infected plants in systemic colonization of tomato plants when inoculated via unwounded roots (Saile et al., 1997; Araud-Razou et al., 1998). In that sense, EPS I would be contributing to minimize or avoid the recognition of bacterial surface structures such as pili and/or lipopolysaccharide by plant defense mechanisms during earlier stages of the infectious process (Duvick and Sequeira, 1984a; Young & Sequeira, 1986; Denny, 1995; Araud-Razou et al., 1998).

7.4 Protein secretion systems

Protein secretion plays an important role in virulence of many bacterial pathogens of plant and animals. *R. solanacearum* displays a remarkable ability for protein secretion since more than 100 proteins can be identified in the cell-free supernatant of wild-type *R. solanacearum* cultured in minimal medium (Poueymiro & Genin 2009). Analysis of the R. *solanacearum* GMI1000 genome sequence revealed that all the major secretion pathways described in Gram-negative bacteria are present in the model strain GMI1000. However, the nature of the substrates for most of these pathways remains unknown and/or their contribution to pathogenicity is undetermined (Table 5 and Figure 6).

7.5 Plant cell wall degrading enzymes and proteins secreted through Type II secretion system

In *R. solanacearum* the plant cell wall degrading enzymes are secreted by the Type II secretion system, also named as the General Secretory Pathway, a widely conserved Sec-dependent secretion pathway (Pugsley, 1993). The Type II secretion system is essential for pathogenesis because mutants defective in either system are severely impaired in colonization ability and multiplication *in planta* (Kang et al., 1994). After its entry in the plant, the bacterium must rapidly find nutrients to multiply and disseminate in the plant leaves. *R. solanacearum* produces different wall degrading enzymes, which are described below.

7.5.1 Cellulolytic enzymes.

R. solanacearum produces an endoglucanase, Egl, that can hydrolyze the β-1,4 glycosidic linkage of cellulose (Roberts et al., 1988). Egl mutants appear to be reduced in their ability to colonize the stems of infected plants but remain pathogenic

(Roberts et al., 1988). Another exoglucanase, a β-1,4-exocellobiohydrolase, CbhA, that releases cellobiose from the non-reducing ends of the chains and it contribute almost as much to disease as Egl, substantially in the ability of *R. solanacearum* to systemically colonize tomato plants (Liu et al., 2005).

7.5.2 Pectinolytic enzymes.

R. solanacearum produces one pectin methylesterase (Pme) and three polygalacturonases (PGs). Pme removes methyl groups from pectin, thereby facilitating subsequent breakdown of this primary cell wall component by PGs, which, in turn, degrade the pectin polymers. R. solanacearum has two types of PG: an endo-PG, named PglA or PehA (Schell et al., 1988; Allen et al., 1991), that cleaves the pectin polymer at random releasing large fragments, and two exo-PG, the exo-poly-α-D-galacturonosidase PehB (Allen et al., 1991) and exopolygalacturonase PehC (Gonzalez and Allen, 2003), that release galacturonic acid dimmers and monomers respectively. Inactivation of single genes has shown that none of the exoenzymes is essential for disease, each one contribute to the wild type's ability to cause wilt (Denny et al. 1990; Huang and Allen, 2000). More recently, the engineering of a mutant lacking the six major plant cell wall degrading enzymes showed that it wilted host plants more slowly than the wild-type but remained pathogenic (Liu et al., 2005). It can be concluded that other Type II secretion system secreted proteins contribute subsequently to the ability of *R. solanacearum* (wild-type) to systemically colonize tomato plants. It was also shown that pectin catabolism does not significantly contribute to bacterial fitness inside the plant (González and Allen, 2003), indicating that cellulase and pectinolytic activities are preferably required for host colonization than for bacterial nutrition.

7.5.3 Tek protein.

Tek is a 28-kDa protein which is the most abundant exoprotein found in *R. solanacearum* supernatants and is associated with production of EPS I (Denny et al., 1996). DNA sequence analysis suggested that the coding sequence for this 28-kDa exoprotein is within a gene, designated *tek* that encodes a 58-kDa precursor protein, which is exported out of the cells as a 55-kDa preprotein and processed extracellularly to release the very basic 28-kDa from its C terminus. The position, transcriptional direction, and regulated expression of *tek* suggest that it is co-transcribed with *xps*R, a gene essential for regulating biosynthesis of EPS I, and

reinforces the association of the 28-kDa EXP with virulence. However, since *R. solanacearum* mutants lacking only the 28-kDa EXP produced wild-type amounts of EPS I and were fully virulent, the function of this protein remains unclear (Denny et al., 1996). However, the whole genome sequence analysis recently revealed the presence of a gene located 2 kb downstream of the *tek* gene and encoding a protein highly similar to Tek (75% similarity). This genetic redundancy suggests that the potential involvement of the Tek proteins in virulence should be re-examined through the analysis of a mutant strain in which both genes have been disrupted (Boucher and Genin, 2004).

7.6 The Type III Secretion System

Many plant pathogenic bacteria as *R. solanacearum* use host contact-mediated type III secretion system (T3SS) to optimize the host environment and suppress plant defense responses and this secretion system is encoded by the *hrp* (<u>h</u>ypersensitive <u>r</u>eaction and <u>p</u>athogenicity) gene cluster that translocates effector proteins into plant cells (for reviews Cornelis et al., 2000; He et al., 2004).

7.6.1 *R. solanacearum* hrp genes

Thirty years ago, screening for pathogenicity-deficient mutants after random mutagenesis of complete genomes led to the discovery of *hrp* mutants, unable to induce symptoms in susceptible host plants and a hypersensitive reaction (HR) in resistant plants or non-hosts. The HR is a plant defense mechanism to prevent the spread of pathogen infection to other parts of the plant. It is characterized by the rapid death of cells in the local region surrounding the infection and is associated with plant resistance (Nimchuk et al., 2003). Through the screening of ~8000 *R. solanacearum* Tn5-induced mutants on host plants, Boucher et al. (1985) identified 12 strains altered simultaneously in their ability to cause disease on the tomato host and to induce the HR on the non-host tobacco plant. However, *hrp* genes are not essential to the plant root invasion process since Type III secretion mutants retain the ability to invade the vascular system of naturally infected tomato plants. *hrp* mutant strains could be isolated from the stems of infected plants, although their respective population levels remain very low compared to those reached by the wild-type strain (Trigalet and Démery, 1986; Vasse et al., 2000). This impaired growth of *hrp* mutants
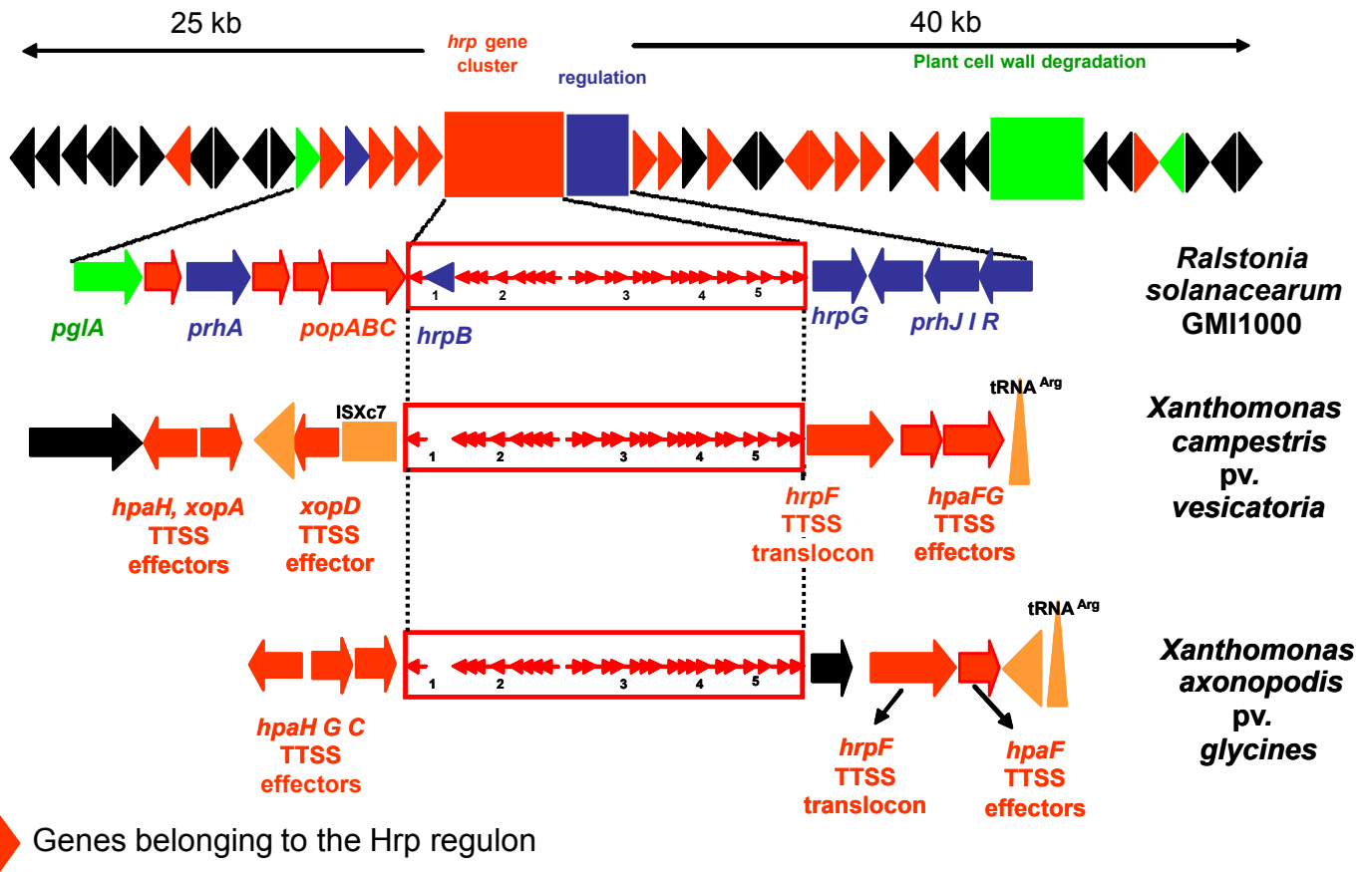
Figure 7. Genetic organization of the Hrp gene cluster of plant pathogenic bacteria from group II

Red box indicates the *hrp* gene cluster. The head arrows with numbers indicate the hrp transcription units and the complete arrows indicate the different genes. Inside the red box of *R. solanacearum* Hrp gene cluster there is *hrpB* transcription regulator which is homologous to *hrpX* in *Xanthomonas* sp

in planta is presumably a consequence of the low availability of nutrients and/or general plant defense responses (Genin and Boucher, 2004).

It is now well established that *hrp* genes are present in the majority of Gram-negative plant pathogens, except *Agrobacterium* sp., *Xylella fastidiosa*, *Xanthomonas albilineans*, and are organized in large gene clusters (Hrp gene clusters) where two lineages have been described based on similarities in *hrp* gene organization and regulation: group I for *Erwinia* sp., *Pantoea stewartii*, and *Pseudomonas syringae*, and group II for *R. solanacearum* and *Xanthomonas* sp. (Alfano & Collmer, 1997; He et al., 2004; Meyer et al., 2006). In group I organisms, several genes are required for the activation of the *hrc* (conserved *hrp* genes), *hrp*, and T3SS effector genes (Merighi et al., 2003), with the final component of the regulatory cascade being HrpL, a member of the ECF family of alternative sigma factors (Xiao et al.,1994; Xiao and Hutcheson, 1994). For bacteria from group II, genetic analyses have shown that transcriptional activation of the T3SS machinery in *R. solanacearum* relies on an unrelated and complex signaling cascade, with the final component being an AraC family regulator named HrpB for this bacterium (Genin et al., 1992) and HrpX for *Xanthomonas* sp. (Wengelnik and Bonas, 1996). The expression of *hrp*, genes is environmentally regulated. These genes are expressed at a low level during growth in complete media, and their expression is induced in plant tissues or in various synthetic minimal media which are thought to mimic conditions found *in planta* (Arlat et al 1992) (Figure 7).

7.6.2 Assembly of the Type III secretion structure.

The *hrp* locus of strain GMI1000 consists of 26 open reading frames organized into seven operons and is located on the megaplasmid (Arlat et al., 1992; Van Gijsegem et al., 1995). The systematical non-polar mutagenesis of the ORFs in the *hrp* gene cluster identified 13 structural genes required for the biogenesis of the T3SS (van Gijsegem et al., 2002), which comprise a set of eight conserved and five non-conserved *hrp* genes. Conserved *hrp* genes (*hrc* genes) are common to other bacterial plant pathogens and encode components of the T3SS which are present in animal pathogenic bacteria (Gough et al., 1992; Bogdanove et al., 1996). With the exception of HrcC, an outer membrane protein which belongs to the secretin family (Genin and Boucher, 1994), Hrc proteins share sequence similarity with components

of the flagellar protein export system (Van Gijsegem et al., 1995). Thus, the flagellar assembly apparatus probably represents an evolutionary ancestor of the T3SS (Aizawa, 2001). T3SS structure is composed by an extracellular appendage called Hrp pilus mainly composed of the HrpY protein. This Hrp pilus has a diameter of 7mm and it is essential for Type III secretion in vitro but dispensable for attachment to plant cells, contrarily to the polar fimbriae located at the same pole of the bacterium which are expressed independently from the *hrp* genes (van Gijsegem et al., 2000). In addition to the Hrp pilus, the *R. solanacearum* injectisome comprises also the products of the two *popF* genes, *popF1* and *popF2*, that encode the T3SS translocators presumed to form pores across the host cytoplasmic membrane (Meyer et al., 2006). PopF1 and PopF2 are secreted by the T3SS, are presumably assembled at the tip of the Hrp pilus and are required for the translocation of T3E proteins such as AvrA into the cytoplasm of plant cells (Meyer et al., 2006).

7.6.3 Type III effector proteins (T3E).

The T3SS allows the delivering of effector proteins into the cytosol of plant cells (Cornelis & van Gijsegem, 2000). Effector proteins are presumed to act in the invasive stages of the infection in order to favour bacterial development by either inhibiting plant defenses or inducing nutrient release from the host cell (Poueymiro & Genin 2009). There are two main classes of T3E, those which were historically designed as 'avirulence' proteins since they induce a HR on non-host or resistant plants, and the set of remaining effectors that also transit through the Type III Secretion system. *R. solanacearum* avirulence proteins identified to date will be discussed in a specific paragraph below.

The genome-wide identification of T3Es in *R. solanacearum* was realized on two main criteria:

i. The first exploited the property that T3Es are transcriptionally controlled by HrpB, a member of the AraC family of transcriptional regulator (Cunnac et al., 2004a). This allowed to mine the GMI1000 genome for promoters containing the hrpB-responsive element, named the hrpII box (TTCGn16TTCG) (Cunnac et al., 2004b) and, to analyze the transcriptomic profile of an *hrpB*-deficient mutant (Occhialini et al., 2005).
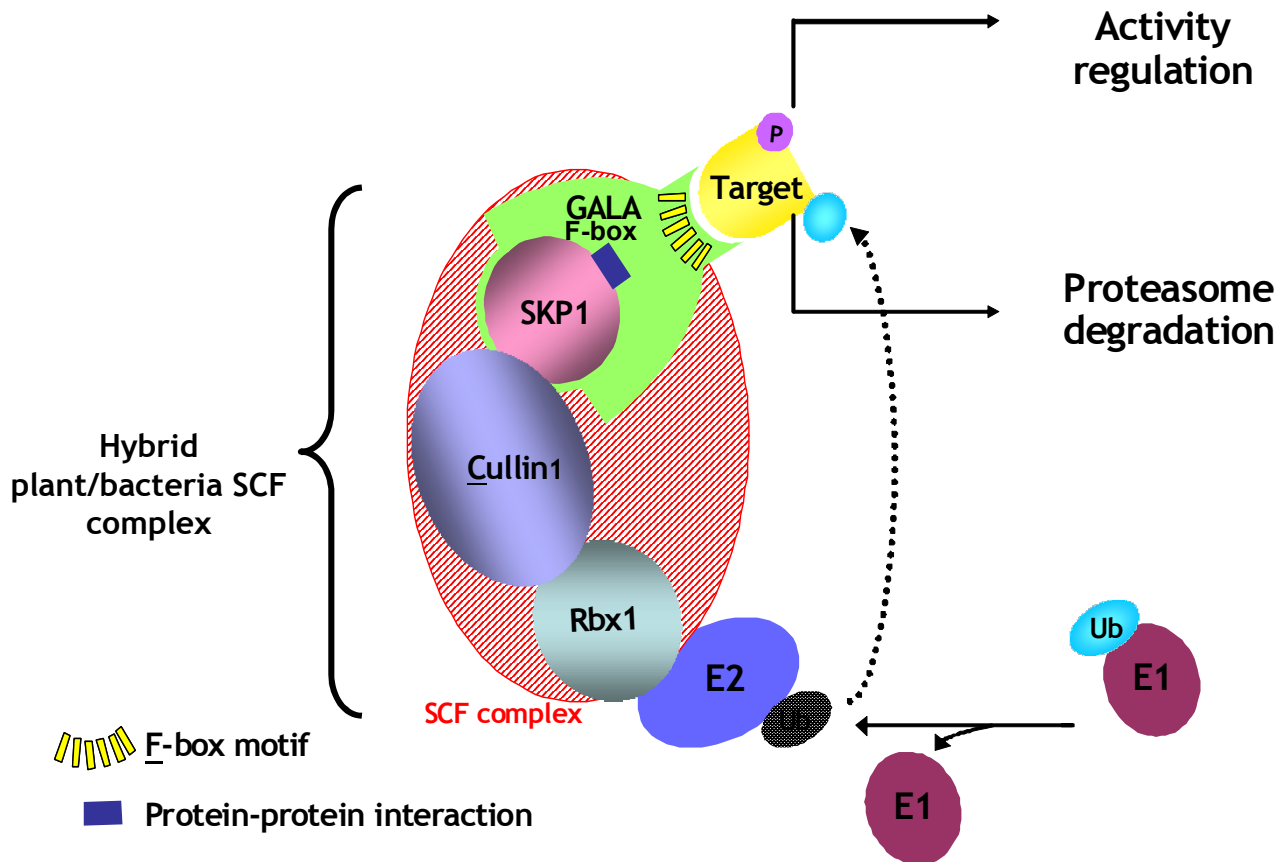
Figure 8. Model and behavior of GALA protein function inside plant cells.

The F-box domain presents in *R. solanacearum* GALA F-box proteins allows the directly interaction with the SKP1-like plant proteins which interact with Cullin1 and, all together, they form the SCF complex-type E3 ubiquitin ligase. These proteins recognize specific target proteins via protein–protein-interaction domains and recruit these target proteins into the SCF complex, thanks to their interaction with the core SKP1-like proteins. Subsequent ubiquitinylation of the target proteins can modulate their activity or lead to their proteasome-dependent degradation, depending on the nature of the ubiquitin chain [from Angot et al., 2006]

ii. The second was based on genetic screens for *hrpB*-regulated genes (Mukaihara et al., 2004) or for proteins translocated inside plant cells through the T3SS in strain RS1000 (Mukaihara and Tamura, 2009; Mukaihara et al., 2010).

These approaches yielded a total of approx. 75 proteins in both strains, which is the largest repertoire described to date in a bacterial plant pathogen (reviewed in Poueymiro & Genin 2009). Functional analysis of this repertoire was carried out on specific genes (Arlat et al., 1994; Guéneron et al., 2000; Lavie et al., 2002, Angot et al., 2006) or in a larger scale (Cunnac et al., 2004b; Mukaihara et al., 2004).

Among the many proteins secreted by *R. solanacearum* T3SS into the extracellular medium are PopA, PopB, PopC which are encoded within a single operon (Guéneron et al., 2000). PopA produces a hypersensitive-like response (HR) when it is infiltrated into plant tissue at high concentration (Arlat et al., 1994). It has been suggested that this protein may allow nutrient acquisition in planta and/or the delivery of effector proteins into plant cells (Lee et al., 2001). PopB has a nuclear localization signal which enables this protein to be transported to the plant cell nucleus (Guéneron et al., 2000). PopC, is a protein harbouring leucine-rich-repeats (LRR), analogous to those of some plant resistance gene products (Guéneron et al., 2000). In addition, a group of seven genes was identified on the basis of homologies with plant-specific leucine-rich repeats (LRR) has been identified and they are designated "GALA" proteins after a conserved GAXALA sequence in their LRR. GALA6 was translocated into plant cells (Cunnac et al., 2004b). *R. solanacearum* has effectors that contain both a LRR and an F-box domain. The F-box is a short domain (48aa) characteristic of the eukaryotic F-box proteins, allowing them to interact directly with the SKP1-like proteins. The Cullin 1 and SKP1-like proteins interact to form the SCF-type E3 ubiquitin ligase complex and control specific ubiquitinylation. The ubiquitin-tagged proteins have either modified properties or are doomed for degradation by the 26S proteasome. A *R. solanacearum* strain in which all of the seven GALA effector genes have been deleted or mutated lost the pathogenicity entirely on *Arabidospsis* or became less virulent on tomato. In addition, GALA7, a host specificity factor was essential for pathogenicity on *Medicago truncatula* plants. Since the F-box domain is required for virulence function of GALA7, it was hypothesized that these effectors may act by hijacking their host SCF-type E3 ubiquitin ligases to interfere with their

host ubiquitin / proteasome pathway to promote disease (Angot et al., 2006) (Figure 8).

Several *R. solanacearum* T3Es are homologous to already demonstrated T3SS-dependent substrates in other bacterial species such as *P. syringae* or *Xanthomonas* Avr proteins; but a large group composed of 34 T3E genes encode hypothetical proteins with no homologies to other proteins or sharing identity to proteins of unknown function (Cunnac et al., 2004b). A comparative genomic hybridization approach with a set of strains representative of the *R. solanacearum* polymorphism was used to study the distribution of T3Es within the species (Guidot et al., 2007; M. Elbaz & S. Genin, personal communication). Nearly half of the GMI1000 effector repertoire (34/74) appears to be conserved within the species, and thus are deemed ancient or stably inherited along with the core genome (Poueymiro & Genin, 2009). This was also confirmed by the comparison of T3E genes and their surrounding genes between RS1000 (race 1, biovar, 4 phylotype I) and GMI1000 (race 1, biovar 3, Phylotype I) (Mukaihara et al., 2010).

Systematical T3E disruption analysis conducted in strains GMI1000 or RS1000 revealed that in most cases a mutation in a single effector gene shows little or no effect on bacterial virulence on tomato or Arabidopsis, whereas defects in the Hrp T3SS completely abolish the pathogenicity of the pathogen (Cunnac et al., 2004b; Mukaihara et al., 2004). This clearly indicates the existence of functional overlap between effector proteins delivered into plant cells via the Hrp T3SS. Supporting this hypothesis is the finding that cumulative disruption of the seven GALA T3E genes strongly affects virulence of GMI1000 on *Arabidopsis* and, to a lesser extent, on tomato (Angot et al., 2006). There is also indication that the individual contribution of a given T3E can vary depending on the host and that a single T3E is able to extend the host range of the pathogen. The GALA7 gene was indeed found to be essential for pathogenicity of strain GMI1000 toward *Medicago truncatula* but not on other hosts (Angot et al., 2006).

7.7 Avirulence proteins: type III effectors recognized by plant resistance genes

The best studied Type III effectors in plant pathogens are probably the products of avirulence (*avr*) genes which were discovered 20 years ago without knowing that they encode T3SS-substrates. *avr* genes were identified by functional assays,

because a cloned *avr* gene can convert a virulent strain into an avirulent one when tested on a resistant (or non-host) plant that carries the appropriate resistance gene (reviewed in Leach and White, 1996). *avr* genes are naturally present in avirulent strains, but in fact the original function of the Avr proteins must have been to promote disease in susceptible plants that lack the corresponding disease resistance genes.

7.7.1 AvrA.

The first cloned *avr* determinant in *R. solanacearum* was *avrA*, which confers avirulence towards tobacco at the host species level (Carney & Denny, 1990). More recently it was shown that resistance of tobacco (*Nicotiana* spp.) to GMI1000 is determined by two T3Es, AvrA and PopP1 (Poueymiro et al., 2009). Both AvrA and PopP1 elicit the hypersensitive response (HR) on three tobacco species (*N. tabacum*, *N. benthamiana*, and *N. glutinosa*) although in different manners; AvrA is the major determinant recognized by *N. tabacum* and *N. benthamiana*, while PopP1 appears to be the major HR elicitor on *N. glutinosa*. Only the double inactivation of the *avrA* and *popP*1 genes allowed GMI1000 to wilt tobacco plants, showing that these two T3Es jointly control host specificity toward tobacco species. (Poueymiro et al., 2009). *avrA* is a widely conserved gene in the species which appears to be subjected to various DNA insertions or rearrangements, and this probably allows *R. solanacearum* to evade the recognition and defense response of tobacco (Robertson et al., 2004; Poueymiro et al., 2009). To date, the tobacco resistance gene involved in this HR response has not been identified.

7.7.2 PopP2.

PopP2, which belongs to the YopJ/AvrRxv protein family, is the avirulence protein recognized by RRS1-R, a protein whose structure combines the TIR-NBS-LRR domains found in several R proteins and a C-terminal WRKY motif characteristic of some plant transcriptional factors. The physical interaction between PopP2 and RRS1-R triggers the resistance in Arabidopsis plants (Deslandes et al., 2003). The nuclear localization of RRS1-R was shown to be dependent on the presence of nuclear localization signals borne by the PopP2 effector. The search for plant PopP2-interacting partners in Arabidospis identified several candidates; the best characterized interactor is the cysteine protease RD19, a protein localized in the
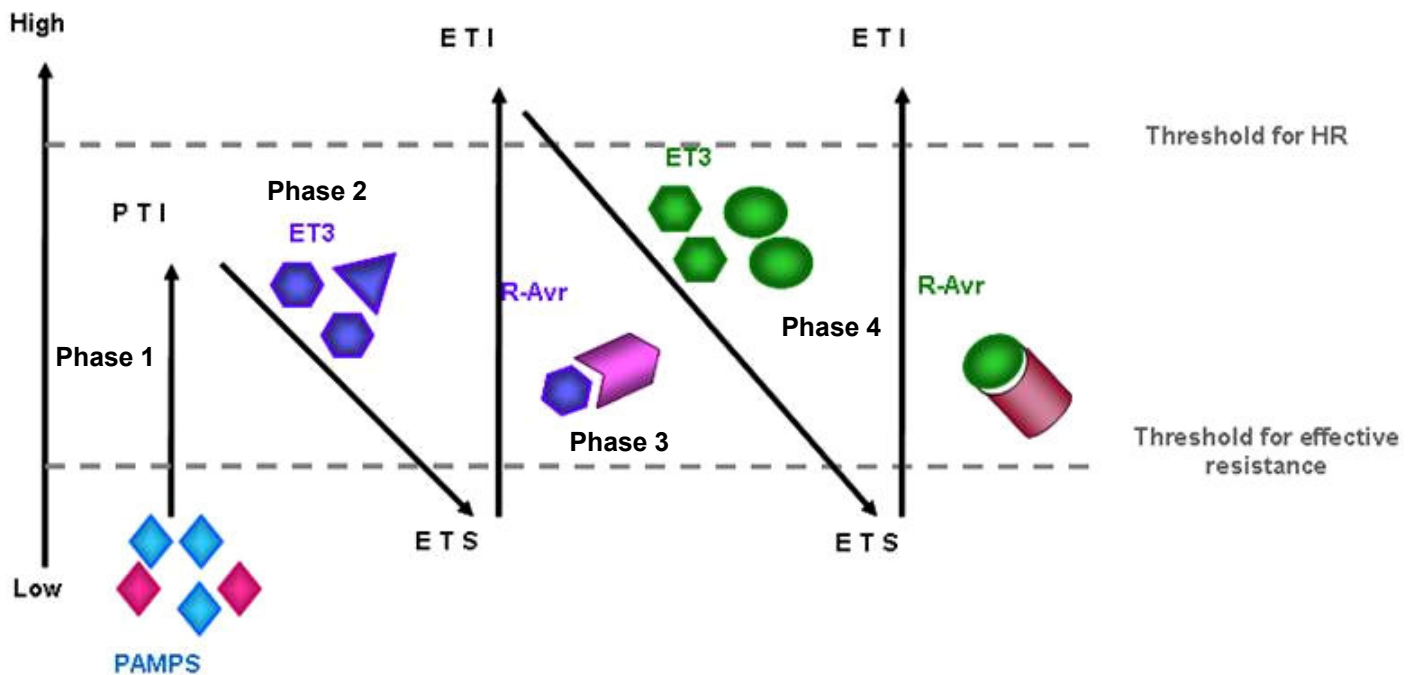
Figure 9. Molecular dialog during plants-bacteria interactions, *Ralstonia solanacearum* –Tobacco model

The zigzag model illustrates the quantitative output of the plant immune system. In phase 1, plants detect microbial/pathogen-associated molecular patterns (MAMPs/PAMPs, pink diamonds) via PRRs to trigger PAMP-triggered immunity (PTI). In phase 2, successful pathogens deliver effectors that interfere with PTI, or otherwise enable pathogen nutrition and dispersal, resulting in effector-triggered susceptibility (ETS). In phase 3, one effector (blue hexagon) is recognized by an NB-LRR protein, activating effector-triggered immunity (ETI), an amplified version of PTI that often passes a threshold for induction of hypersensitive cell death (HR). In phase 4, pathogen isolates are selected that have lost the blue hexagon effector, and perhaps gained new effectors through horizontal gene flow (in green)—these can help pathogens to suppress ETI. Selection favours new plant NB-LRR alleles that can recognize one of the newly acquired effectors, resulting again in ETI. [From Jones and Dangl, 2006] In the case of interaction between *R. solanacearum* and their specific host tobacco, the resistant gene AvrA is recognized by a specific Resistant protein in the host in the phase 3. This recognition induces the effector-triggered immunity (ETI) and induction of an hypersensitive response (HR) in the tobacco plant.

plant lytic vacuole and which is relocalized in the plant nucleus upon interaction with PopP2 (Bernoux et al., 2008)., The recognition event of PopP2/RD19 interaction by RRS1-R activates defence genes through its WRKY domain and leads to effector-triggered immunity. Because loss of resistance in the absence of RD19 is only partial, it is probable that other plant factors participate in the RRS1-R-mediated resistance (Poueymiro & Genin, 2009).

7.7.3 PopP1.

As PopP2, PopP1 is a T3E belonging to the AvrRxv/YopJ family. PopP1 was shown to be an avirulence factor recognized by resistant Petunia plants (Lavie et al., 2002) and which acts as a host-specificity determinant towards tobacco together with AvrA (Poueymiro et al., 2009).

7.8 The molecular dialog during plants-bacteria interactions, model *R. solanacearum* –Tobacco

At the infection moment, when the bacteria interact with plant host, the plant recognizes pathogen-associated molecular patterns (PAMPs) via receptor-like proteins known as pathogen recognition receptors (PRRs). Responses induced by PRRs, often referred to as PAMP-induced immunity (PTI), are generally "low-impact", and are a basal defense which confers resistance to most pathogens. However, host adapted pathogens are able to overcome PTI mechanisms through the deployment of effector proteins delivered to the host cytoplasm via the various secretion mechanisms of bacteria, suppressing plant defense response, so the effector trigger susceptibility (ETS) and plant disease develop. However, certain plants have receptor-like proteins (R) that are more specific in their recognition spectra. These plant proteins recognize specific effector proteins and induce a much more drastic suite of "high impact" defense responses, often referred to as effector-triggered immunity (ETI), culminating in the induction of a type of programmed cell death known at the hypersensitive response (HR). On the other hand, bacteria also inject effector proteins which suppress the ETI, since the host doesn't have the protein receptor to this kind of effector protein, resulting again in ETS and disease develops (Jones and Dangl, 2006). T3Es proteins can determine the host range. The issue of the interaction depends on the pool of PAMP/effectors of the bacterium and on the

pool of receptors and Resistance proteins of the plant. In the case of interaction between *R. solanacearum* and their specific host tobacco, the resistant gene AvrA is recognized by a specific Resistant protein in the host. This recognition induces the effector-triggered immunity (ETI), thus an hypersensitive response (HR) in the plant (Figure 9).

7.9 Other candidate pathogenicity determinants

7.9.1 Lipopolysaccharide (LPS).

The recognition between *R. solanacearum* and the host has long been thought to implicate an interaction between bacterial LPS, a component of the outer membrane, and plant lectins (Whatley et al., 1980; Baker et al., 1984; Hendrick & Sequeira, 1984). *R. solanacearum* LPS has three parts: the lipid A, the oligosaccharide core and the O-specific antigen (Baker et al., 1984). The core structure is composed of rhamnose, glucose, heptose, and 2-ketodeoxy-octonate, whereas the O-specific antigen is a chain of repeating rhamnose, N-acetylglucosamine, and xylose in a ratio of 4:1:1 (Baker et al., 1984). Presence or absence of the O-specific antigen differentiated respectively between smooth and rough LPSs, appearing in *R. solanacearum* strains which were respectively negative and positive HR-inducers (Whatley et al., 1980; Baker et al., 1984). However, subsequent research indicated that a specific interaction between *R. solanacearum* rough LPS and a plant cell wall receptor was not enough to initiate the HR, although many of the mutations in the LPS also affected virulence (Hendrick & Sequeira, 1984). In *R. solanacearum*, smooth LPS is apparently required to prevent agglutination by certain plant lectins (Sequeira & Graham, 1977). Furthermore, *R. solanacearum* LPS and EPS are somehow related, since a gene cluster was found to be required for the biosynthesis of both cell surface components (Kao & Sequeira, 1991).

7.9.2 Lectins

Two genes encoding lectins have been characterised in *R. solanacearum* (Sudakevitz et al., 2004; Kostlanova et al., 2005), presumably with a function in adhesion to plant surfaces, which is important for *R. solanacearum* pathogenicity during the early stages of infection. In fact, it was found that these lectins bind L-fucose and interact with the plant xyloglucan polysaccharide belonging to the hemicellulose fraction of plant primary cell walls (Kostlanova et al., 2005). However,

no mutant in the corresponding genes was generated so their impact in pathogenesis remains unknown.

8 Regulation of pathogenicity gene expression

Expression of virulence factors in *R. solanacearum* is regulated in the early and late stages of infection and it is controlled by a complex regulatory network that responds to environmental conditions, the presence of host cells, and bacterial density (Schell, 2000).

8.1 PhcA, a global regulator controlling phenotypic conversion.

At the center of the regulation network is PhcA (Phc, phenotype conversion), a LysR family transcriptional regulator which directly or through intermediary regulatory genes, coordinates the expression of several processes (Brumbley et al., 1993); it simultaneously activates diverse virulence genes such as those of EPS biosynthesis, plant cell wall degrading enzymes as Pme and Egl exoproteins, swimming motility or Type IV pili, and represses others such as *hrp* genes and those related to production of polygalacturonases, siderophores, and motility (Huang et al., 1995; Schell, 2000; Kang et al., 2002; Genin & Boucher, 2002).

Spontaneous or induced mutations in *phcA* result in pleiotropic changes, but phenotypically appear as conversion of fluidal colonies to afluidal, a phenomenon referred as phenotypic conversion (PC), and described 50 years ago by Kelman (Kelman, 1954; Denny et al., 1994). PC-type mutants are easily recognized on agar medium containing tetrazolium chloride because their colonies are round, red and non mucoid (butyrous), which contrast with the irregularly round, white or pink, fluidal parent colonies (Kelman, 1954). This change in colony type is due to the loss of one or more components of EPS I (Orgambide et al., 1991) and a strong reduction in endoglucanase activity but an increase in that of endopolygalacturonase (Schell, 1987; Brumbley & Denny, 1990). Furthermore, cell motility has been shown to be increased in PC-type variants (Kelman & Hruschka, 1973; Brumbley & Denny, 1990). Although these variants are impaired in wilting host plants (Kelman, 1954), they are infective and can grow in planta (Denny & Baek, 1991), causing disease symptoms such as stunting, stem necrosis and adventitious root formation (Husain & Kelman, 1958; Denny & Baek, 1991). Several PC-type variants carrying localized mutations
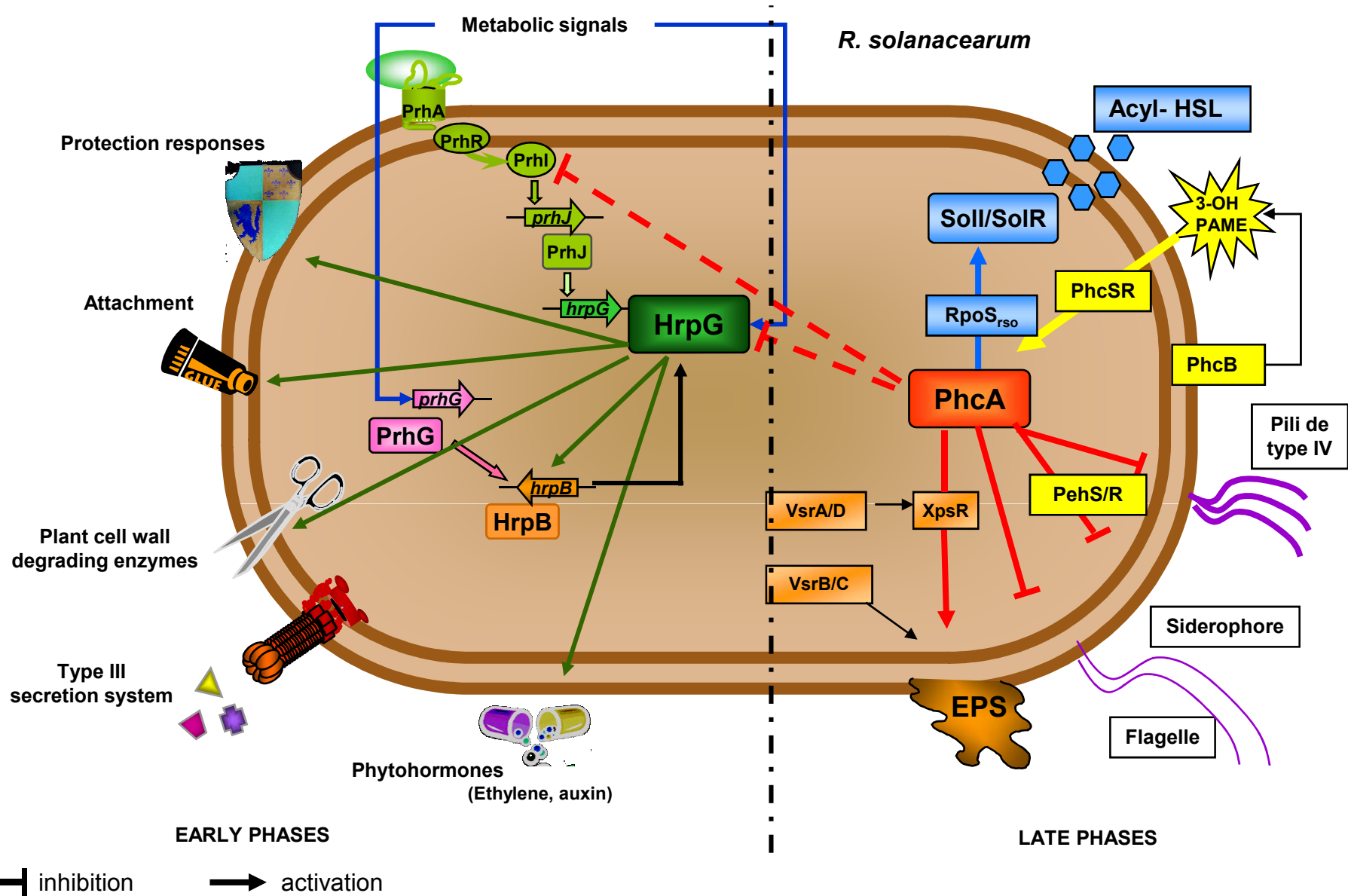
Figure 10. Regulation network of virulence functions in *Ralstonia solanacearum*

The pathogenicity of *R. solanacearum* is differently regulated in the early and late stages of infection in response to environmental conditions, such as presence of host plant cells and bacterial population densities

within phcA were able to revert from PC to wild type *in planta*, and also in vitro when in presence of root exudates of susceptible hosts (Poussier et al., 2003).

8.2 Activation of PhcA

The level of active PhcA is regulated in response to cell density by a quorum sensing –dependent mechanism that involves the specific autoinducer molecule 3-hydroxy palmitic acid ester (3-OH PAME) (Flavier et al., 1997a). At low density in culture, presumably corresponding to saprophytic life and early plant colonization, PhcA is not expressed, leading to expression of "early" disease virulence factors, including several polygalacturonases and both twitching and swimming motility. At a later stage of infection, at high cell density as in the xylem vessels of the host, the extracellular accumulation of 3-OH PAME leads to activation of PhcA and, subsequently, production of EPS, repression of motility, and potent plant cell wall-degrading enzymes (cellulases and pectin methylesterase) will be actived (Genin et al., 2005). Figure 10

8.3 3-OH PAME, an endogenous signal molecule essential to pathogenesis

3-OH PAME is synthesized by PhcB, a membrane-associated protein, from S-adenosyl methionine. When extracellular 3-OH PAME accumulates above threshold concentrations, 5 nM, (i.e. at high cell density in a restricted space, such as the plant vascular system) the signal activates a two component regulatory system encoded by PhcS, a histidine kinase sensor, and PhcR, a response regulator (Clough et al., 1997). When inactive, this two-component system represses the production of PhcA (Flavier et al., 1997b; Schell 2000). Therefore, when bacterial cells are in low density or are dispersed in the soil, levels of 3-OH PAME are low, consequently the two component system is inactive and PhcA levels are low. This results in the lack of expression of late virulence genes (EPS, cellulases) and the induction of expression of siderophore, pili and flagellar movement. On the other hand, when *R. solanacearum* cells are in high concentrations (e.g. in intercellular spaces, xylem vessels or pit membranes), 3-OH PAME accumulates above the threshold. This in turn activates PhcS and PhcR, and consequently raises the levels of PhcA in all cells. Cells with elevated levels of active PhcA produce copious amounts of EPS I and exoenzymes and are highly virulent. Therefore, production of EPS I and cell wall-

degrading enzymes, triggering the occlusion of the host xylem vessels (Genin et al., 2005; Schell, 2000) (Figure 10).

8.4 Acyl homoserine lactone: a second Quorum sensing molecule

Acyl-homoserine lactones are autoinducers taking part in the quorum sensing (QS) system, a well-known mechanism of bacterial cell-cell communication that activates the expression of the virulence genes only when bacteria are in high population levels (Fuqua et al., 2001). In the *R. solanacearum* regulatory network, PhcA positively controls the production of a second QS molecule, an Acyl homoserine lactone (acyl-HSL) dependent autoinduction system consisting of *luxR* and *luxI* homologues, designated *solR* and *solI* respectively. *R. solanacearum solI* mutants, incapable to produce acyl-HSL are not altered in their production of virulence factors in culture or for virulence *in planta* (Flavier et al., 1997b).

However the SolI/SolR system is not only induced by PhcA, but also by RpoS$_{rso}$ sigma factor. *rpo*S$_{Rso}$ mutants showed an alteration in production of virulence factor and virulence in tomato plants contrarily to *solI* mutants. This could be explained due to the low production of acyl-AHL in the *rpo*S$_{Rso}$ mutant which is necessary for the expression of, *solR*, whose product in turn is a required transcriptional activator of the acyl-AHL synthase gene, *solI* (Flavier et al., 1998).

9 A global regulation network of virulence functions

9.1 Regulation of virulence functions, the case of Eps

Expression of the *eps* locus and genes encoding extracellular enzyme is dependent of PhcA by an indirect way, and comprises several couples of two-component regulatory systems which can potentially integrate various environmental signals, whose nature still remains unknown (Boucher and Genin, 2004). PhcA first binds to the promoter of an intermediate regulator (*xps*R) and activates its transcription (Huang et al., 1998; Schell, 2000). Then XpsR, in conjunction with VsrC activates *eps* transcription. Expression of *xpsR* is also controlled by the VsrA/VsrD two-component system (Huang et al., 1998; Schell, 2000). Thus, the levels of active PhcA and active VsrD, which are controlled by the Phc system and VsrA, respectively, in response to their cognate signals, synergistically control levels of

XpsR and hence *eps* (Schell, 2000). However, high level transcription from the *eps* promoter (P_{eps}) requires input not only from the Phc system and *vsrA/vsrD* (via *xpsR*), but also from VsrB/VsrC (Figure 10).

9.2 Regulation of pectinolytic enzymes

Product the PehSR two-component regulatory system was found to control the production of pectinolytic enzymes (Allen et al., 1997). The described model for environmental control of polygalacturonase *pglA* is as follows: when the 3-OH PAME concentration is low, *pehSR* is highly expressed. If the unidentified cognate signal for the PehS sensor kinase is present, PehS will phosphorylate PehR, stimulating it to activate transcription of *pglA* directly or via an intermediate. When 3-OH PAME is abundant (e.g. cells are confined), *pehSR* expression (and hence PglA production) is repressed by PhcA, irrespective of the presence of the signal for PehS. Since the VsrB/VsrC two-component system represses PglA production by eightfold independently of *pehSR* or *phcA* (Huang et al., 1993; Schell, 2000), its activation by its cognate signal could further repress *pglA* expression (Schell, 2000). It is also described that PehSR also regulates bacterial swimming motility and production of Type IV pili which participate in important aspects of the *R. solanacearum* life cycle (Allen et al., 1997; Kang et al., 2002, Boucher and Genin, 2004) (Figure 10).

9.3 *hrp* gene activation in response to plant cell contact

The perception of plant cells by *R. solanacearum* is mediated by PrhA, a bacterial outer membrane receptor, which transduces this signal through a complex regulatory cascade involving the PrhR, PrhI, PrhJ, HrpG and HrpB regulators. PrhI is an ECF sigma70 factor that, together with the plant signal receptor PrhA and the PrhR protein, forms a signal transduction module traversing three compartments (outer membrane, PrhA, periplasm, PrhR, and cytoplasmic membrane, PrhI,) (Marenda et al., 1998; Aldon et al., 2000; Brito et al., 2002). In response to a signal from the plant cell surface, this module rapidly activates transcription of the target gene *prhJ*. The PrhJ regulator, in turn, activates HrpG, an OmpR-related regulatory protein, that activates HrpB, the major pathogenicity regulator controlling the expression of the *hrp* and T3SS effector promoters via the hrp$_{II}$ box (an imperfect 'plant inducible promoter' element) (Brito et al., 1999, 2002; Cunnac et al., 2004b; Wengelnik and Bonas, 1996). PrhA, PrhR, PrhI and PrhJ proteins are involved only in transduction of the

plant signal, whereas HrpG is required for transcriptional activation of *hrpB* in response to both plant and nutritional signals (Brito et al., 1999; Brito et al., 2002) (Figure 10).

The *hrp* genes that encode components of T3SS are not expressed when the bacteria are grown in complete medium, only their expression is induced after growth in apoplast-mimicking minimal media (Arlat et al., 1992). In this condition only *hrp*B and *hrp*G, are required for *hrp* gene induction (Plener et al., 2010).

9.4 Connection with the PhcA-dependent network.

PhcA negatively regulates the expression of the T3SS in complete medium and this PhcA-mediated repression is triggered by complex nitrogen sources (Genin et al., 2005). Since the activity of PhcA is controlled by the autoinducer molecule 3-OH PAME (mentioned above), it implies that *hrp* genes are repressed also at high cell density when this diffusible chemical signal accumulates. When *R. solanacearum* perceives high cell density and complex growth environment signals as indicators of nutrient sufficiency, the bacterium suppresses the expression of T3SS for acquisition of nutrients from plant cells (Genin et al., 2005).

9.5 HrpG, another master regulator of pathogenicity functions.

HrpG does not only activate HrpB transcription and T3SS expression but also controls the expression of other virulence determinant such as plant cell wall degrading enzymes, exopolysaccharides, or ethylene and auxin phytohormone biosynthesis genes (Valls et al., 2006). The discovery that production of these phytohormones is controlled by an *hrp* master regulatory gene, whose activity is induced in presence of plant cells strongly, suggested that these molecules play a key role during the early steps of infection in addition to the T3SS (Valls et al., 2006). This was recently confirmed by a study showing that a GMI1000 mutant defective for ethylene production was less competitive than the wild-type strain when co-inoculated in eggplant leaves (Macho et al., 2010). Amongst the other determinants dependent on *hrp*G they are genes involved in protective functions such as the catalase enzyme (*katE*) and polyamines synthesis genes. Finally, another example of genes positively controlled by HrpG are the genes implied in the adhesion to plant surfaces such as lectin genes which potentially plays an important role during the

early steps of plant infection (Valls et al., 2006). Valls et al. provided evidence that the T3SS-independent functions controlled by HrpG are necessary for pathogenicity on tomato plantlets, therefore showing that some important functions that remain to be identified are collectively important for pathogenesis beyond type III secretion.

Recently, a new regulatory gene encoding a protein highly similar to HrpG, called *prh*G, was identified. This new identified protein PrhG appears to be specifically involved in the control of the *hrpB* gene in response to environmental signals encountered by the bacteria when they are grown under minimal medium conditions (Plener *et al.*, 2010) but since the corresponding mutant is not significantly altered in virulence, its role during the pathogenic process remains obscure.

CHAPTER II

PRESENTATION OF PhD PROJECT

CHAPTER II

1 *Ralstonia solanacearum* race 3 strain, the causative agent of potato Brown Rot

1.1 Taxonomical classification

On the basis of taxonomical studies, *R. solanacearum* is considered as a complex species due to its very large infra-specific diversity. Molecular and genomic analyzes made possible to define four monophyletic groups called phylotypes which can be associated with their geographical origin (Fegan and Prior, 2005). Recent phylogenetic evidence indicated that strains that fit with the definition of the potato brown rot agent were placed into the phylotype IIB sequevar 1 (i.e., the biovar 2 Andean strains of *R. solanacearum* historically known as race 3, biovar 2). These strains are highly pathogenic to potato and tomato and adapted to highland temperatures. Strains that clustered into three of the four phylotypes of *R. solanacearum* can wilt potato; however, it is the phylotype IIB sequevar 1 strains (IIB1 strains) that are the most persistent and potentially the most destructive for potato (Guidot et al., 2009).

Although classically described as 'narrow host range' strains, 'race 3' strains are not exclusively associated with the potato host and were subsequently reported to possess a significantly broader host range spectrum (including eggplant, geranium, pepper, cabbage and even non-solanaceous herbaceous weeds) (Elphinstone, 2005; Alvarez et al, 2008a). One of the reason for explaining this 'paradox' is that artificial inoculations in controlled conditions certainly overestimates the natural host range of a strain since these inoculations are performed under rather high bacterial inoculum (10^7 to 10^8 cfu/ml) in the 'natural' drenching inoculation method or by direct stem-injection, conditions which are rarely reached in natural conditions in the field.

The origin of *R. solanacearum* is not clear, but Hayward (1991) suggests it predates the geological separation of the continents as the bacterium has been found in virgin jungle in South America and Indonesia. However *R. solanacearum* race3 strains are considered as a very homogenous group; isolates from around the world have been
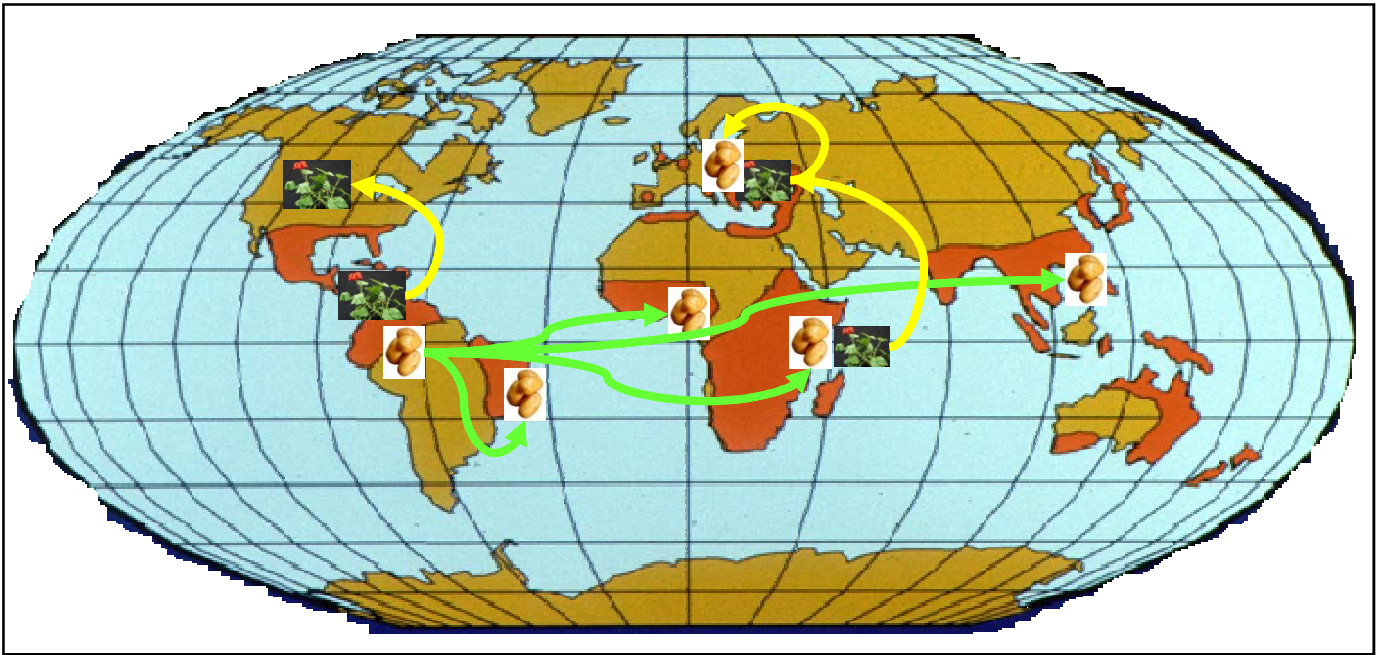
Figure 11. Origin and dissemination of *R. solanacearum* R3

Green arrows indicate the distribution of Race 3 from their place of origin in the Andes (south America) in potato tubers and yellow arrows indicate the introduction of Race 3 in geranium cuttings into the North of Europe and The USA.

shown to be nearly genetically and phenotypically identical, suggesting that this race 3 was originated in the Andes with potato and it was distributed from South America in a latent state within the tubers (Fegan and Prior, 2005; Gabriel et al., 2006, Milling et al., 2009).

1.2 Dissemination of the pathogen and epidemiology of the potato brown rot disease

For the European and Mediterranean Plant Protection Organization region (**EPPO**), it is mainly *R. solanacearum* race 3 biovar 2, the causal agent of wilting disease (brown rot) of potato (primary host) and bacterial wilt on tomato, which is of importance, since this so-called low-temperature strain is adapted to cooler climates in the highlands of the tropics and in the Mediterranean area (EPPO 2004). Its occurrence has now also been reported from temperate zones, and in particular this race 3 has been observed in regions of several European countries such as The Netherlands, Belgium, France, Sweden, Spain and the United Kingdom (Janse 1996; Janse et al., 1998; van Elsas et al., 2000; Caruso et al., 2005, Elphistone, 2005) (See Distribution map of *R. solanacearum* race 3 (Figure11). The dissemination of the organism from such infested soil areas into surface water and sediment and the weedy species *Solanum dulcamara* (bittersweet), which grows along waterways, may have contributed to the establishment of this organism in temperate climate zones (Janse et al., 1998; Elphinstone et al., 1998). In particular bittersweet may have catalyzed the spread of the organism, given its capacity to serve as a colonisable host (Wenneker et al., 1999; Alvarez et al. 2008a) and illustrates the difficulty to eradicate R3 from the northern Europe. Despite widespread detection of the pathogen in northern Europe, direct losses of potato to disease have been limited to a few outbreaks during unusually hot summers (Priou et al., 2006). Nonetheless the economic impact of the additional quarantine related testing has been significant (Champoiseau et al., 2010). Adaptation to cold, long term soil persistence and its threat for major economical plants such as potato has led to the decision to list *R. solanacearum* R3 as a high-concern quarantine pathogen in Europe and North America and as a Select Agent in the Agricultural Bioterrorism Protection Act 2002 (Lambert, 2002).

1.3 Introduction to Europe and North America of *R. solanacearum* race 3

R3 strains often forms symptomless or latent infections, principally in the cool tropical highlands, that facilitate unknowing pathogen spread in seed potato tubers or plant cuttings, but when infected seed tubers are planted in warmer lowland fields, the resulting plants quickly wilt and die (Allen et al., 2001; Swanson et al., 2005). Thanks to their latent property, it is suggested that worldwide distribution of *R. solanacearum* R3 started from potato tubers from the Andes (South America), the geographical area where the host plant originates, since isolates from around the world have showed to be genetically and phenotypically identical (Milling et al., 2009; Swanson et al., 2005).

Recent introductions of R3 in Europe and North America proceeded through geranium cuttings. Geraniums sold in Europe and The United States are commonly grown from cuttings produced in the highland tropics of Africa and Central America, where R3 is endemic (Swanson et al., 2005). Into northern Europe it was introduced in the late 1980s causing both economic and political problems when it appeared on potatoes in 1995 (Janse et al., 1996). In December 1999, R3 was detected for the first time in the UK on imported geraniums cuttings, *Pelargonium zonale*, produced in Kenya for their propagation for the European market. During the following years, symptoms produced by *R. solanacearum* R3 were detected in several *Pelargonium* nurseries in other European countries as Belgium, Germany and The Netherlands (Janse et al., 2005). Despite ongoing eradication efforts, the bacterium is still present in a number of fields and waterways of northern Europe, although it has caused only minor direct crop losses. In 1979, R3 entered into the United States through the latently infected geranium cuttings (Janse et al., 2005; Swanson et al., 2005). Years latter, on several occasions in 1999 and 2000, imported geranium cuttings were positive for R3 (Kim et al., 2002 and 2003, Williamson et al., 2002). Nevertheless, during 2001 and 2002 there were no reported cases but in February 2003, the bacterium was again identified in geraniums introduced from Kenya, and a reintroduction of this R3 also occurred in December 2003 from new cuttings that came from Guatemala (O'Hern, 2004).

Bacterial wilt epidemiological cycle of potato race 3 strains in temperate climates was proposed to start with the water effluents resulting from domestic or industrial processing of this material which leads to contamination of water streams. This initial

inoculum is multiplied by infecting asymptomatically the weed *Solananum dulcamara* that is commonly found along rivers and that forms aquatic, adventitious roots, therefore prompting the release of bacteria in the water stream and infection of additional plants downstream. Use of such contaminated waters for irrigation of plots devoted to potato seed production is the most probable source of primary potato infections. Because potato can develop asymptomatic latent infection, infected potato seed thus obtained became a main source of R3 dissemination among many western European countries (Champoiseau et al., 2010).

2 Genome sequence projects of *R. solanacearum* 'race 3' strains

2.1 Strain IPO1609

2.1.1 Origin and physiological studies

R. solanacearum race 3 (biovar 2) strain 1609 (there after named IPO1609) was isolated in 1995 from potato, *Solanum tuberosum* L. cv. *Bartina* by the Dutch plant protection service (JD Janse, Plant Protection Services, Wageningen, The Netherlands). At the time, the strain aggressiveness was regularly tested on tomato plants in this laboratory, consistently resulting in wilting of 100% (4 out of 4 plants) of tomato plants in up to two weeks (van Elsas et al., 2000). Different studies have been published using this strain, principally focused in the population dynamics and the physiological response under temperate climate conditions in habitats such as agricultural soils and irrigation water used in potato cropping practices (van Elsas et al., 2000; 2001). These studies showed that population densities of IPO1609 strain declined progressively over time in the soil and into drainage water to levels below the respective limits of detection, due to the presence of indigenous organisms and the presence of sediments (in water). Nevertheless, the effect of temperature on strain IPO1609 cells was stronger than any other biotic or abiotic factor. At 12 or 15 and 20°C, a gradual decline of the population densities was observed in three different kind of soils, occasionally bordering the limit of detection (10^2 CFU g^{-1} of dry soil), in periods of approximately 90 to 210 days (van Elsas et al., 2000). In water, the
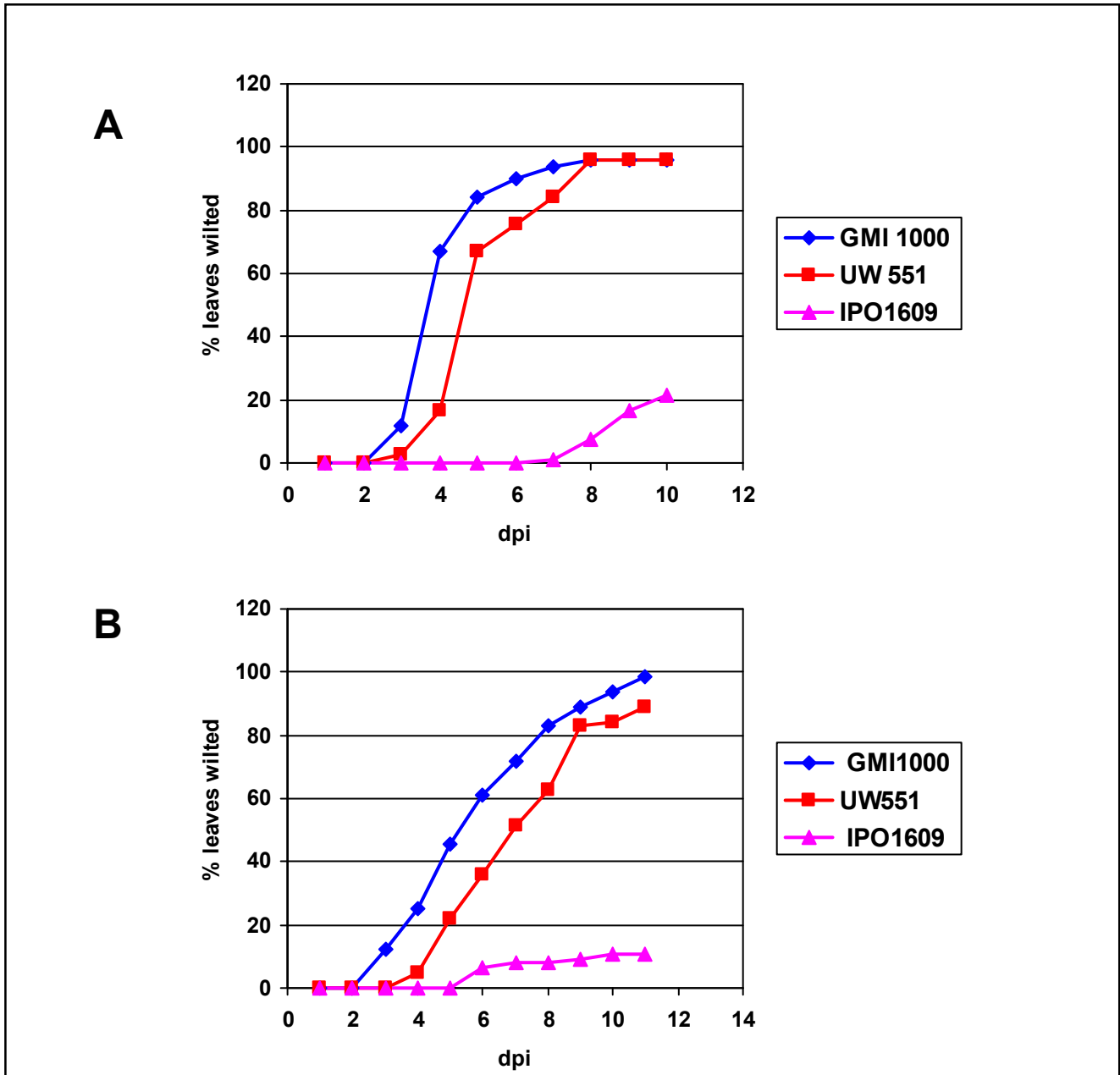
Figure 12. **Virulence of *Ralstonia solanacearum* GMI1000, UW551 and IPO1609 on tomato (A) and potato plants (B)**

R. solanacearum strains GMI1000, UW551 and IPO1609 were tested for pathogenicity on 24 tomato plants of 4-week-old (*Solanum lycopersicum* cv Supermarmande) with 50 ml of bacterial suspension of 1 x 10^8 cfu ml-1 per plant and on potato plants (*Solanum tuberosum* cv. Monalisa) by infiltration with 10 μl of bacterial suspension of 1 x 10^7 cfu/ml. Disease development was scored daily by using a disease index where 0 indicates no disease, 1 indicates 1 to 25% of leaves wilted, 2 indicates 25 to 50% of leaves wilted, 3 indicates 51 to 75% of leaves wilted, and 4 indicates 76 to 100% of leaves wilted. Plants were kept at 28°C, 75% humidity during 10-12 days. Results are representative of three or more experiments.

population survival was maximal at 12, 20 and 28°C (van Elsas et al., 2001). However a progressive reductions in the sizes of culturable populations where observed in the soil and irrigation water at low temperatures as 4°C reflected in an accelerated decline of CFU counts, to undetectable numbers (Van Elsas et al., 2000; 2001). At this cold condition, substantial nonculturable *R. solanacearum* IPO1609 populations where shown to be metabolically responsive and therefore viable since they were in the physiological state known as viable but non-culturable (VBNC). Other characteristic of *R. solanacearum* IPO1609 strain was its great capacity to grow when present in low numbers in sterile ultrapure water, even upon several serial transfers to new ultrapure water, this growth was still observed (van Elsas et al., 2001). This capability of *R. solanacearum* IPO1609 to actively grow at very low substrate concentrations is revealing a possible life strategy of this organism in oligotrophic environments (van Elsas et al., 2005).

2.1.2 Genome sequence

Recently work in the laboratory of C. Boucher and the French National Center Genoscope established a draft genome sequence for the Phylotype II 'race 3' strain IPO1609 which is adapted to potato. Although closure of the genome could not be achieved, assembling led to a relatively limited number of supercontigs. Nineteen supercontigs of 10kb or more are sufficient to cover more than 98% of the IPO1609 available sequence (Guidot et al., 2009). By comparison with strain GMI1000, supercontig IPO_001 which spans 3,372,855 bases covers most of the chromosome-born core genes previously identified (Guidot et al., 2007) whereas supercontig IPO_002 which spans 1,870,194 bases covers most of the GMI1000 megaplasmid-born core genes.

Although the IPO1609 was originally described as pathogenic on tomato by van Elsas et al. (2000), pathogenicity assays conducted in our laboratory using the sequenced isolate of IPO1609 revealed that the strain was almost non-pathogenic on tomato and on potato (Figure 12). Strain IPO1609 was scored as HR negative when infiltrated in tobacco leaves but this behaviour was shown to be dependent on the structure of the Type III effector *avrA* which encodes the major HR elicitor on several tobacco species (Poueymiro et al., 2009). Additional experiments have shown that

IPO1609 expressing the *avrA* gene from GMI1000 elicits an HR similar to the one elicited by the wild-type GMI1000 (S. Genin, unpublished). This latter result suggested that the Type III Secretion System in strain IPO1609 is functional and that the lack of virulence of the strain was not due to a T3SS-defective genotype. Accordingly, sequence analysis of the *hrp* cluster in strain IPO1609 revealed that all the T3SS genes are present and highly conserved to those of GMI1000.

2.2 Strain UW551

2.2.1 Origin and physiological studies

R. solanacearum UW551 strain was isolated in Winsconsin, The United States, in 2003 from the stem of a wilted geranium (*Pelargonium hortorum*) grown from a cutting originating from Kenya (Swanson et al., 2005). UW551 commonly forms latent infections in geraniums, thus wilt symptoms are not detected despite a large content of bacteria cells in their tissue. UW551 is therefore considered as a non aggressive pathogen of geranium, contrarily to its effect on potato and tomato plants (Swanson et al., 2005). One of the most relevant characteristic of Race 3 strains is their cold tolerance, thus ecological traits of UW551 has been evaluated and compared in cold conditions with the American strain K60 (phylotypes II, race 1, bv1) and tropical strain GMI1000 (Phylotype I, race 1 bv3) (Milling et al., 2009). Surprising in water at 4°C K60 strain remained culturable the longest (up to 90 days), whereas tropical strain remained culturable for the shortest time (≈40 days). However, inside potato tubers, UW551 survived more than 4 months at 4°C, whereas North American strain K60 and tropical strain GMI1000 were undetectable after less than 70 days in tubers. GMI1000 and UW551 grew similarly in minimal medium at 20 and 28°C and, although both strains wilted tomato plants rapidly at 28°C, UW551 was much more virulent at 20°C, killing all inoculated plants under conditions where GMI1000 killed just over half. Thus, these results demonstrate that UW551 (and presumably other R3 strains) has no special adaptation to survive cold temperatures in water under controlled conditions, nor did it grow much faster than a tropical strain when cultured at the moderately cool temperature of 20°C. However, the presence of host plant tissue appears to mediate distinctive cold adaptation for R3bv2, both in terms of survival at near-freezing temperatures and ability to wilt plants at cool temperatures

(Milling et al., 2009).

2.2.2 Genome sequence

An 8× draft genome was obtained and annotated for strain UW551 by Gabriel et al, (2006). The draft UW551 genome consisted of 80,169 reads resulting in 582 contigs containing 5,925,491 base pairs, with an average 64.5% GC content. Annotation revealed a predicted number of 4,454 protein coding open reading frames (ORFs), 43 tRNAs, and 5 rRNAs; 2,793 (or 62%) of the ORFs had a functional assignment. This genome sequence showed a level of 71% synteny in comparison with the GMI1000 (phylotype I, Asian, R1bv3) genome. In addition, all major classes of genes currently known or predicted to be involved in pathogenicity, including plant-degradative enzymes, type III effectors, and some additional ORFs known to be upregulated in plants, were highly conserved. Genomic comparisons revealed the gene differences in alcohol oxidation responsible for biovar determination. Among the 402 genes present in UW551 but absent in GMI1000, few appeared to be involved in pathogenicity and none obviously involved in cold tolerance. Therefore, pathogenic and cold adaptation variability within the species may be determined by regulatory genes, genes of unknown function, or to genetic variation found among conserved genes. Physically, the most obvious difference between the compared genomes was the presence of a cluster of 38 probable prophage genes specific to UW551. PCR analyses revealed that this cluster of genes were present in all R3bv2 strains tested from a wide variety of geographical sources. These data confirmed that R3bv2 strains are highly clonal and that UW551 is representative of the group (Gabriel et al., 2006).

3 Preliminary indication that strain IPO1609 may carry a large deletion on its megaplasmid

A recent work established the list of genes that are specific to potato brown rot IIB1 strains of *R. solanacearum* using the Comparative Genomic Hybridization (CGH) technology (Guidot et al., 2009). This work used a microarray representing genes of the reference phylotype I strain GMI1000 and of the phylotype IIB1 strain IPO1609. This analysis provided indication that a large region of the megaplasmid of strain
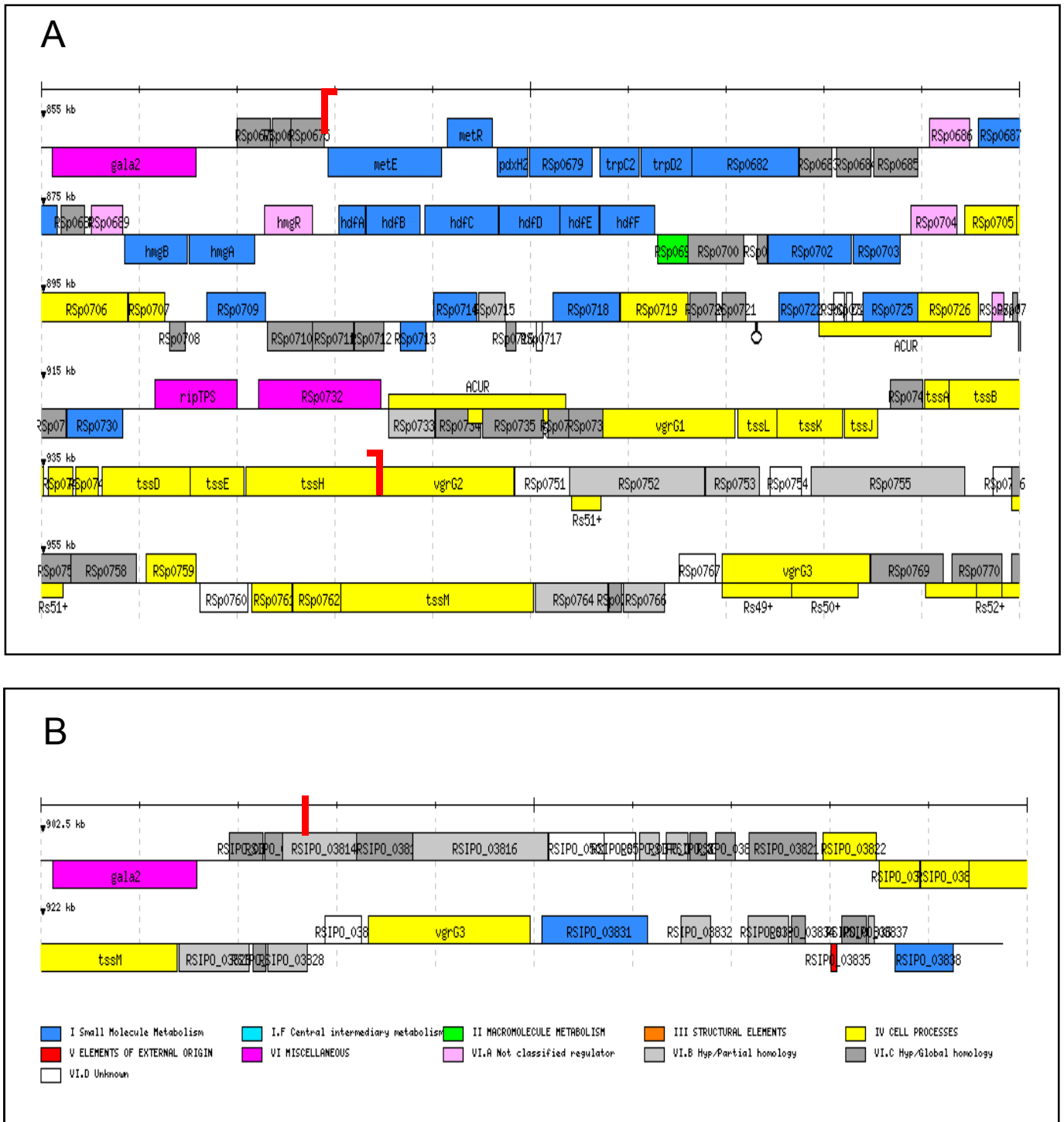
Figure 13. **Genomic map of the megaplasmid region in strain GMI1000 (A) and IPO1609 (B) containing the large set of genes predicted to be absent in strain IPO1609 from the CGH study** (see Table 6).

The red bars on the GMI1000 map delineates the region absent in IPO1609 and the red bar on the IPO1609 indicates the predicted deletion point. Each line correspond to 20kb of DNA; colour indicates genes with similar functional assignement. Genes above or below the horizontal lines are transcribed from left to right or right to left, respectively. Thin yellow bars symbolize ACUR (Alternate Codon Usage Regions) or Repeated Regions (Rs+) in the GMI1000 genome.

IPO1609 comprising 68 genes (corresponding to GMI1000 RSp0676 to RSp0749) was predicted to be absent since no hybridization was detected for most of the IPO1609 genes in this region (see Table 6 as an annex at the end of this chapter). Similar CGH experiments performed with four other phylotype II strains (including another 'race 3' strain, CIP301) revealed that this region was specifically missing in IPO1609.

The comparison of the genomic maps of annotated genes in this region of the megaplasmid in strains GMI1000 and IPO1609 confirmed this difference (Figure 13). In order to rule out the possibility of an assembling mistake or a genomic rearrangement, homology searches were performed with the IPO1609 sequence for each gene in the predicted missing region. Results were negative, except for one block of genes (RSp0693 to RSp0698) which appears to be located elsewhere on the chromosome of strain IPO1609; this observation also correlates with the CGH data (Table 6).

4   PhD objective

The genome annotation of strain IPO1609 and its analysis revealed that all the pathogenicity determinants described in the literature (T3SS, EPS biosynthesis, plant cell wall degrading enzymes etc…) were present in the sequenced genome. The morphological characteristics of strain IPO1609 on BG medium plates were indistinguishable from other *R. solanacearum* strains, indicating that IPO1609 was not significantly altered for EPS production or growth properties *in vitro*.

Further genomic sequences comparisons revealed that the DNA sequences from strains IPO1609 and UW551 were almost identical and most often exceeded 97% identity. Global comparisons were hampered by the fact that the two genome sequences are draft sequences with unassembled contigs, each containing multiple stretches of nucleotide gaps. At the level of the protein products predicted from the sequences, some discrepancies between the two strains were observed but reflected differences in the prediction of ORFs in the two genome annotations. The exact conservation of the position of most of the mobile genetic elements (Insertion

Sequences, transposases) in the two genome sequences also support the view that UW551 and IPO1609 are very closely related taxonomically.

The objective of this PhD work was to determine what causes the striking difference of pathogenicity observed between IPO1609 and UW551 on the host plants potato and tomato, given the strong genomic relatedness of the strains, and to identify the molecular determinants responsible for this trait. This question was attractive since all the major *R. solanacearum* pathogenicity determinants previously described appeared to be conserved in both strains, suggesting that this difference in the pathogenicity trait could rely on novel pathogenicity gene(s). Since strain IPO1609 appeared to carry a large deletion estimated to 70-80kb on its megaplasmid compared to other phylotype II strains, we first focused our interest on this particular region. Our first goal was therefore to evaluate the relevance of this deletion on the pathogenicity phenotype. In a second step, we carried out a functional analysis of this region to identify the molecular determinants involved in this phenotype.

ANNEX 1

Table 6. Detection of gene distribution in strain IPO1609 and four other strains from Phylotype II using comparative gene hybridization technology with the GMI1000 microarray. The table details the data obtained for a region covering approx. 90kb of the GMI1000 megaplasmid (oligonucleotides RSp0664-RSp0767 on the microarray). Total DNA from each strain was hybridized and quantification of the signals from individual arrays was done using ImaGene 5.6.1 software and analyzed using Genesight 3.5.2 software. The pair median ratio represents the normalized ratio of the hybridization signal of the tested strain to that of the reference strain GMI1000 which is $\log_2$ transformed. In yellow are indicated the values (and the genes) below the cut-off value of –2 corresponding to genes predicted to be absent/divergent, based on the methodology developed by Guidot *et al.* (2007).

Gene ID GMI1000	IPO1609 Phyl IIB Potato Netherlands	NCPPB3987 Phyl IIB Tomato Brasil	CFBP2957 Phyl IIA Tomato Martinique	CIP301 Phyl II Potato Peru	UW20 Phyl II Banana Honduras
	Pair Median Ratio	**Pair Median Ratio**	**Pair Median Ratio**	**Pair Median Ratio**	**Pair Median Ratio**
RSp0664	-0,476347	-0,870944654	-0,6530668	-0,2457303	-0,821938
RSp0665	0,1086895	-0,172865708	0,17016713	0,2258463	-0,053788
RSp0666	0,7757995	-0,405116607	0,63343306	0,9254335	0,4678112
RSp0667	0,7235624	-0,069442393	0,47374926	0,540798	0,7513614
RSp0668	-0,647871	-1,383666277	-1,63245748	-1,0604121	-0,351194
RSp0669	-0,337895	-1,002614366	-5,01834541	-6,4940042	-0,251215
RSp0670	0,5974088	0,246503507	-3,61190646	-3,7518413	0,6477916
RSp0671	-0,027821	-0,233127715	-0,00955332	0,328231	0,154464
RSp0672	0,1448509	-0,053464423	0,62698458	0,0391635	-3,514095
RSp0673	-0,937233	-0,698479812	-0,99827803	-1,8119309	-0,965959
RSp0674	-0,681683	-0,006952774	-0,05207508	0,1372871	-0,488851
RSp0675	0,391652	0,641128526	0,88351726	1,0295205	0,6788294
RSp0676	-4,219403	-0,577993785	-0,6634391	-0,0857742	-0,895095
RSp0677	-4,439364	-0,388984065	-0,50120543	-0,2353167	-0,202047
RSp0678	-4,927953	0,068285439	1,05227776	0,4581142	-0,104979
RSp0679	-6,578064	-0,506796245	-0,37321773	-0,7024212	-0,535067
RSp0680	-3,43529	-3,405540668	-4,22909165	-4,4826805	-3,893731
RSp0681	-3,315004	-0,288716986	0,20555881	-0,3515486	-0,136613
RSp0682	-3,194073	0,299377019	0,84793048	0,683572	0,2827971
RSp0683	-5,914642	-3,51071068	-5,60716867	-3,3555663	-2,832877
RSp0684	-2,254291	-0,032068631	-0,63139676	-0,6600332	-0,330355
RSp0685	-3,306083	-0,457083191	-1,03413847	-1,3890228	-0,659856
RSp0686	-3,969992	-1,771275254	-3,56219097	-4,3063274	-4,495633
RSp0687	-3,681674	-0,94338081	-0,59598146	-0,6207625	-1,143395
RSp0688	-4,58934	-0,336799004	-1,42060877	-1,2318375	-0,607798
RSp0689	-5,158623	0,080224841	-0,32312429	0,2044696	0,0131533
RSp0690	-3,606194	-0,326405792	-0,83429401	-0,5782763	-0,476946
RSp0691	-3,270809	-0,10369976	-0,10898481	-0,2636189	-0,156591
RSp0692	-3,458818	-0,85218456	-0,09363626	0,1903327	-2,649094
RSp0693	-0,235425	-0,357998957	0,80379316	0,4667158	-0,202242
RSp0694	0,2557611	0,234170618	0,06449924	0,2122757	0,2390278
RSp0695	-5,309751	-4,323973624	-7,29010861	-7,2582193	-5,837472
RSp0696	-4,335862	-3,005922454	-5,19454928	-6,22738	-3,331973
RSp0697	0,7374388	0,691727471	0,1463154	0,2223634	0,7781612
RSp0698	-0,051587	0,053677191	-1,02818082	-0,2611537	0,079468
RSp0699	-1,000449	-0,202911054	-0,07616791	-0,1071645	0,0364948
RSp0700	-5,098961	-0,886238212	-2,5908234	-2,2432936	-1,340186

RSp0701	-6,358316	-1,858228904	-0,94740731	-0,6884249	-0,690151
RSp0702	-6,15112	0,180089364	-0,42766462	-0,2745489	0,25533
RSp0703	-5,633702	0,451557869	-0,44645952	-0,7817917	0,5294268
RSp0704	-3,094557	0,006792806	0,13499857	0,6492013	0,2233728
RSp0705	-4,613403	-0,089390994	0,24570756	0,5797166	-0,026335
RSp0706	-0,852311	0,687441461	0,52590913	0,9180873	0,9108633
RSp0707	-3,60907	0,59764978	0,16778208	0,4882437	0,2301249
RSp0708	-1,959392	-0,410486832	-0,77757674	-0,7390758	-0,614441
RSp0709	-3,744762	-0,287685773	-0,21628222	-0,017107	-0,319545
RSp0710	-2,23385	-0,23574316	-1,32941894	-1,0931034	-0,331106
RSp0711	-3,485107	0,17407504	-3,08445503	-2,6236413	0,030846
RSp0712	-4,277694	-0,078862896	-0,59580488	-0,2813432	-0,34322
RSp0713	-2,065765	-0,657107722	-1,30474552	-1,2507795	-1,41646
RSp0714	-4,178853	-1,098124337	-1,23357398	-1,1761418	-0,950853
RSp0715	-8,564433	-0,513125226	-0,9446235	-0,4151598	-0,494563
RSp0716	-5,356296	-0,660640118	-0,97358434	-0,4968474	-0,576421
RSp0717	-2,5127	-1,396409211	-3,10426685	-3,8723379	-1,851318
RSp0718	-2,750959	0,102456516	-1,56442994	-0,3269109	-0,035278
RSp0719	-2,989364	-2,057122545	-5,93625798	-5,1656086	-3,059817
RSp0720	-6,020979	-1,11938505	-2,04306255	-1,9343639	-1,290171
RSp0721	-1,096988	-1,751195285	-3,09916214	-2,7451956	-3,161551
RSp0722	-4,276509	0,169563528	0,14874335	0,3296305	0,1301541
RSp0723	-3,514556	-4,589651527	-8,35223221	-8,0304242	-6,08837
RSp0724	-5,126675	-4,677002706	-6,08952411	-7,1029906	-6,005329
RSp0725	-3,795555	-3,894199193	-4,5450606	-4,1746743	-2,919336
RSp0726	-5,829621	0,118258249	0,26863232	0,4459297	-0,360957
RSp0727	-4,568383	-2,668858655	-6,16726	-6,0343846	-4,380572
RSp0728	-6,906601	-1,076413969	-0,77703567	-0,3923771	-0,953646
RSp0729	-8,720351	0,433160894	-0,07572988	-0,0487355	0,1529354
RSp0730	-4,256554	0,273261531	0,09600712	0,0478443	0,1560151
RSp0731	-3,634412	-1,968412118	-2,28221697	-2,2904823	-1,856616
RSp0732	-8,362249	-2,034179172	-5,39158902	-3,7741144	-3,037072
RSp0733	-6,885435	-5,629943971	-3,60344897	-7,4997849	-6,045299
RSp0734	-8,1034	-16,99145096	-3,97893721	-8,2450452	-5,398757
RSp0735	-4,734912	-5,566028671	-4,71476076	-4,7182968	-5,090651
RSp0736	-6,930124	-6,578140676	-5,78695142	-6,7072026	-6,757362
RSp0737	-6,134063	-5,603971551	-6,60785841	-4,674513	-6,202295
RSp0738	-5,706929	-5,409795879	-5,58141564	-2,7708735	-6,884379
RSp0739	-2,339511	-0,228421343	-0,29105815	-0,5013074	-0,027823
RSp0740	-6,453885	-0,253985312	0,09854279	-0,012521	-0,117505
RSp0741	-5,689108	0,122924527	-0,56032021	-0,6059262	-0,445327
RSp0742	-5,16171	-1,10743727	-1,0947435	-0,6567083	-0,916218
RSp0743	-2,851303	-0,634868706	-0,65837326	-0,6616439	-0,766744
RSp0744	-5,554219	-0,453614123	-0,51152144	-1,6754201	-0,646204
RSp0745	-6,889272	-0,134343825	0,27907112	0,1086651	-0,684732
RSp0746	-3,43038	-0,09926707	0,26446645	0,0176964	-0,460388
RSp0747	-4,654026	0,088593291	0,63023059	0,7031793	0,2072129
RSp0748	-4,063453	-0,786481207	-2,62941049	-2,3271503	-0,868058
RSp0749	-5,292201	-0,700404237	-0,62555202	-0,2590535	-0,222063
RSp0750	-0,236443	-0,329179256	-0,32242647	-0,6368059	-0,258452
RSp0751	-7,776346	-5,578551247	-7,4567756	-8,0412369	-6,729759
RSp0752	-2,872997	-5,006150995	-1,98301213	-7,4511872	-2,215272
RSp0753	-5,216787	-2,961193896	1,96263326	-3,9706467	0,1413318
RSp0754	-2,502343	-4,497988323	-7,13453046	-8,2055689	-5,948822
RSp0755	-5,854095	-5,42348808	-5,7757634	-7,2286821	-6,462315
RSp0756	-3,658971	-3,423070812	-6,03429643	-3,3283915	-4,775667
RSp0757	-1,24458	-0,974788815	-1,62920422	-1,1582655	-1,123756
RSp0758	0,1519111	-0,035051556	0,15702856	-0,211136	-0,002785
RSp0759	-1,566686	-0,989621446	-1,29584525	-0,4417447	-1,688168
RSp0760	-4,972579	-9,502911264	-7,89846612	-9,3442693	-7,367774
RSp0761	-0,189496	-0,142414947	-0,02945208	0,1868259	-0,294732
RSp0762	0,5764314	0,316040263	0,45722842	0,7692879	0,1050594
RSp0763	0,5717376	0,105668285	0,37407054	0,7746962	0,5583849
RSp0764	-0,200095	-0,592318619	-0,02574792	0,6265149	-0,293095

RSp0765	-0,293947	-0,679787706	-0,27398423	-0,060221	-0,320521
RSp0766	0,2449837	-0,215463349	0,11026472	0,7027418	0,0699721
RSp0767	-1,577685	-1,613188145	-1,96917091	-2,2618486	-0,316083

CHAPTER III

RESULTS

CHAPTER III

RESULTS
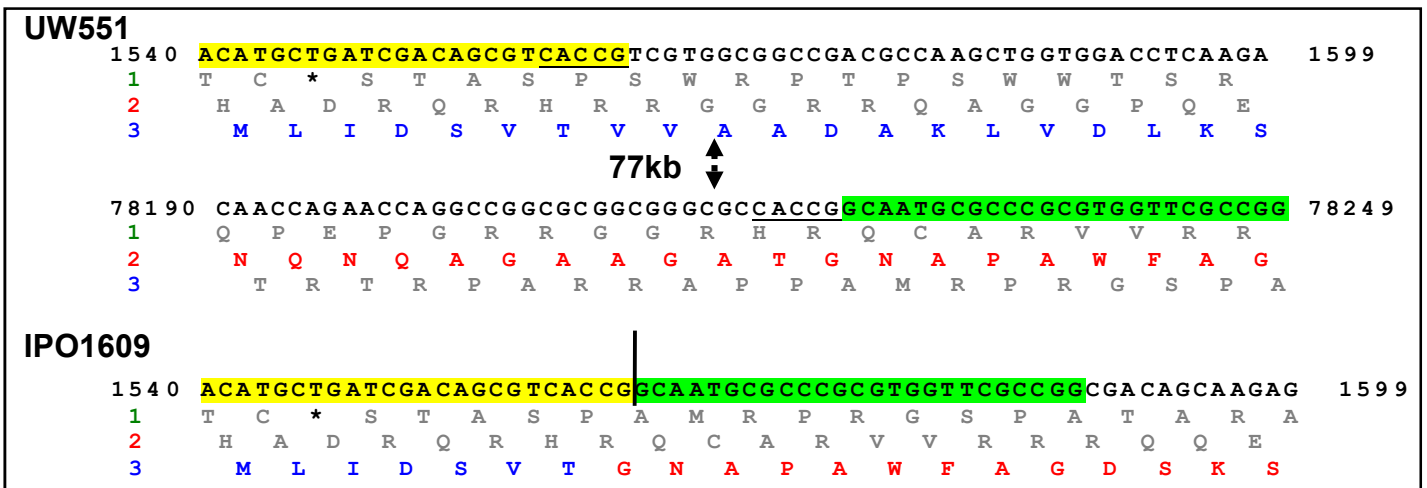
Figure 14 (A) Nucleotide sequence of the regions surrounding the 77 kb deletion point in strains UW551 and IPO1609. In strain UW551, the sequence is shown upstream and downstream the predicted deletion endpoint mapped in strain IPO1609. The yellow and green colours show the DNA sequences directly upstream and downstream the deletion point, respectively, and which are identical in both strains. The deletion point is symbolized by a black vertical bar. Below the nucleotide sequences are shown the predicted amino acids within the three possible reading frames. In strain IPO1609, the deletion creates an in-frame protein fusion between UW551 proteins RRSL_02267 and RRSL_01740 that corresponds to protein RSIPO_03814 (third reading frame) in strain IPO1609. A direct five nucleotide repeat just upstream the deletion endpoint is underlined.
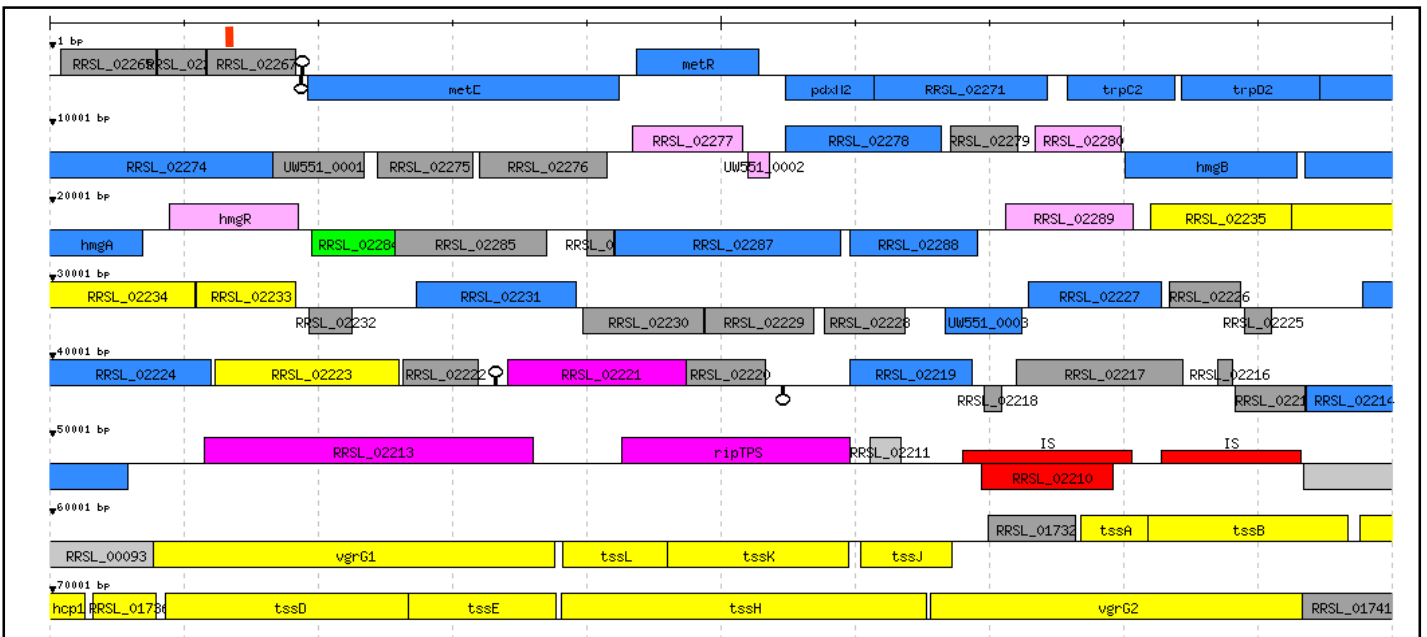
Figure 14 (B). Genomic map of the 77 kb region assembled from UW551 contig sequences. The red bars delineate the deleted region in strain IPO1609. Each line corresponds to 10kb of DNA. Junction of the UW551 contigs was realized between genes RRSL_02289 and RRSL_02235, *tssK* and *tssJ*, and the two Insertion Sequences (IS). Colour code represents functional classes for annotated genes: Small Molecule Metabolism (blue), Secretion & Transport processes (yellow), transcriptional regulators (pink), Transposable elements (red), Type III-dependent effectors (magenta) and proteins of unknown function (grey). Small bars topped by an empty circle symbolise predicted transcriptional terminator sequences. Gene nomenclature is the one from UW551 as deposited to GenBank, except for three genes (UW551_0001 to UW551_0003) not predicted from the annotation performed by Gabriel et al. (2006) and for the Type VI Secretion System, which follows the one used for *Burkholderia mallei* (Schell et al., 2007).

CHAPTER III

1 Comparison of the genome sequences of UW551 and IPO1609 strains identifies a large deletion on the megaplasmid of strain IPO1609.

We performed an analysis of the assembly of contigs from the UW551 draft genome published by Gabriel et al. (2006) in order to determine whether the ~70 contiguous genes found to be absent in IPO1609 were present or missing. All the genes predicted to be absent in IPO1609 were present in strain UW511 but were distributed on four different UW551 contigs. Comparison of the genomic maps of GMI1000 and IPO1609 indicated that the deletion probably occurred very closely to the RSp0675– homologue gene (Figure 13). We therefore examined the nucleotide sequence of this homologue in strain UW551 (named RRSL_02267) and compared it to the IPO1609 DNA sequence (Figure 14A). DNA sequence of the RRSL_02267 gene was 100% identical to the RSIPO_03814 gene only over the 394 first nucleotides. The 3' part of the RSIPO_03814 was homologous to UW551 RRSL_01740 (Figure 14B). The RSIPO_03814 gene therefore appears to be an in-frame fusion gene between RRSL_02267 and RRSL_01740 which are distant by >70kb on the GMI1000 megaplasmid. The exact deletion point was mapped in between the positions 394 bp downstream the start codon of RRSL_02267 and 1117bp upstream of the end codon of RRSL_01740 (Figure 14B). This observation at the DNA sequence level correlated perfectly with the results of the CGH analysis which predicted the absence of 68 genes in the same region, making unlikely that this deletion was due to an incorrect genome assembling in IPO1609. Finally, analysis of the immediate upstream and downstream sequences to the predicted deletion point revealed the existence of a direct repeat of five nucleotides CACCG which could be indicative of a remnant of a putative recombination event (Figure 14A).

Table 7. Inventory and function of the UW551 genes absent in strain IPO1609.
Genes are listed in accordance with the gene organization as shown on Figure 13. Homology (percentage of identity at the protein level) to GMI1000 orthologues is indicated; absence of value indicates that the corresponding gene is absent in GMI1000.

UW551 gene	Gene name	Protein size (AA)	Description	Homology to GMI1000
RRSL_02267		223	Putative Lipoprotein	I=91%
RRSL_02268	*metE*	776	Homocysteine methyltransferase (Methionine synthase)	I=87%
RRSL_02269	*metR*	304	Methionine Biosynthesis Transcriptional Regulator	I=91%
RRSL_02270	*pdxH2*	219	Pyridoxine/Pyridoxamine 5'-phosphate oxidase	I=86%
RRSL_02271		428	Putative Diaminopimelate decarboxylase	I=91%
RRSL_02272	*trpC2*	268	Indole-3-glycerol phosphate synthase	I=81%
RRSL_02273	*trpD2*	344	Anthranilate phosphoribosyltransferase	I=91%
RRSL_02274		733	Trimethylamine/Histamine dehydrogenase	I=87%
UW551_0001		266	Conserved hypothetical protein	I=86%
RRSL_02275		238	Conserved hypothetical protein	I=87%
RRSL_02276		315	Transmembrane hypothetical protein	I=74%
RRSL_02277		274	Transcription regulator	I=87%
UW551_0002		54	N-terminal fragment of a LysR transcription regulator (probable pseudogene)	
RRSL_02278		385	Acyl-CoA dehydrogenase	I=94%
RRSL_02279		165	Transmembrane protein	I=78%
RRSL_02280		212	FMN-binding negative transcriptional regulator	I=91%
RRSL_02281	*hmgB*	426	Fumarylacetoacetate hydrolase	I=92%
RRSL_02282	*hmgA*	448	Homogentisate 1,2-dioxygenase	I=95%
RRSL_02283	*hmgR*	321	Transcription regulator	I=86%
RRSL_02284		207	Peptide chain release factor homolog protein	I=84%
RRSL_02285		379	Conserved hypothetical protein	I=88%
RRSL_02286		68	Conserved hypothetical protein	
RRSL_02287		558	FAD-dependent monooxygenase	I=86%
RRSL_02288		319	Beta-lactamase, type II	I=97%
RRSL_02289		320	Transcription regulator	I=91%
RRSL_02235		350	Peptide transport system, ABC transporter, permease protein	I=73%
RRSL_02234		608	Peptide transport system, ABC transporter, permease protein	I=77%
RRSL_02233		247	Peptide transport system, ABC transporter, ATP-binding protein	I=95%
RRSL_02232		105	Signal peptide hypothetical protein	
RRSL_02231		398	Cytochrome P-450-like monooxigenase	I=89%
RRSL_02230		303	Transmembrane hypothetical protein	I=86%
RRSL_02229		270	Transmembrane hypothetical protein	I=72%
RRSL_02228		319	Beta-lactamase, type II hydrolase	I=97%
UW551_0003		190	N-acetyltransferase protein	I=69%
RRSL_02227		330	D-alanine aminotransferase	I=84%

2   Establishment of a physical map and annotation of the 77kb region of UW551 deleted in strain IPO1609

Genes predicted to be absent in IPO1609 were found to be distributed on four contigs of UW551 (Cont0556, Cont0555.1, Cont0477 and Cont0547, as deposited in GenBank) which all appear to contain multiple genes homologous to GMI1000 genes clustered in the RSp0676-RSp0752 region. Assembling of the UW551 contigs covering the IPO1609 deleted region was therefore realized based on the global synteny with the GMI1000 genome. During this assembling, we suspected that one contig (Cont0551.1) was chimaeric or misassembled since it contained 29kb of megaplasmid-homologous genes and 42kb of a region predicted to be located on the GMI1000 chromosome, both region being separated by the presence of a transposable element. In order to follow the synteny with the gene organisation in strains GMI1000 or Molk2, we decided to assemble only the 30kb half of Cont0551.1 corresponding to the megaplasmid-borne region in other sequenced strains. Each junction point of the resulting assembled sequence (between RRSL_02289 and RRSL_02235, RRSL_02210 and the neighboring Insertion Sequence, and between the *tssK* and *tssJ* genes) was verified by PCR amplification using a pair of primers hybridizing on each contig border (data not shown), thus confirming the physical structure of the region shown in Figure 14. The mapping of the deletion point identified in strain IPO1609 within the genes RRSL_02267 and RRSL_01740 (*vgrG2*) indicated that the deletion has an exact size of 76.653 bp. We then performed a manual annotation of this 77kb region using the ORF prediction tool FrameD (Schiex et al., 2003) and the iANT annotation interface (Salanoubat et al., 2002). The 77kb deleted region is predicted to carry 66 genes, including two IS/Transposase elements. Three genes (named UW551_0001, UW551_0002, and UW551_0003) detected by FrameD were not predicted from the analysis of Gabriel et al. (2006) but encode proteins displaying homology with other known proteins (see Table 7).

A rapid analysis of the distribution of the 66 genes by functional classes reveals that the majority of them belongs to three main groups, those contributing to the Small Molecule Metabolism (18 genes), to Traffic & Secretion processes (16 genes) and Hypothetical Conserved proteins of unknown function (21 genes). In addition, five

RRSL_02226		175	Signal peptide hypothetical protein	I=76%
RRSL_02225		66	Conserved hypothetical protein	I=90%
RRSL_02224		472	Cytochrome C peroxidase	I=87%
RRSL_02223		458	Phosphate-selective porin	I=82%
RRSL_02222		186	Signal peptide hypothetical protein	I=92%
RRSL_02221		444	Type III secretion system effector protein with lipase domain	
RRSL_02220		196	Transmembrane protein	
RRSL_02219		301	L-aspartate dehydrogenase	I=76%
RRSL_02218		45	Conserved hypothetical protein	
RRSL_02217		413	Transmembrane transporter	I=76%
RRSL_02216		39	Signal peptide hypothetical protein	I=89%
RRSL_02215		172	Conserved hypothetical protein	I=87%
RRSL_02214		408	Sterol desaturase	I=90%
RRSL_02213		818	Type III secretion system effector protein	I=76%
RRSL_02212	ripTPS	565	Type III secretion system effector protein with trehalose-6-phosphate activity	I=78%
RRSL_02211		77	Hypothetical protein	
RRSL_02210		328	Transposase	I=90%*
RRSL_02209			Insertion sequence element	
RRSL_00093		477	Hypothetical protein	
RRSL_01728	vgrG1	995	Type VI secretion system Vgr substrate family protein	I=72%
RRSL_01729	tssL	262	Component of the type VI protein secretion system	I=91%
RRSL_01730	tssK	448	Component of the type VI protein secretion system	I=94%
RRSL_01731	tssJ	226	Component of the type VI protein secretion system	I=82%
RRSL_01732		215	Tetratricopeptide repeat domain protein	I=92%
RRSL_01733	tssA	170	Component of the type VI protein secretion system	I=88%
RRSL_01734	tssB	496	Component of the type VI protein secretion system	I=92%
RRSL_01735	hcp1	167	Type VI secretion system effector, Hcp1 family	I=80%
RRSL_01736		157	Type VI secretion system lysozyme-related protein	I=94%
RRSL_01737	tssD	616	Component of the type VI protein secretion system	I=90%
RRSL_01738	tssE	367	Component of the type VI protein secretion system	I=91%
RRSL_01739	tssH	908	Type VI secretion ATPase, ClpV1 family	I=86%
RRSL_01740	vgrG2	923	Type VI secretion system Vgr substrate family protein	I=75%
RRSL_01741		384	Conserved hypothetical protein	I=69%

* Homology detected with a GMI1000 protein but outside of the 77kb-homologous region

genes are predicted to encode transcriptional regulators, and three genes to code for Type III-dependent effectors.

Type III effectors. Two of these effectors have counterparts in GMI1000: RRSL_02213 is 86% identical to RSp0732, a translocated effector of unknown function (Cunnac et al., 2004), and RipTPS (79% identity with GMI1000$_{RipTPS}$) which codes for a trehalose-6-phosphate synthase, also translocated into plant cells (Poueymiro 2009). The enzymatic activity of RipTPS was demonstrated by functional complementation of a yeast *tps* mutant (Poueymiro 2009), and trehalose-6-phosphate appears to be an essential signal molecule in plants controlling sugar metabolism and development (Paul et al., 2008). The third Type III effector, RRSL_02221, corresponds to a candidate carrying a hrp$_{II}$ box in its promoter region and a N-terminal domain having features of T3SS-translocated substrates; interestingly, it possesses a lipase domain but has no orthologue in the GMI1000 genome (Poueymiro & Genin, 2009).

Type VI Secretion System. The high number of genes functionally assigned to secretion processes within the 77kb region is mainly explained because it contains part of a Type VI Secretion System (T6SS) gene cluster (genes RRSL_00093 to RRSL_01741 and beyond). The T6SS is a recently characterized protein secretion system that is composed of 20-25 proteins whose function are not well understood (for reviews: Cascales, 2008; Filloux et al., 2008; Pukatzki et al., 2009). T6SS landmarks include an AAA+ *Clp*-like ATPase (encoded by the *tssH* gene, Figure 14), IcmF/DotU-like proteins that are homologous to T4SS membrane components, and the secreted Hcp-like and VgrG-like proteins, proposed effectors that are also essential components of the secretion machinery (Pukatzki et al., 2009). Interestingly, T6SS form injectisomes that have the potential to translocate effector proteins into eukaryotic host cells. T6SS clusters have been implicated in the virulence of certain mammalian pathogens such as *Pseudomonas aeruginosa* (Mougous et al., 2006) or *Vibrio cholerae* (Pukatzki et al., 2006) but also of some plant pathogenic bacteria, including *Agrobacterium tumefaciens* (Wu et al., 2008) and *Pectobacterium atrosepticum* (Liu et al., 2008). In addition to pathogenesis, T6SS may modulate root colonization/nodule formation by the nitrogen-fixing plant symbionts/mutualists *Mesorhizobium loti* and *Rhizobium leguminosarum* (Bingle et

al., 2008). Recent *in silico* analyses of prokaryotic genomes have further revealed that T6SS are widespread among Proteobacteria, suggesting that they may be involved in yet unknown pathogenic or symbiotic lifestyles or other types of cell-cell communication (Jani & Cotter 2010; Sarris et al., 2010).

Contrary to some other bacteria shown to carry multiple T6SS clusters (Sarris et al., 2010), only one megaplasmid-borne T6SS gene cluster was identified in the genome of the different *R. solanacearum* sequenced strains. The 77kb deletion is predicted to remove 17kb of the T6SS gene cluster, including several components essential to secretion in other bacteria such as *tssH* or *tssJ*, but also putative substrates (Hcp1 and VrgG1-encoding genes). It is therefore likely that this T6SS is not functional in strain IPO1609.

Auxin/Indole biosynthesis. The 77 kb region also contains one or several operons (comprising seven genes: RRSL_02276 to *pdxH2*) putatively involved in pathogenesis since it was proposed to encode an auxin/indole biosynthetic pathway (Valls et al., 2006). *R. solanacearum* is known to produce auxin (Phelps & Sequiera, 1968) and GMI1000 was shown to produce different types of indole-derived diffusible molecules (Valls et al., 2006; Delaspre et al., 2007). Interestingly, homologues of the RRSL_02276-*pdxH2* gene cluster in GMI1000 are under the transcriptional control of HrpG, the master regulator of pathogenicity and other host adaptation functions (Valls et al., 2006), which is suggestive of a role during the infectious process.

Sterol desaturation. Gene RRSL_02214 is predicted to encode a sterol desaturase, and some preliminary experiments indicate that its expression is also *hrpG*-regulated. Sterols are indispensable compounds in plants and other eukaryotes because they are structural constituents of membranes, in which they regulate fluidity and permeability. It was recently reported that C22 desaturation of the predominant Arabidopsis phytosterol β-sitosterol via a cytochrome P450 and the concomitant accumulation of stigmasterol are significant metabolic processes *in P. syringae*-inoculated Arabidopsis leaves (Griebel & Zeier, 2010). It thus has been proposed by the authors of this study that pathogen-induced changes in the sterol composition of leaf membranes influence plant disease resistance and affect the outcome of particular plant–pathogen interactions.

Methionine biosynthesis. In *R. solanacearum* different genes are involved in methionine biosynthesis, however two genes, *metE* and *metR*, are inside of the 77 kb region and are part of the *met* regulon described in *E. coli* and other gram negative bacteria (Maxon et al., 1989; Cai et al., 1989; Fritsch et al., 2000). *metR* encodes a LysR-type transcriptional regulator, and *metE* encode to an enzyme involved in the last step of methionine biosynthesis, converting L-homocysteine into L-methionine. Both *metE* and *metR* are located one next to the other and their transcription is in opposite sense. Transcriptomic analyses indicated that *metE* expression is controlled by HrpG, but *metR* was not considered as target of this master regulator (Valls et al., 2006).

Degradation of aromatic amino acids. Three genes (*hmgA*, *hmgB* and *hmgR*) belong to the homogentisate pathway that converts homogentisate to maleylacetoacetate in the pathway of phenylalanine and tyrosine catabolism (Arias-Barrau et al., 2004). *hmgA* encodes homogentisate dioxygenase that opens the aromatic ring of homogentisate (2,5-OH-phenylacetate), which is isomerized to fumarylacetoacetate. Then fumarylacetoacetate is hydrolyzed by a specific hydrolase encoded by *hmgB* to form fumarate and acetoacetate, which are two compounds of the central metabolism. *hmgR* encodes an IclR-type regulator that acts as a repressor of the aromatic catabolic pathway, and study of genetic organisation of the cluster in many bacterial species indicate that *hmgR* is found next to *hmgA* and transcribed divergently (Arias-Barrau et al., 2004).

Among the genes with a fairly predictable function listed above, orthologues of few of them had been studied in the reference strain GMI1000 before the beginning of this work. Disruption mutants were performed in GMI1000 Type III effector genes Rsp0732 (RRSL_02213 homologue) and *ripTPS* but none of them were significantly altered in pathogenicity on tomato or Arabidopsis (Cunnac et al., 2004; Poueymiro, 2009). Another mutant carrying a deletion of six genes (ΔRSp0679-RSp0685, corresponding to UW551 genes in-between RRSL_2271 and RRSL_02276 on Figure 14) within the putative auxin/indole biosynthetic operon was also constructed (M. Valls & S. Genin, unpublished data). The resulting mutant strain retained full
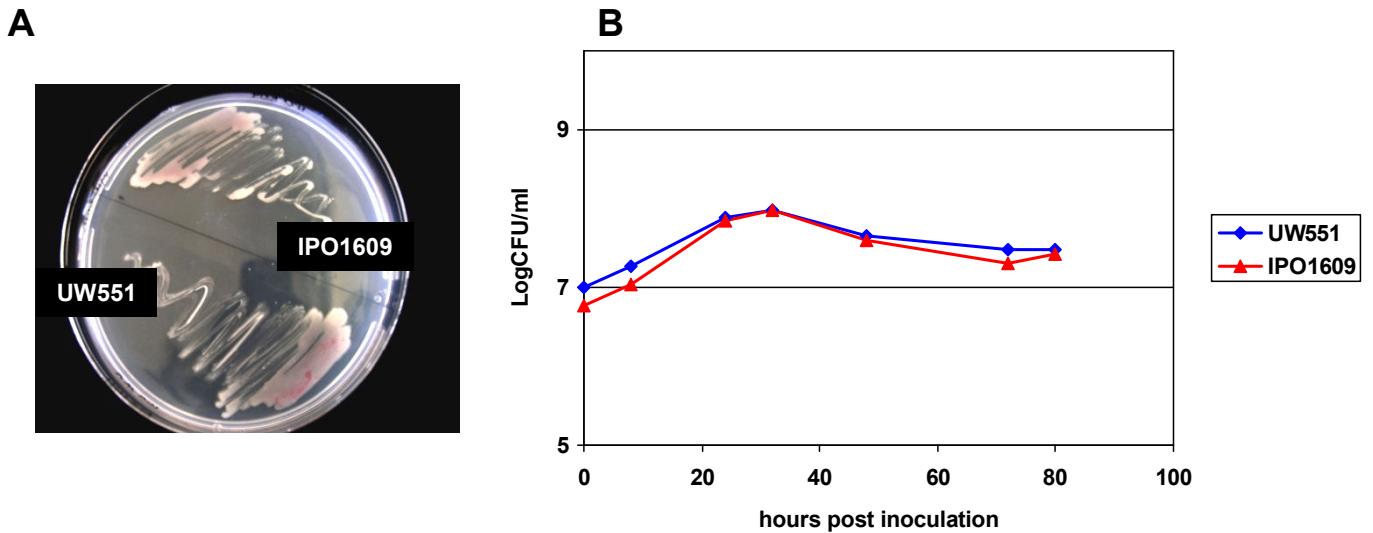
Figure 15. Growth of *R. solanacearum* race 3 strains on minimal medium supplemented with glucose 0.2%

(A) Strain IPO1609 is able to grow normally on a Minimal Medium agar supplemented with glucose as sole carbon source, and without any addition of Met as UW551 strain. This clearly established that IPO1609 is not auxotrophic for Methionine. (B) Growth of UW551 and IPO1609 strains at 28°C in liquid minimal medium supplemented with glucose. After inoculation of minimal culture medium supplemented with glucose by 1x10^7 cfu the cell count was performed by dilution plating in CB medium at different time points. Both strains showed a similar growth in this condition
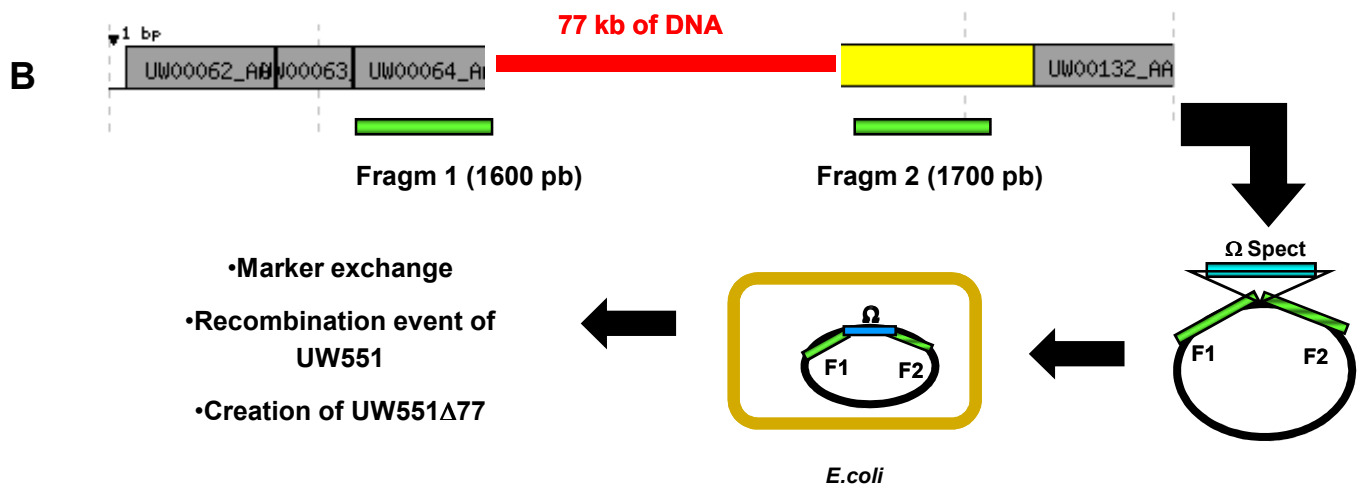
Figure 16. Deletion of the 77 kb region in strain UW551.

Two DNA fragments of approximately 1.0kb in size on each border of the deletion were PCR-amplified. Both fragments were cloned on a plasmid vector and the Ω interposon conferring Spectinomycin resistance was cloned in between the 'upstream' and 'downstream' fragments. The resulting plasmid was introduced in *R. solanacearum* UW551 by natural transformation and the recombinant clones were selected after growth in BG medium supplemented with spectinomycin. The presence of the deletion in the resulting strain named UW551Δ77 was verified by PCR by amplifying a DNA fragment using primers on each border of the deletion point.

virulence on tomato but its ability to produce auxin or indole-related molecules has not yet been examined.

3 Comparison of growth rates of strains IPO1609 and UW551 *in vitro*

Since the 77kb region deleted in IPO1609 appeared to carry many genes involved in metabolic pathways, we first investigated whether the strong decrease in pathogenicity was due to growth deficiency. In particular, because some methionine (Met) auxotrophic mutants were described as non-pathogenic on tobacco (Coplin et al., 1974), we sought to determine whether the deletion of the *metE* and *metR* genes caused auxotrophy and could therefore contribute to the hypovirulent phenotype. Contrary to some Acridine Orange sensitive mutants that carry large deletion in their megaplasmid and which are Met auxotrophs (Boucher et al., 1988), strain IPO1609 is able to grow normally on a Minimal Medium supplemented with glucose as sole carbon source, and without any addition of Met (Figure 15A). This clearly established that the strain is not auxotrophic for Met. We then evaluated the growth kinetics of both strains in liquid Minimal Medium supplemented with glucose over 96 hours. No significant difference in OD_{600} between the two strains was observed over the time course, and this was confirmed by enumeration of bacteria after plating (Figure 15B). We concluded from this experiment that strain IPO1609 was not impaired in growth compared to UW551, despite the fact that many genes in the deleted 77kb region were predicted to encode proteins involved in metabolic functions.

4 Engineering of an UW551 strain derivative carrying the 77kb deletion and evidence that this region contains genes essential for pathogenicity

To determine whether the 77kb region is involved in the loss of pathogenicity of strain IPO1609, our strategy was to engineer a UW551 strain carrying the 77 kb deletion identified in strain IPO1609. Two DNA fragments of approximately 1.0kb in size on each border of the deletion were PCR-amplified (Figure 16). Both fragments were
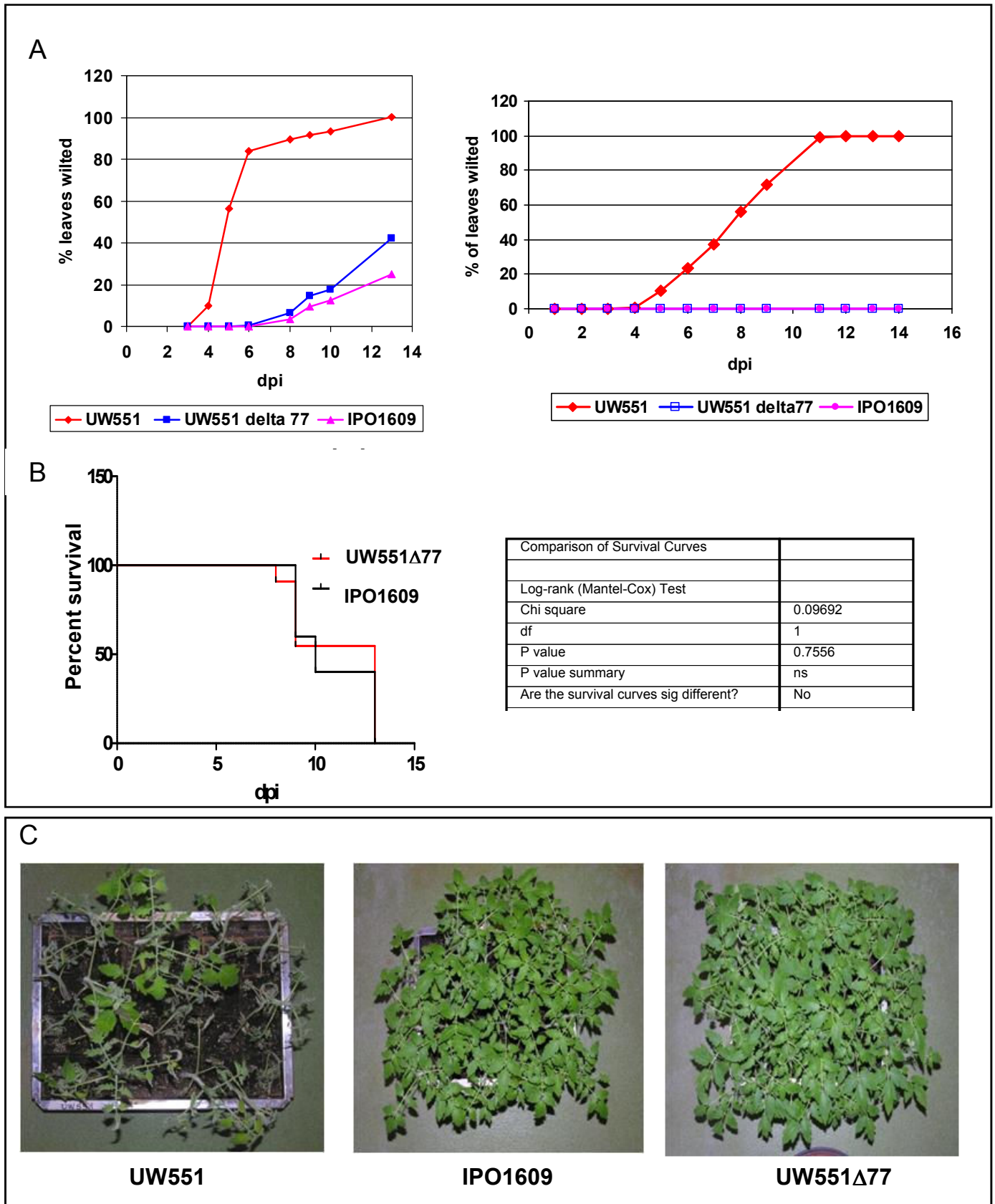
Figure 17. Virulence of *R. solanacearum* UW551, IPO1609 and the mutant UW551Δ77 on tomato plants. (A) Percentage of leaves wilted during the time of tomato plants inoculated by soaking the soil to a final bacterial population of 1×10^8CFU/ml (right) and 1×10^7CFU/ml per plant (Left) ; (B) Comparison by Log-Rank test of survival curves of tomato plants inoculated with IPO1609 and the mutant UW551Δ77. P value=0.75, thus, there was not significant difference between the percentage of plant living treated with IPO1609 and the mutant strain; (C) Plant wilting phenotype of tomato plants after 6 days post inoculation. Results are representative of three or more experiments

cloned on a plasmid vector and the Ω interposon conferring Spectinomycin resistance was cloned in between the 'upstream' and 'downstream' fragments (see Material & Methods). This construct was used to transform UW551 in order to select for a double recombination event using the Spectinomycin selection marker that would lead to re-create the 77kb deletion in the recipient strain. The presence of the deletion in the resulting strain named UW551Δ77 was verified by PCR by amplifying a DNA fragments using primers on each border of the deletion point.

Pathogenicity of the UW551Δ77 mutant was compared to the wild-type UW551 parental strain by drenching inoculation of tomato plants and by stem inoculation of tomato and potato plants. Remarkably, the UW551Δ77 mutant strain exhibited a similar phenotype as IPO1609, being severely attenuated in virulence on both plants using a high inoculum level (10^8 cfu/ml). Wilting symptoms appearance in plants treated by the UW551Δ77 mutant or by IPO1609 strain only started six days after inoculation, whereas plants inoculated with wild-type UW551 already showed more than 50% of wilting at this time (Figure 17). After fifteen days post-inoculation, disease symptoms did not progress further for strains IPO1609 and UW551Δ77 and never exceed 50% of wilting (data not shown). We applied a Log-Rank statistical test to determine whether the disease curves obtained with the UW551Δ77 mutant and the wild-type IPO1609 strain were not significantly different under these conditions (P value=0.75) (see Material & Methods). When a lower bacterial inoculum (10^7 cfu/ml) was used, the difference between strains UW551 and IPO1609 or UW551Δ77 was even more spectacular since the two latter strains did not produce wilting symptoms on tomato plants whereas UW551 remained fully pathogenic (Figure 17) This was first evidence that the 77kb deletion dramatically reduces pathogenicity of strain UW551 in tomato and potato plants, and strongly suggests that absence of this region in IPO1609 is involved in its reduced virulence.

The inoculation method by soil drenching used above allows a comparison of strain pathogenicity over the different steps of infection (including passage in soil and reaching plant roots, root infection, cortex colonization and endodermis crossing, and finally xylem vessels colonization). We therefore performed a pathogenicity test using another inoculation method by direct stem-injection of the bacteria in the vascular system. This procedure will therefore bypass all the early stages of the *R. solanacearum* infection process. When this inoculation method was applied, both the
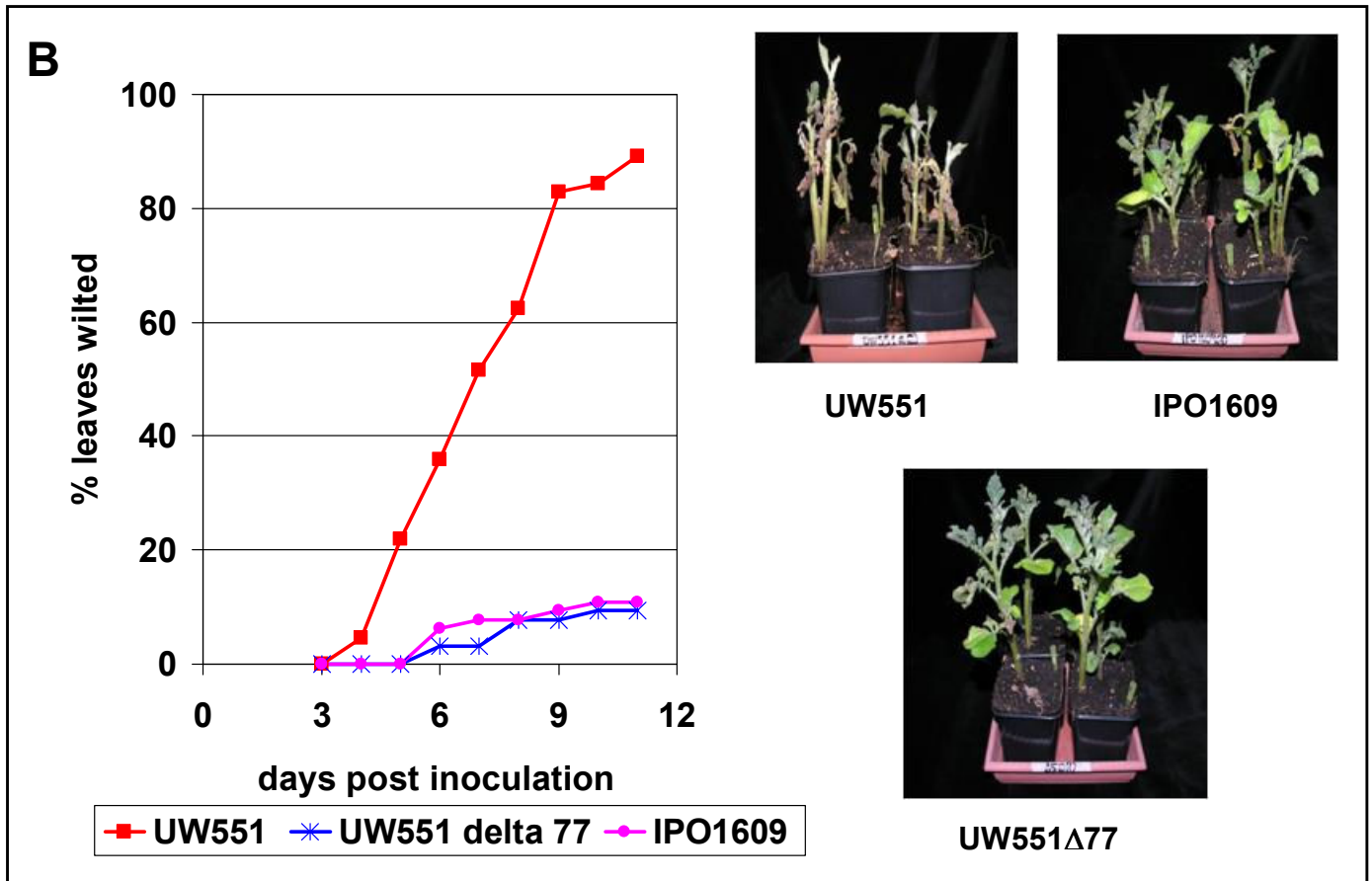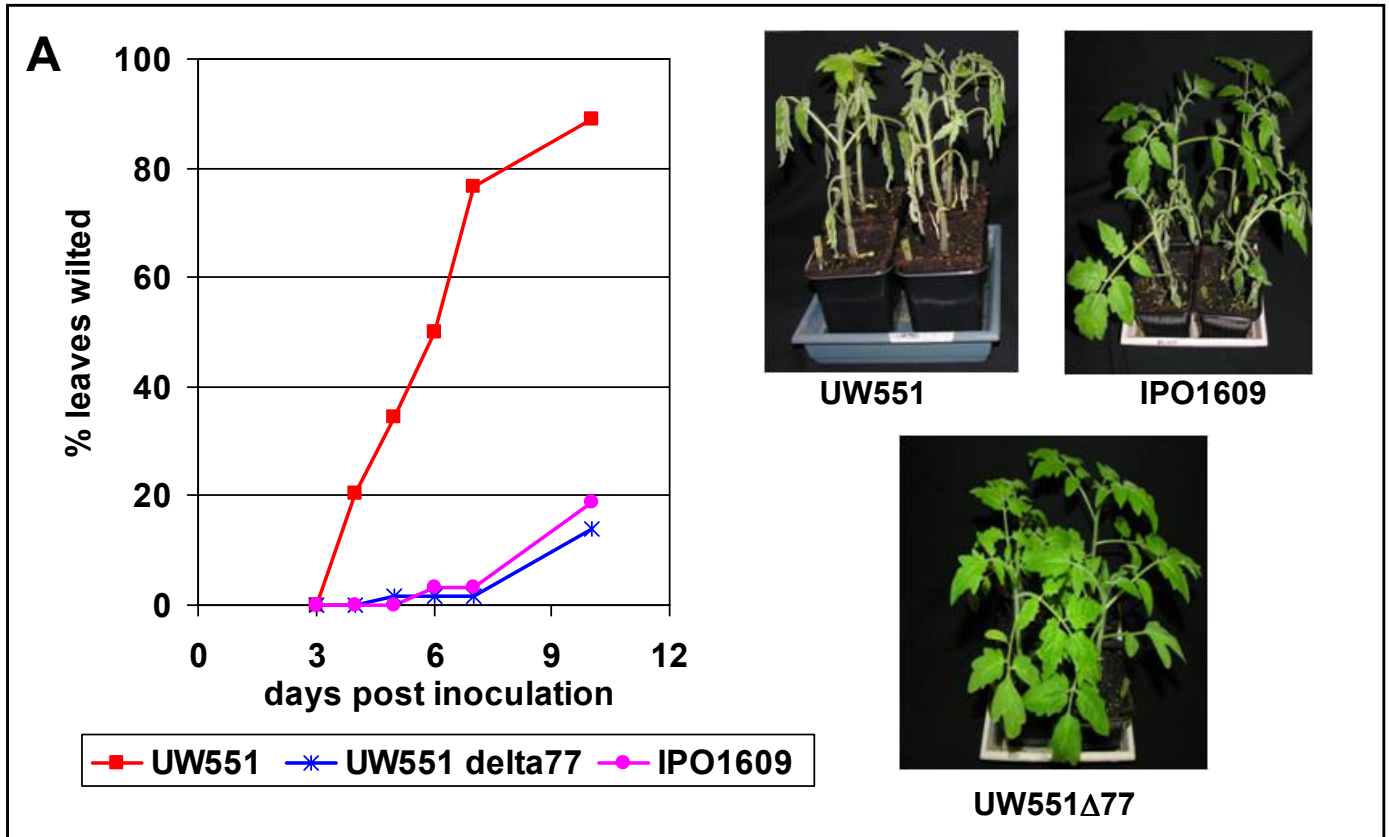
Figure 18. **Virulence of *R. solanacearum* UW551, IPO1609 and the mutant UW551Δ77 by stem infiltration of 10 μl of bacterial suspension of 1x10^7 CFU/ml per plant.** (A) Percentage of leaves wilted during the time and plant wilting phenotype of tomato plants after 6 days post inoculation. (B) Percentage of leaves wilted during the time and plant wilting phenotype of potato plants after 6 days post inoculation. Results are representative of three or more experiments

UW551Δ77 and IPO1609 strains were scored hypovirulent as following soil drenching inoculation method (Figure 18) thus indicating that the defect in pathogenicity is not due to specific early step(s) of plant infection but can be also observed when the bacteria are directly injected into the xylem compartment.
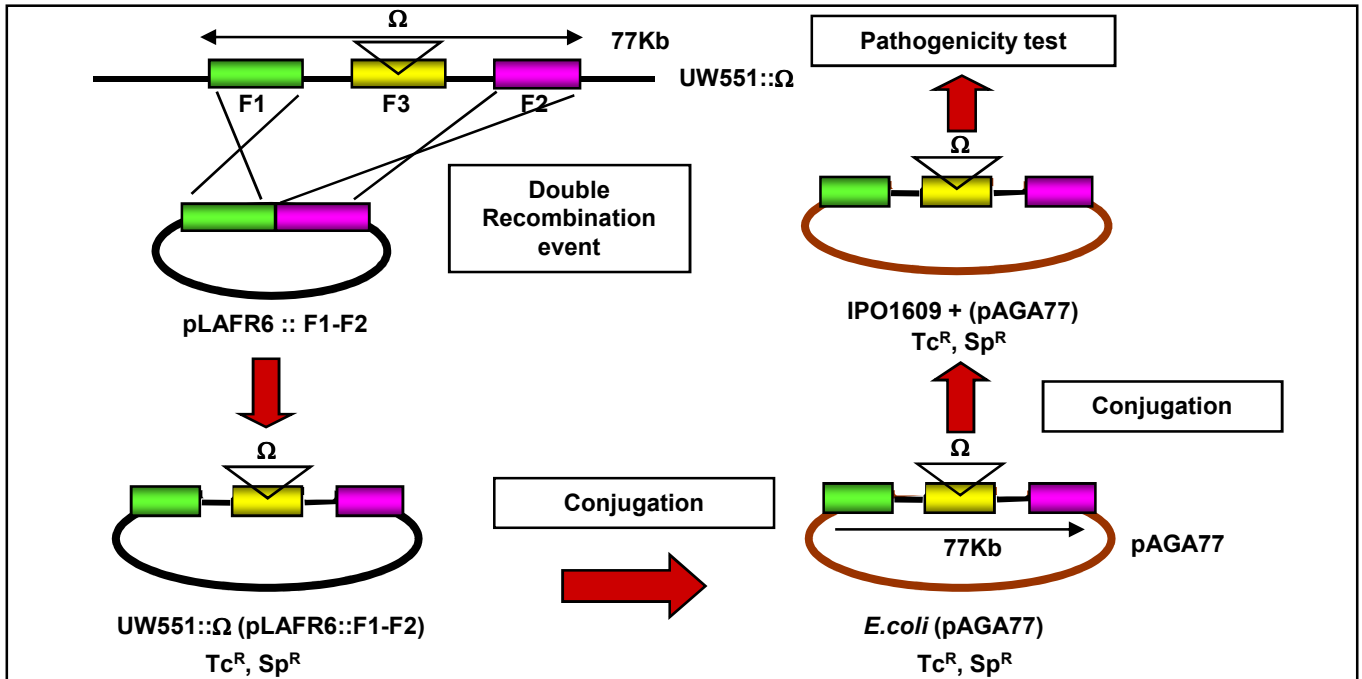
Figure 19. Cloning of the 77 Kb region from *R. solanacearum* UW551 and complementation of IPO1609 strain.

The Ω interposon was cloned into F3 (RRSL_0225 and RRSL_02224 genes) of 77kb region and this construct was recombined into UW551 to generate strain UW551::Ω. Border fragments F1 and F2 from 77kb region of UW551 were jointly cloned in pLAFR6 and the resulting plasmid (pLAFR6::F1-F2) was introduced by electroporation in UW551::Ω and by a double recombination marker exchange event the resulting clones UW551::Ω (pLAFR6::F1-F2) were selected on Tc and Sp plates. The pLAFR6::F1-F2 construction, called pAGA77, was introduced in *E. coli* DH5α by triparental mating conjugation and finally it was mobilized to *R. solanacearum* IPO1609 strain by conjugation. The recombinant clones were recovered in B medium supplemented with Tetracycline and Spectinomycin.

Comparison of Survival Curves	
Log-rank (Mantel-Cox) Test	
Chi square	0.09390
df	1
P value	0.7593
P value summary	ns
Are the survival curves sig different?	No
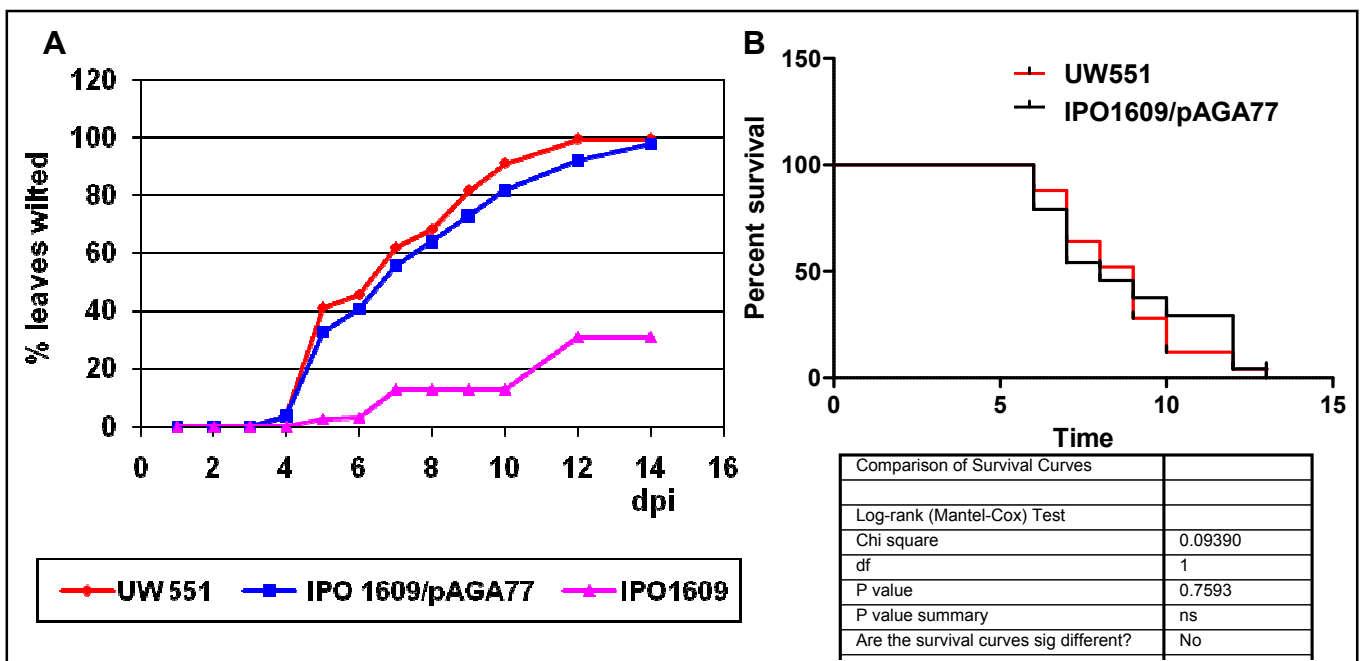
Figure 20. **Virulence of *R. solanacearum* UW551, and the complemented strain IPO1609/pAGA77 on tomato plants inoculated by soaking the soil to a final bacterial population of 1x10^{8}CFU/ml per plant**.
(A) Percentage of leaves wilted during the time, (B) Comparison by Log-Rank test of survival curves of tomato plants inoculated with IPO1609 and the complemented strain IPO1609/pAGA77. P value=0.94, thus, there was no significant difference between the percentage of plant living treated with UW551 and IPO1609/pAGA77 strain. Results are representative of three or more experiments.

5 Cloning of the 77kb region from strain UW551 and functional complementation of strain IPO1609

Once confirmed the hypovirulent phenotype on plants of the UW551 strain deleted for the 77kb region, our purpose was to confirm that this deletion is involved in the low virulence of IPO1609 strain. Cloning of the 77kb region was performed in different steps summarized on Figure 19: first, a selectable marker (the Ω interposon carrying a Spectinomycin resistance character) was recombined in the UW551 genome into the 77kb region between the RRSL_02225 and RRSL_02224 genes; second, the joining border fragments named F1 and F2 (*i.e* those described above to create the deletion) were both jointly cloned in a pLAFR6 replicative vector, and third, this resulting plasmid was introduced into the UW551 mutant strain marked with the Ω interposon to select for a double recombination marker exchange event. To do this, this *R. solanacearum* UW551::Ω / pLAFR6::F1-F2 strain was conjugated with *E. coli* cells and trans-conjuguants that acquired a pLAFR6 vector carrying the Ω interposon were selected on LB+Spectinomycin+Tetracycline plates (see Figure 19 and details in the Material & Methods section). Such a recombinant plasmid was obtained, named hereafter pAGA77, and PCR verifications were made to confirm that genes known to be present in the 77kb region could be amplified using pAGA77 as sole template.

The pAGA77 plasmid was then introduced into strain IPO1609 by conjugation and pathogenicity of the resulting IPO1609/pAGA77 strain was compared to the IPO1609 and UW551 wild-types. Results presented on Figure 20 clearly show that the pAGA77 plasmid restored full pathogenicity of strain IPO1609 to a level comparable to the one of UW551. Statistical analyses using the Log-Rank test confirmed that the disease curve progress of strain IPO1609/pAGA77 was similar to the one of UW551 (P value=0.94). This result therefore demonstrated that hypovirulence of strain IPO1609 was dependent upon the 77kb deletion identified on its megaplasmid and that this 77kb region carries important genes for *R. solanacearum* pathogenicity.
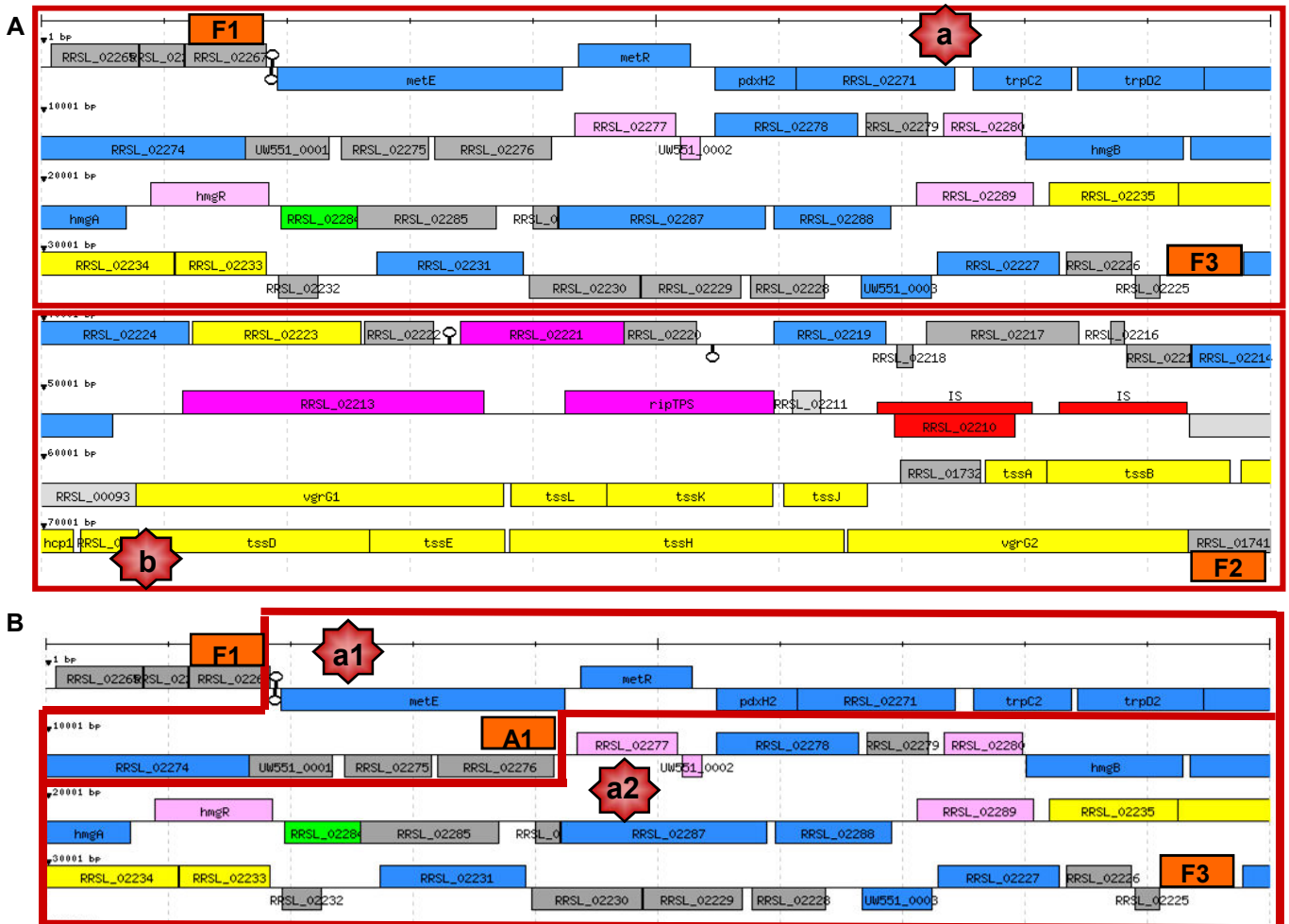
Figure 21. Genomic map of 77kb region with delimited areas selected to construction of UW551Δ77-derived mutant strains. (A). The region "a" involved the first half of the 77kb region delimited by F1-F3 and disrupted by a Ω cassette to generate the mutant UW551Δ77a; and region "b" involved the second half delimited by F3-F2 and disrupted by a Ω cassette to generate the mutant UW551Δ77b. B. Region "a1" involved the first part of region "a" delimited by F1-A1 and disrupted by a Ω cassette to generate the mutant UW551Δ77a1 and region "a2" involved the second part of "a" region delimited by A1-3 and disrupted by a Ω cassette to generate the mutant UW551Δ77a2
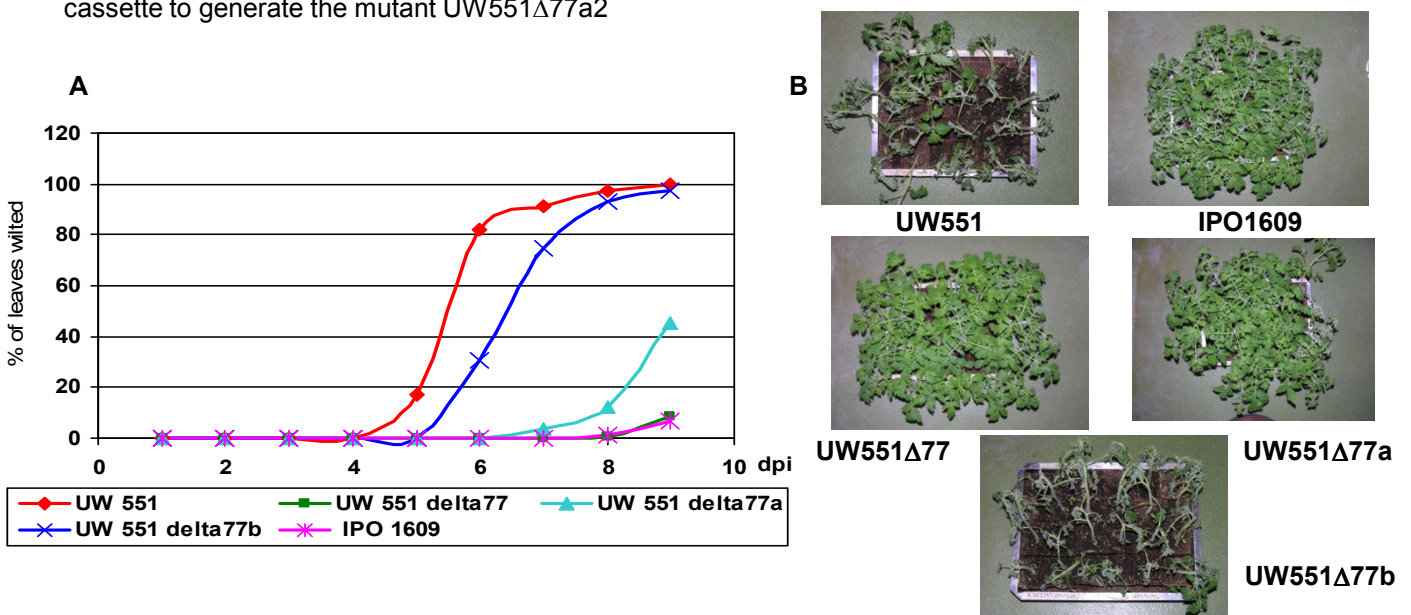
Figure 22. Virulence of *R. solanacearum* UW551, IPO1609 and the mutants UW551Δ77, UW551Δ77a and UW551Δ77b by soaking the soil to a final bacterial population of 1x10⁸CFU/ml per plant. (A) Percentage of leaves wilted during the time. Delayed wilting symptoms were observed after inoculation with IPO1609 and the mutants UW551 Δ77 and UW551 Δ77a. In contrast, the mutant UW551 Δ77b displayed the same virulent phenotype as the wild-type UW551, thus the genes responsible of the decreased pathogenicity of UW55Δ77 are found in the first part of the 77kb region mutant strains (B) Phenotype of tomato plants at 8 days post inoculation. Results are representative of three or more experiments

6 Mapping of the genes from 77kb contributing to UW551 pathogenicity

Once confirmed that 77kb deletion dramatically reduces pathogenicity of strain UW551 and in order to identify the gene(s) responsible for this phenotype, we first performed other deletion experiments in strain UW551 and constructed mutants with different deleted parts of this region.

6.1 The right-hand half of 77kb region does not contribute to pathogenicity

The 77 kb region was divided into two halves: a 37 kb left-hand half from the *metE* to the RRSL_02225 gene, and a 40kb right-hand half from RRSL_02224 to *tssH* (see Figure 21). A DNA fragment (designed F3) in the intergenic region between the RRSL_02225 and RRSL_0024 genes was PCR amplified and was subsequently used to generate plasmid constructs in combination with the other fragments F1 and F2 previously used. These plasmids were used as marker-exchange constructs for deletion for each the left-hand and right-hand halves in UW551 (see Material & Methods and Figure 16). The resulting mutant strains were designed UW551Δ77a (carrying a 37kb deletion corresponding to the first half) and UW551Δ77b (carrying the 40kb deletion corresponding to the second half). A virulence test on tomato and potato plants was then carried out between UW551 and both mutant strains (Figure 22). The mutant strain UW551Δ77b displayed the same virulent phenotype as the wild-type UW551, being normally pathogenic on tomato and potato plants (Log-Rank test P value=0.69). This clearly established that the genes lying in this second half do not have a significant impact on the pathogenicity of UW551, although this region contains genes classically associated with pathogenesis, namely three Type III effector genes (RRSL_02221, RRSL_02213 and *ripTPS*). This observation further confirms the existence of functional overlap between *R. solanacearum* effector proteins delivered into the plant cells via Hrp T3SS (Angot et al., 2006). In addition, it reveals that the UW551 Type VI secretion genes probably plays little role during the plant infection process since the 40kb deletion strain UW551Δ77b misses several predicted essential components from this secretion system (Cascales, 2008; Pukatzki et al., 2009). This conclusion can be reached since only one T6SS appears to be present in all the *R. solanacearum* strains sequenced to date, contrary to what observed in other plant pathogens such as *P. syringae* (Sarris et al., 2010) or some
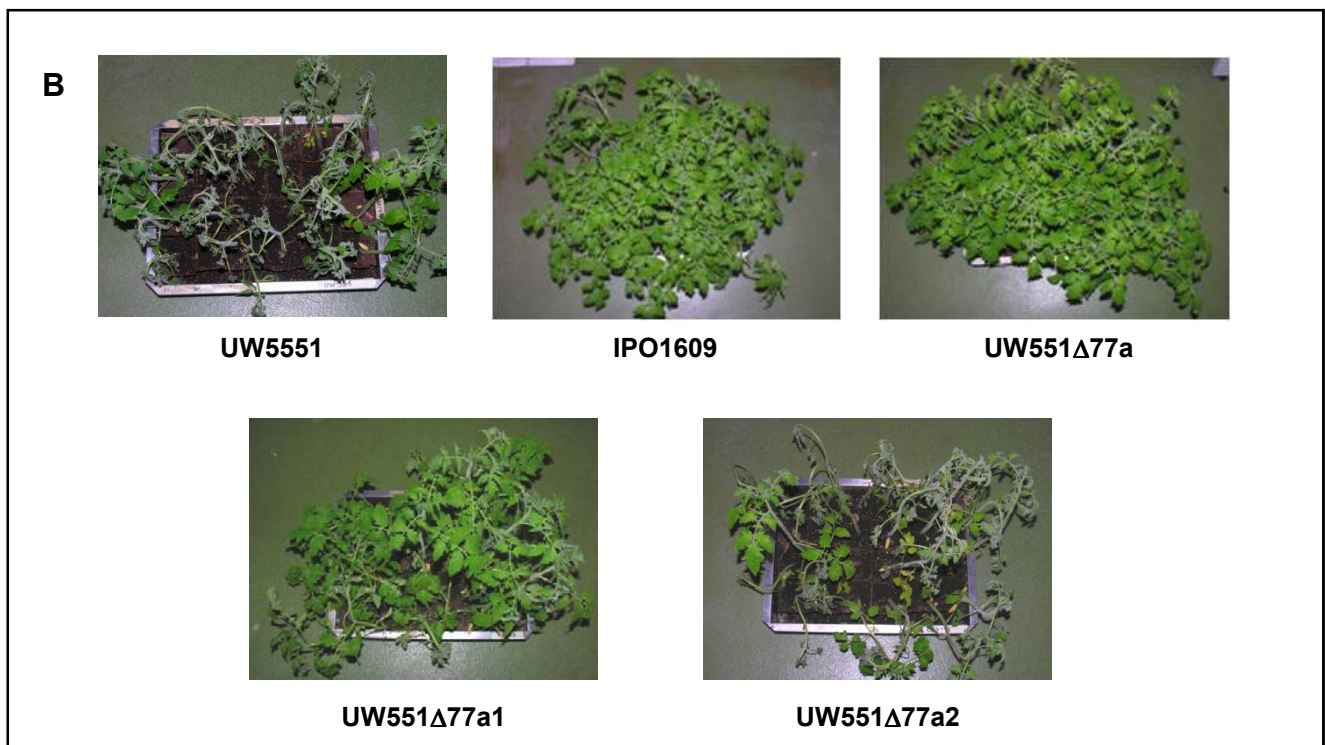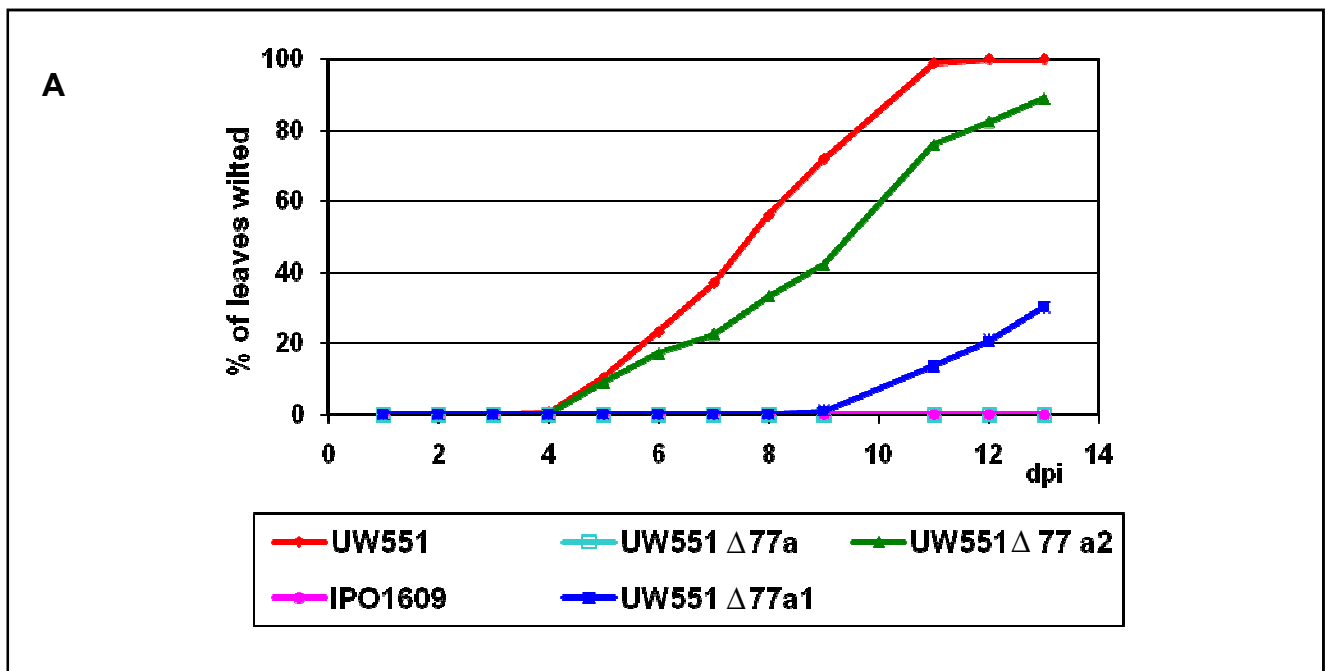
Figure 23. Virulence of *R. solanacearum* UW551, IPO1609 and the mutants UW551Δ77a, UW551Δ77a1 and UW551Δ77a2 by soaking the soil to a final bacterial population of 1x10⁷CFU/ml per plant. (A) Percentage of leaves wilted during the time. Delayed wilting symptoms were observed after inoculation with IPO1609 and the mutants UW551 Δ77 and UW551 Δ77a. In contrast, the mutant UW551 Δ77b displayed the same virulent phenotype as the wild-type UW551, thus the genes responsible of the decreased pathogenicity of UW55Δ77 are found in the first part of the 77kb region mutant strains (B) Phenotype of tomato plants at 8 days post inoculation. Results are representative of three or more experiments

other β-Proteobacteria (Schwarz et al., 2010) harboring multiple T6SS clusters. Recent studies have shown that the contribution of the T6SS may not occur only during interactions with eukaryotes host and that T6S-dependent inter-bacterial interactions are likely relevant in the environment (Jani & Cotter 2010; Schwarz et al., 2010).

6.2 The genes controlling pathogenicity of UW551 on tomato and potato are located in the first half from the 37kb region

Contrarily to the mutant strain carrying the deletion of the second half of the 77kb region, the strain carrying the deletion of the first half between region F1 and F3 (UW551Δ77a) presented a phenotype on plants similar to strains IPO1609 or UW551Δ77 deleted for the whole region (Figure 22). This result confirmed that the hypovirulent phenotype of strain IPO1609 was associated with the loss of genes located within the 37kb region corresponding to the first half of the deletion. To dissect further this region, two other deletions were performed within this region 'a': a deletion named a1 comprised a 12kb region from the *metE* to RRSL_02276 genes, and a second one named a2 comprised a 25kb region from the RRSL_02277 to RRSL_02225 genes. Pathogenicity assays were then conducted on tomato plants at low density inoculum (10^7 cfu/ml) since this concentration allowed a clear distinction between strains deleted in the first part of 77kb region and fully virulent strains UW551 and UW551Δ77b. Pathogenicity assays on tomato plants shown on Figure 23 indicated that the impact of the a1 deletion was significantly much stronger than deletion of region a2. Deletion of region a2 appeared to lead to a slight decrease in pathogenicity compared to the wild-type UW551 parent (P value = 0.4) whereas deletion of region a1 was associated with hypovirulence of the corresponding strain, although the rate of wilting symptoms was significantly higher than strains IPO1609 and UW551Δ77. Altogether, these results showed that genes involved in hypovirulence of IPO1609 are distributed on both in the a1 and a2 regions, but that the greatest impact on pathogenesis was due to determinant(s) bound to the a1 12 kb region.
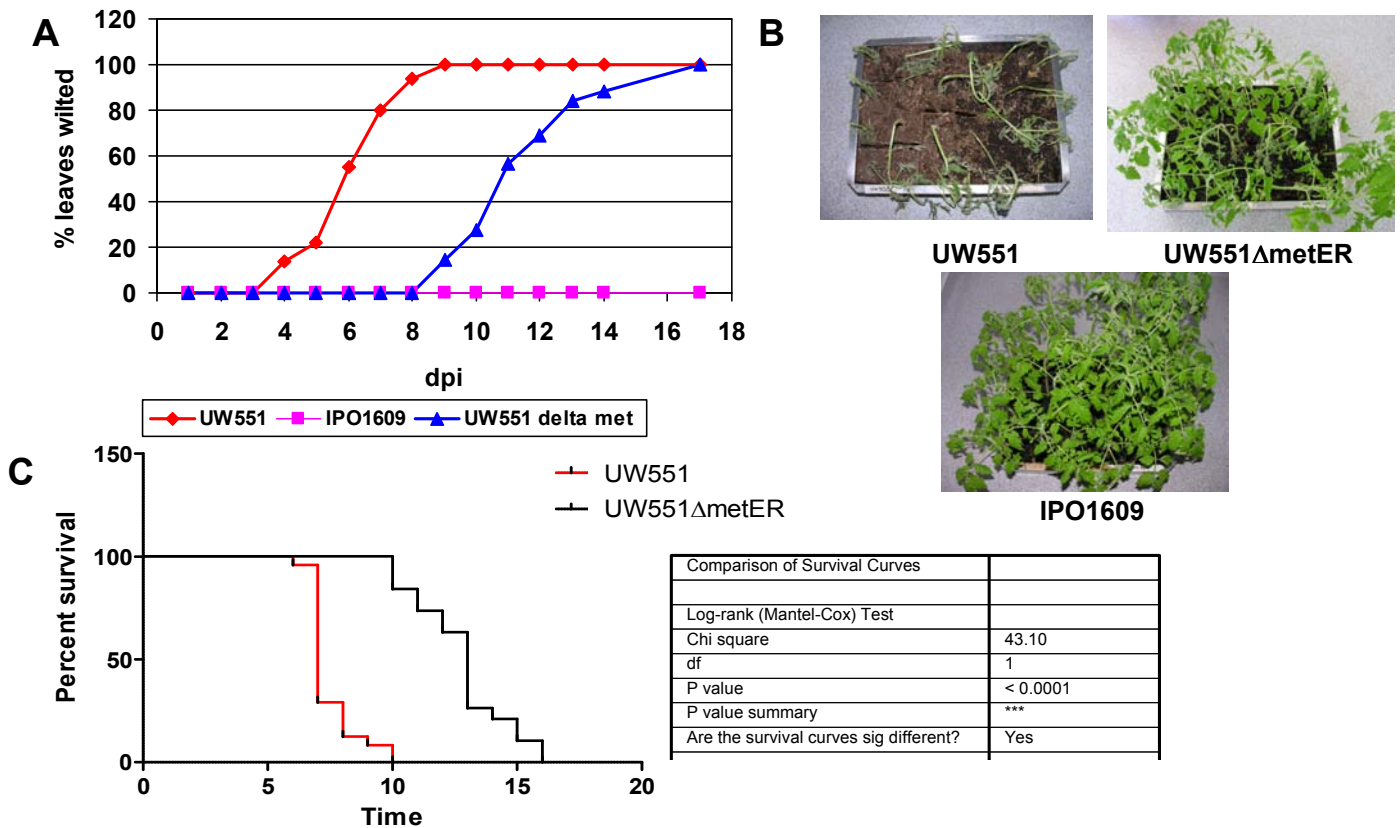
Figure 24. **Virulence of the metER deleted mutant strain on tomato plants.** Plants were inoculated by soaking the soil to a final bacterial population of 1 x 10^7 cfu ml^{-1} per plant. (A) Percentage of leaves wilted during the time. Delayed wilting symptoms were observed after inoculation with the mutant strain UW551Δ*metER* compared to the wild type strain UW551. There was no symptoms appearance on plants treated with the strains IPO1609. (B) Plant wilting phenotype of tomato plants at 11 dpi. (C) Comparison by Log-Rank test of survival curves of tomato plants inoculated with UW551 and the mutant strain UW551Δ*metER* (P value <0.0001) showing a significant difference between the percentage of living plants treated with UW551 and UW551Δ*metER* mutant. Results are representative of three or more experiments.
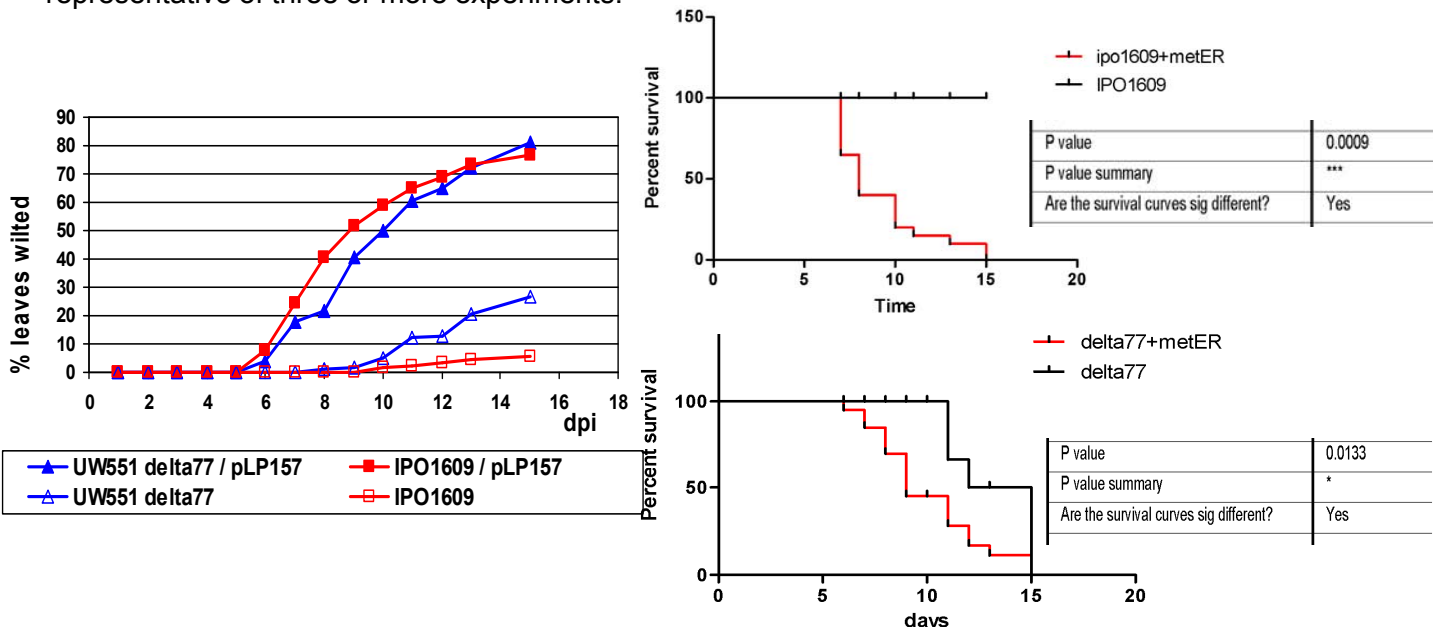
Figure 25. **Virulence of *R. solanacearum* strains IPO1609, UW551Δ77, and the complemented strains carrying pLP157 (carrying functional *metER* genes) on tomato plants inoculated by soaking the soil to a final bacterial population of 1x10^{8}CFU/ml per plant.** (A) Percentage of leaves wilted during the time. The expression of the *metER* genes in IPO1609, and mutant UW551Δ77 significantly enhanced pathogenesis since symptoms appeared four days earlier in the transconjugants and reached higher rates of wilting. (B) Statistical differences in pathogenicity of strains carrying or not the pLP157 plasmid supported by comparison of Survival Curves using the Log-Rank test (P value <0.05).

7 Evidence that the *metE-metR* methionine biosynthesis genes are required for full pathogenicity of strain UW551

Since the strongest impact on pathogenicity was associated with the deletion of the a1 region, we decided to focus our study on this 12kb region. This region comprises the *metE* and *metR* genes and seven other genes organised in one (or possibly two) operon(s) which were proposed to encode an indole/auxin-related molecule (Valls et al., 2006). This hypothesis was made since two genes were predicted to encode an Indole-3-glycerol phosphate synthase and an Anthranilate phosphoribosyltransferase, respectively, whereas some other genes displayed homology to oxidoreductases or dehydrogenases. However, since a GMI1000 mutant deleted for six genes of this putative indole/auxin biosynthetic pathway was not significantly altered in virulence (M. Valls, unpublished data), we first focused our analysis on the *metE* and *metR* genes. We therefore created a mutant strain carrying a deletion of both *met* genes, UW551Δ*metER* (Δ*metE-metR::Ω*), and its virulence was tested on tomato plants (Figure 24). A reduction in the rate of symptom appearance was observed since disease symptoms in the *metER* mutant only started 8 days post-inoculation and at this time, the wild-type UW551 strain already killed 100% of the plants. Although the *metER* mutant remained able to wilt tomato plants on a longer period (17 days post-inoculation), which is different to the behaviour of IPO1609, this was a first indication that the methionine biosynthesis genes could be implied in the hypovirulent phenotype of strain IPO1609.

To confirm the implication of the *metER* in pathogenicity, we cloned the *metER* genes from UW551 on the broad-host range vector pLAFR6 in order to perform complementation assays. The resulting plasmid, named pLP157, was introduced by electroporation in the strains IPO1609, UW551Δ77 and UW551Δ*metER* and each transconjugants was compared in pathogenicity assays on tomato with the corresponding parental strain without plasmid. Results on Figure 25 are shown for strains IPO1609 and UW551Δ77. The expression of the *metER* genes in these strains significantly enhanced pathogenesis since symptoms appeared four days earlier in the transconjugants and reached higher rates of wilting (up to 80% whereas parental strains produced 5 to 25% of leaves wilted at this stage). Statistical differences in pathogenicity of strains carrying or not the pLP157 plasmid were supported by the Log-Rank test (P value <0.05). Pathogenicity of strains IPO1609
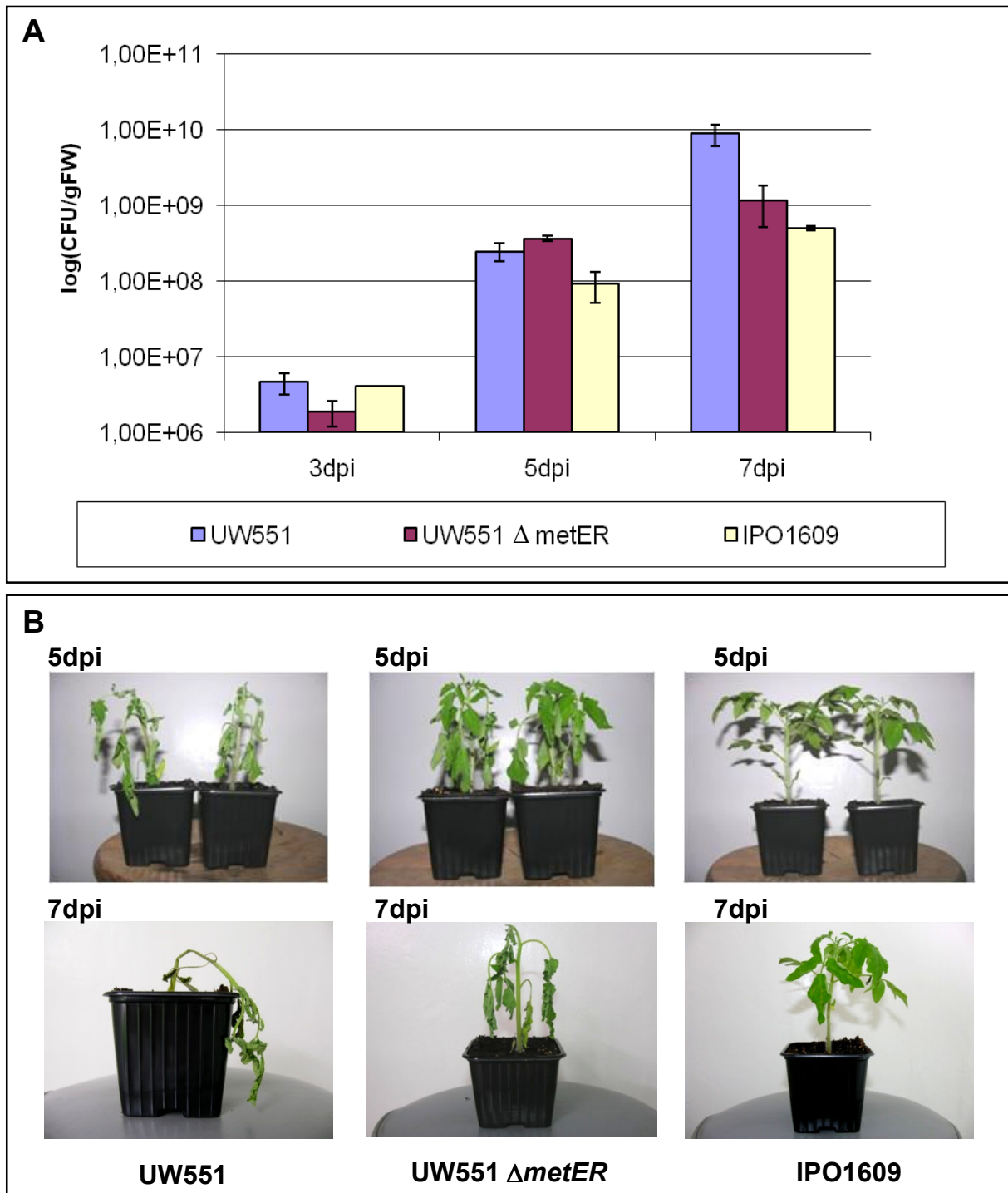
Figure 26. Determination of bacterial growth *in planta* of strains UW551, IPO1609 and the mutant UW551Δ*metER*.

(A) 10μl of a bacterial suspension of 10^6 cfu/ml of wild type strains UW551, IPO1609, and the mutant UW551ΔmetER were injected directly into the stem of three tomato plants of 4-week-old. Three, five and seven days post-inoculation (dpi), the aerial part of the plant were cut from their crown stem and the cut sections were placed in clear water within 15-30 minutes to allow the exit of the viscous bacterial white slime. Bacteria were enumerated by plating serial dilutions onto BG medium with and without antibiotic and the results were expressed in Log of colony number by gram of fresh matter log(CFU/gFW). Bars represent the standard error between the averages obtained for three plants.

(B) Phenotype of tomato plants at 5 and 7 dpi. Wilting symptoms were already observed with strain UW551 and, to a lesser extent, with the UW551Δ*metER* double mutant at 5 dpi

and UW551Δ77 was not fully complemented by pLP157 compared to UW551 since symptoms appearance is faster and the rate of wilted leaves reaches 100% with this strain at the density of inoculum used (10^8 cfu/ml). This observation confirms that other determinants within the left-hand half of the 77kb deletion are probably involved in the fitness of strain IPO1609 *in planta* but demonstrates that a clear contribution to pathogenicity is conferred by the *metE* and *metR* genes.

8 The delayed pathogenicity of the UW551Δ*metE-metR* mutant does not only result from a growth defect in the host

Since strains IPO1609 or UW551Δ77 were not auxotrophic for methionine on minimal medium *in vitro*, but appeared to be limited in growth in a minimal medium lacking methionine, we examined whether these strains and the UW551Δ*metER* double mutant were impaired for growth inside the plant. Internal growth curves (IGC) were performed on tomato plants after stem inoculation of 10^4 bacteria and bacterial populations were enumerated 3, 5 and 7 days post-inoculation. Results of one experiment are presented in Figure 26. This shows that all strains actively grew inside the plants to reach high population levels at 7dpi (up to 10^9 to 10^{10} cfu/g of fresh weight). It seems however that at 7dpi bacterial populations of strains IPO1609 and UW551Δ*metER* were reduced from 10 to 12-fold compared to UW551. At 5 dpi, this decrease was observed only for strain IPO1609 (5-fold less compared to the other strains). Interestingly, at 5 dpi wilting symptoms were already observed with strain UW551 and, to a lesser extent, with the UW551Δ*metER* double mutant (Figure 26). At 5dpi, this difference in the rate of symptom production was therefore not correlated with a decrease of the UW551Δ*metER* strain in its capacity to multiply efficiently *in planta*.

Although this preliminary result remains to be confirmed, it suggests that the hypovirulence phenotype observed with a strain carrying disruption of the *metER* genes is probably not only due to a reduced capacity to grow in the host. This hypothesis is supported by the fact that the *metER* mutant reaches high populations levels in the plant (5.10^8 cfu/g of fresh weight at 5 dpi). In comparison, a typical avirulent mutant such as the T3SS component *hrcV* mutant strain reaches in identical stem-inoculation and inoculum conditions a population of approx. 5.10^5 cfu/g of fresh

weight at 5 dpi (Macho et al., 2010), which is 1000-fold less than the *metER* mutant. This suggests that methionine may not be an essential growth limiting factor inside the plant, and this is in agreement with the finding that the methionine concentration measured in the apoplasm of tomato plants was determined to be of 24,9 μM (Rico and Preston, 2008), indicating that presence of this amino acid is not scarce in this host. However, methionine biosynthesis certainly contributes to the global bacterial fitness since the *metER* mutant population remains inferior to the one of UW551 over longer periods of time, as observed at 7dpi.

9 Distribution of the genes identified within the 77kb region in the *R. solanacearum* species

Some intriguing questions remaining about the occurrence of the 77kb deletion identified in strain IPO1609 concern the molecular mechanism involved and its possible specificity (as suggested by the existence of a short repeat sequence next to the deletion point) and the biological relevance of this deletion in the species or in the bacterial population. Comparative genomic hybridization studies were conducted on approximately 45 *R. solanacearum* strains (Guidot et al., 2007; A. Guidot, M. Elbaz & C. Boucher, unpublished data) and this 77kb deletion was detected only in strain IPO1609. Analysis of the genome of the six sequenced strains to date (and representative of the four main phylotypes of the species) revealed a fairly high level of synteny in this region of the megaplasmid (see Table 8 as annex at the end of this chapter). Orthologues of most of the genes within the 77kb region are found in other strains; among the few differences observed were some probable substrates of the Type VI Secretion System in the RRSL_0093 region (which all appear to be strain-specific) and for some type III effectors (RRSL_02221 and RRSL_02213). In conclusion, this comparative analysis shows that, although it does not contain any essential gene, this 77kb region is very well conserved in terms of genetic organisation and gene content in the *R. solanacearum* species.

10 Materials and Methods

10.1 Strains and media

The characteristics of the plasmids and bacteria used in this work are listed in table 9. *Escherichia coli* strain was grown at 37°C in Luria –Bertani medium (Ausubel et

al., 1989). *R. solanacearum* strains were grown in complete BG medium or in MP minimal medium supplemented with 0.2% glucose. The composition of BG medium is as follows (g liter^{-1}): Bacto peptone, 10; Casamino Acids, 1; yeast extract, 1. For agar plates, BG medium was supplemented with glucose (5 g liter^{-1}) and triphenyltetrazolium chloride (0.05 g liter^{-1}). The composition of MP minimal medium is as follows (g liter^{-1}): FeSO$_4$. 7H2O, 1.25 x 10^{-4}; (NH$_4$)$_2$SO$_4$, 0.5; MgSO$_4$. 7H$_2$O, 0.05; KH$_2$PO$_4$, 3.4. The pH was adjusted to 7 with KOH (Plener et al., 2010). When needed, antibiotics were added to the media at the following final concentrations (mg liter^{-1}): spectinomycin, 40 *for R. solanacearum*; tetracycline, 10; ampicillin, 100 for *E. coli*.

10.2 Natural transformation of *R. solanacearum* strains

R. solanacearum was grown in MP medium supplemented with glycerol (20g l^{-1}) until reach an OD$_{600nm}$ between 0.5 -1.0. 15 µl of bacterial suspension was mixed with 3-5 µg of linear plasmid and the mixture was placed on a 0.45 µm cellulose nitrate filter unit placed on a BG agar. After 48 hours of incubation at 28°C the bacteria were resuspended from the filter in 1 ml of BG medium and 100 µl was spread on LB plates supplemented with the antibiotic marker in order to select recombinant clones.

10.3 Conjugation by triparental mating

Mating to transfer a plasmid from *R. solanacearum* into *E. coli*

Overnight liquid cultures of donor strain *R. solanacearum*, helper strain *Escherichia coli* K2013 (Km^r) and recipient strain *E. coli* DH5α were made using the appropriate antibiotics. 1 ml of each recipient and helper strains were mixed with 0.6 ml of donor *R. solanacearum* strain and all together were centrifuged at 10.000 rpm during 1 minute. The pellet was resuspended into 0.1 ml of CB medium and was placed on a 0.45 µm cellulose nitrate filter unit placed on a CB agar. The plate was incubated for 3 hours at 28°C. Bacteria were resuspended from the filter in 1 ml of CB medium and 100 µl was spread on LB plates supplemented with the antibiotic marker of mobilized plasmid and incubated at 37°C overnight.

Plasmid transfer from *E. coli* into *R. solanacearum*

Overnight liquid cultures of donor strain *E. coli* DH5α, helper strain *Escherichia coli* K2013 (Km[r]) and recipient strain *R. solanacearum* were made using the appropriate antibiotics. 1 ml of each donor and helper strains were mixed with 0.6 ml of recipient *R. solanacearum* strain and all together were centrifuged at 10.000 rpm during 1 minute. The pellet was resuspended into 0.1 ml of CB medium and was placed on a 0.45 μm cellulose nitrate filter unit placed on a CB agar. Then, the filter unit was transferred to a test tube with 5 ml of CB medium and bacteriophage T4 was added to counterselect *E. coli* interspecific matings. The reaction was incubated by 2 hours at 28°C. Finally, 100 μl of serial dilutions were plated on CB medium supplemented with the antibiotic marker of the mobilized plasmid and the plates were incubated by two days at 28°C.

10.4 Assembling and annotation of the 77kb region of strain UW551

To assemble contig sequences from the draft genome sequence of strain UW551 deposited in Genbank corresponding to the 77kb region deleted in strain IPO1609, we first identified four UW551 contigs (Cont0556 [GenBank accession AAKL01000018.1]; Cont0555.1 [GenBank accession NZ_AAKL01000025.1], Cont0488 [GenBank accession NZ_AAKL01000172.1] and Cont0547 [GenBank accession NZ_AAKL01000031.1]) containing homologues of GMI1000 genes predicted to cover the 77kb region by a former CGH study (Guidot et al., 2007).These four contigs were jointly assembled as follows: (1) Cont0556 from position 55400 to 83486, (2) Cont0555.1 from position 72014 to 42177, (3) Cont0448 from position 8003 to 1, and (4) Cont0547 from position 1 to 13324.

Contig555.1 was split into two parts at position 42177 after a transposase gene since we suspected a local assembling mistake at this position. The final assembling of the 77kb region from UW551 was checked by PCR.

Gene prediction was performed under the iANT software environment using program FrameD (Schiex et al., 2003) that can be described as a conditional random field (CRF) gene finder. A gene is defined by a coding sequence composed of one or more regions coding in different frames (according to possible indels), surrounded by non-coding regions. The features used in the CRF model include interpolated Markov models to estimate the coding/non-coding potential of a region, existence of START

and STOP codons, existence of a similarity with known proteins and possible existence of frameshifts (Schiex et al., 2003). Predicted Open Reading Frames were reviewed individually by a gene annotator for start codon assignment and BlastP analysis of the predicted products was also expertized to generate the proposed annotations. Proteins were classified according to Riley's rules (Karp et al., 1999).

10.5 Creation of the 77kb deletion in strain UW551 to determine whether this region is involved in the loss of pathogenicity of IPO1609

Upstream (border fragment named F1) and downstream (border fragment named F2) from 77kb deletion region in UW551 were PCR-amplified using primers F1XbaI and F1R1 and F2R1 and F2SacI listed in Table 10 respectively. The PCR program used for that is as follows: 94°C by 2 min, 35 cycles of denaturation at 94°C by 30 sec, annealing at 55°C by 30 sec, extension at 72°C by 1 min, and final incubation at 72°C by 5 min. Both fragments were cloned into pGEM®-T vector (Promega) and disrupted by insertion of Ω cassette in the *Eco*R1 site (Prentki et Krisch, 1984) conferring spectinomycin resistance. The resulting plasmid was introduced in *R. solanacearum* UW551 by natural transformation of bacteria grown in MP medium supplemented with glycerol (20g I^{-1}) with 3-5µg of linear plasmid and the recombinant clones were selected after growth in BG medium supplemented with spectinomycin. The confirmation of the homologous recombination event of the incoming DNA at the target locus was performed by PCR analyses using primers F1Xba-ΩDw and ΩUp-F2SacI to verify the creation of UW551Δ77 strain (F1-F2::Ω).

10.6 Construction of UW55Δ77 derivated mutant strains carrying different deleted parts of the 77 kb region

The 77kb region was divided in two halves: a 37 kb left-hand from the *metE* and RRSL_02225 gene, named "a" and a 40 kb right-hand half from RRSL_02224 to *tssH* gene named "b" (Figure 21). Upstream (border fragment F1) and downstream (intergenic fragment F3) of "a" region was PCR amplified using primers F1Xba -F1R1 and F3R1-F3SacI. Fragments F1 and F3 obtained from "a" half were both jointly cloned into pGEM®-T (Promega) vector and a Ω cassette was inserted in the unique *EcoRI* site between both fragments. The resulting plasmid was linearized by *Nsi*I

used to naturally transform strain UW551 and a homologous recombination event was selected using the Spectinomycin resistance of the Ω cassette. Recombinant clones were isolated and the confirmation of the homologous recombination event of the incoming DNA at the target locus was performed by PCR using primers F1Xba-ΩDw and ΩUp-F3SacI for region "a" The resulting strain was called UW551Δ77a (F1-F3::Ω). For the construction of the deletion of the region "b", upstream (intergenic fragment F3) and downstream (border fragment F2).of this one were both jointly cloned into pGEM®-T (Promega) vector and a Ω cassette was inserted in the unique *EcoRI* site between both fragments. The resulting plasmid was linearized by NsiI used to naturally transform strain UW551 and a homologous recombination event was selected using the Spectinomycin resistance of the Ω cassette. Recombinant clones were isolated and the confirmation of the homologous recombination event of the incoming DNA at the target locus was performed by PCR using primers F3SphI -ΩDw and ΩUp-F2SacI for region "b". Additionally, a Southern blot analysis was performed using as probe a 2.3 kb *Eco*RI fragment comprising the *metR,* gene located in the region a from 77 Kb deleted region.

For the constructions of mutants of the left-hand from 77kb region, this one was divided in two parts, a region named a1 from the *metE* to RRSL_02276 gene and a2 from RRSL_02277 to RRSL_02225 gen. UW551Δ77a$_1$ (F1-A1::Ω) and UW551Δ77a$_2$ (A1-F3::Ω) strains which carry deleted the first half of 77kb region the same methodology was used. Upstreams and downstreams of region a1 limited by border fragment F1 and intergenic fragment A1 and the region a2 limited by intergenic fragment A1 and intergenic fragment F3 (figure 21) were PCR amplified using the primers F1Xba -F1R1 and A1R1a-A1SacI for a1 and the primers A1Xba- A1R1b and F3R1-F3SacI for a2 region. Fragments F1 and A1 obtained from a1 region were both jointly cloned into pGEM®-T (Promega) vector and a Ω cassette was inserted in the unique *EcoRI* site between both fragments. The resulting plasmid was linearized by XbaI used to naturally transform strain UW551 and a homologous recombination event was selected using the Spectinomycin resistance of the Ω cassette. Recombinant clones were isolated and the confirmation of the homologous recombination event of the incoming DNA at the target locus was performed by PCR using primers F1Xba-ΩDw and ΩUp-A1SacI for region a1. The resulting strain was

called UW551Δ77a$_1$ (F1-A1::Ω). For the construction of the deletion of the region a2, upstream fragment F3 and downstream fragment F2.of this one were both jointly cloned into pGEM®-T (Promega) vector and a Ω cassette was inserted in the unique *EcoRI* site between both fragments. The resulting plasmid was linearized by NsiI used to naturally transform strain UW551 and a homologous recombination event was selected using the Spectinomycin resistance of the Ω cassette. Recombinant clones were isolated and the confirmation of the homologous recombination event of the incoming DNA at the target locus was performed by PCR using primers A1Xba - ΩDw and ΩUp-F3SacI for region a2. The resulting strain was called UW551Δ77a$_2$ (A1-F3::Ω)

Additionally, upstream and downstream fragments of genes *metE* and *metR* located in the a1 part of 77kb region, were PCR-amplified using the primers metEXbaI-metER1 for metE and metRR1-metRSacI for metR. Both fragments were jointly cloned into pGEM®-T (Promega) vector and a Ω cassette was inserted in the unique *EcoRI* site between them. The resulting plasmid was linearized by SacII used to naturally transform strain UW551 and a homologous recombination event was selected using the spectinomycin resistance of the Ω cassette. Recombinant clones were isolated and the confirmation of the homologous recombination event of the incoming DNA at the target locus was performed by PCR using primers metEXba-ΩDw and ΩUp-metRSacI. The resulting strain was called UW551ΔmetER (*metE- metR::Ω*).

10.7 Cloning of the 77Kb region from UW551 and creation of complemented strain IPO1609/PAGA77

An intergenic region of 1.2 kb called F3 located at the end of first half a between the genes RRSL_02225 and RRSL_02224 of the 77kb region (Figure 21) was PCR amplified using primers F3R1 and F3SacI. The resulting fragment was cloned into pGEM®-T (Promega) vector and the Ω cassette was inserted in the unique *SmaI* site. The resulting plasmid was linearized by *Xba*I and used to naturally transform strain UW551 and a homologous recombination event was selected using the Spectinomycin resistance of the Ω cassette. Recombinant clones were isolated and

confirmation of marker-exchange event in the F3 genomic region was performed by PCR using primers F3R1-ΩDw --ΩUp F3SacI. The resulting strain was called UW551 omega (UW551::Ω).

In parallel the upstream and downstream borders of 77kb region named F1 and F2 respectively (Figure 19) were both jointly cloned from pKS(+) vector (Stratagene) between HindIII and SacI sites of pLAFR6 (tetracycline resistance, Tc[r]) replicative vector. The pLAFR6::F1-F2 resulting plasmid was introduced by electroporation (2.5kv 200W, 25mF, 0.2-cm cuvette gap) into the UW551::Ω strain to select for a double recombination marker exchange event the clones in CB medium supplemented with spectinomycin and tetracycline. The resulting strain was called UW551:: Ω /pLAFR6::F1-F2 (figure 21)

Then by tri parental mating using as the helper strain *E. coli* K2013, carrying the conjugative plasmid pRK2013 (kanamycin resistant Km[r]), and the donor strain *R. solanacearum* UW551:: Ω /pLAFR6::F1-F2 was conjugated with *E. coli* DH5α cells, and trans-conjugants that acquired the pLAFR6 vector carrying the omega interposon, hereafter called pAGA77, were selected on LB+Spectinomycin+Tetracycline plates and the confirmation of recombinant plasmids was made by colony PCR using primers A1R1a-A1SacI which are specific for the **a1** region of the 77kb region, and using as positive control DNA of UW551 strain and as negative control DNA from *E. coli* DH5α. Other confirmation method was by restriction analyzes of pAGA77 plasmid using the enzymes *Eco*RI, *Bam*HI and *Kpn*I comparing the size of the generated fragments with the restriction map made for this region. Finally The pAGA77 plasmid mobilized from *E. coli* DH5α to *R. solanacearum* IPO1609 strain by other tri parental mating using the helper strain *E. coli* K2013 (Figure 19). Clones were selected in CB medium supplemented by tetracycline and spectinomycin.

10.8 Pathogenicity assays on plants

The UW551mutant strains (UW551Δ77, UW551Δ77a, UW551Δ77b) were tested for pathogenicity on 24 tomato plants of 4-week-old (*Solanum lycopersicum* cv Supermarmande) with 50 ml of bacterial suspension of 1×10^7 cfu ml^{-1} and 1×10^8 cfu ml^{-1} per plant and on potato plants (*Solanum tuberosum* cv. Monalisa) by

infiltration with 10 µl of bacterial suspension of 1 x 10^7 cfu/ml. The mutant strains which carries the deletion of the first half of the 77kb region (UW551Δ77a1, UW551Δ77a2, UW551Δ*metER*) were tested for pathogenicity only on tomato plants as previously described. Disease development was scored daily by using a disease index where 0 indicates no disease, 1 indicates 1 to 25% of leaves wilted, 2 indicates 25 to 50% of leaves wilted, 3 indicates 51 to 75% of leaves wilted, and 4 indicates 76 to 100% of leaves wilted. Plants were kept at 28°C, 75% humidity during 10-15 days. The pathogenicity tests were performed as minimum three times.

10.9 Statistical analyses

The pathogenicity results were analyzed by the within-group Kaplan–Meier estimator which estimates the survival function from life-time data. In this case, the test estimates (the total number of plants surviving [i.e., with disease index scores below 3] out of the total number inoculated for each group), considering as a group the *R. solanacearum* strain used in the test. This disease index score of 3 was used to discriminate between live plants (score below 3) and dead plants (score of 3 or above) since it is assumed that a plant reaching a disease symptom score of 3 (*i.e* 75% of the leaves wilted) will eventually lead to plant death. In certain cases, mainly those involving hypovirulent mutants, the threshold to estimate the total number of plants surviving was fixed to a disease score of 2 since pathogenicity of these mutant strains rarely exceed 50% of wilted leaves.

Kaplan Meier test was computed for each time interval in order to build Kaplan-Meier survival curves for each group. Additionally, the Log-rank test was used to perform between-group comparisons, testing the equivalence of the Kaplan-Meier survival curves for a pair of groups. This test was applied to check the null hypothesis that there is no difference between the survival curves produced by the plants inoculated by a specific *R. solanacearum* strain. When p-value was < 0.05 the null hypothesis was refused, thus the survival curves were significantly different. These analyses were made using the software GraphPad Prism, version 5.03.

10.10 Determination of bacterial growth *in vitro*

Overnight culture was used to inoculate a 250-ml flask containing 50 ml of MP medium supplemented with glucose 0,2% to a starting with a bacterial concentration of 1 x 10^7 cfu ml^{-1}. Cultures were incubated at 28°C by 5 days and CFU counts were

determined by dilution plating on CB medium supplemented with Spectinomycin for the mutant strain. As control the wild type UW551 strain was used since it has the 77kb region and consequently the *metE* and *metR* genes are present in it. The experiment was repeated twice.

10.11   Determination of bacterial growth *in planta*

$10\mu l$ of a bacterial suspension of 10^6 cfu/ml of wild-type strains UW551, IPO1609, and the mutant UW551$\Delta metER$ were injected directly into the stem of three 4-week-old tomato plants. Three, five and seven days post-inoculation (dpi), the aerial part of the plant were cut from their crown and the leaves were removed. Cut sections were weighed and disinfested by 70% alcohol solution and were placed in clear water within 15-30 minutes to allow the exit of the viscous bacterial white slime. Bacteria were enumerated by plating serial dilutions onto BG medium with and without antibiotic and the results were expressed in Log of colony number by gram of fresh matter (log(CFU/gFW)). A student T-test was applied to results to compare the differences in growth *in planta* between IPO1609 and the mutant UW551$\Delta metER$ with respect to wild type UW551. A *p*-value <0.05 was considered as significantly different.

ANNEX 2

Table 8. **Comparative analysis of gene content in genomic regions homologous to the 77kb region deleted in IPO1609 in other *R. solanacearum* sequenced strains.** Gene nomenclature follows the one of UW551 as shown on figure 21. The green color indicates that an orthologous gene is found in the corresponding regions of strains representative of the biodiversity of the species: Phylotype II (Molk2 and CFBP2957), Phylotype III (CMR15), Phylotype IV (Psi07) and Phylotype I (GMI1000). Empty rectangles show the genes absent in the corresponding strain.

UW551 gene	Gene name	Description	Molk2	CFBP2957	Psi07	CMR15	GMI1000
RRSL_02267		Putative Lipoprotein	🟩	🟩	🟩	🟩	🟩
RRSL_02268	*metE*	Homocysteine methyltransferase (Methionine synthase)	🟩	🟩	🟩	🟩	🟩
RRSL_02269	*metR*	Methionine Biosynthesis Transcriptional Regulator	🟩	🟩	🟩	🟩	🟩
RRSL_02270	*pdxH2*	Pyridoxine/Pyridoxamine 5'-phosphate oxidase	🟩	🟩	🟩	🟩	🟩
RRSL_02271		Putative Diaminopimelate decarboxylase	🟩	🟩	🟩	🟩	🟩
RRSL_02272	*trpC2*	Indole-3-glycerol phosphate synthase	🟩	🟩	🟩	🟩	🟩
RRSL_02273	*trpD2*	Anthranilate phosphoribosyltransferase	🟩	🟩	🟩	🟩	🟩
RRSL_02274		Trimethylamine/Histamine dehydrogenase	🟩	🟩	🟩	🟩	🟩
UW551_0001		Conserved hypothetical protein	🟩	🟩	🟩	🟩	🟩
RRSL_02275		Conserved hypothetical protein	🟩	🟩	🟩	🟩	🟩
RRSL_02276		Transmembrane hypothetical protein	🟩	🟩	🟩	🟩	🟩
RRSL_02277		Transcription regulator	🟩	🟩	🟩	🟩	🟩
UW551_0002		N-terminal fragment of a LysR transcription regulator (pseudogene)	🟩	🟩	⬜	⬜	⬜
RRSL_02278		Acyl-CoA dehydrogenase	🟩	🟩	🟩	🟩	🟩
RRSL_02279		Transmembrane protein	🟩	🟩	🟩	🟩	🟩
RRSL_02280		FMN-binding negative transcriptional regulator	🟩	🟩	🟩	🟩	🟩
RRSL_02281	*hmgB*	Fumarylacetoacetate hydrolase	🟩	🟩	🟩	🟩	🟩
RRSL_02282	*hmgA*	Homogentisate 1,2-dioxygenase	🟩	🟩	🟩	🟩	🟩
RRSL_02283	*hmgR*	Transcription regulator	🟩	🟩	🟩	🟩	🟩
RRSL_02284		Peptide chain release factor homolog protein	🟩	🟩	🟩	🟩	🟩
RRSL_02285		Conserved hypothetical protein	🟩	🟩	🟩	⬜	🟩
RRSL_02286		Conserved hypothetical protein	🟩	🟩	🟩	🟩	🟩
RRSL_02287		FAD-dependent monooxygenase	🟩	🟩	🟩	🟩	🟩
RRSL_02288		Beta-lactamase, type II	🟩	🟩	🟩	🟩	🟩
RRSL_02289		Transcription regulator	🟩	🟩	🟩	🟩	🟩
RRSL_02235		Peptide transport system, ABC transporter, permease protein	🟩	🟩	🟩	🟩	🟩
RRSL_02234		Peptide transport system, ABC transporter, permease protein	🟩	🟩	🟩	🟩	🟩

Locus	Gene	Annotation					
RRSL_02233		Peptide transport system, ABC transporter, ATP-binding protein	🟩	🟩	🟩	🟩	🟩
RRSL_02232		Signal peptide hypothetical protein	🟩	🟩	🟩	🟩	🟩
RRSL_02231		Cytochrome P-450-like monooxigenase	🟩	🟩	🟩	🟩	🟩
RRSL_02230		Transmembrane hypothetical protein	🟩	🟩	🟩	🟩	🟩
RRSL_02229		Transmembrane hypothetical protein	🟩	🟩	🟩	🟩	🟩
RRSL_02228		Beta-lactamase, type II hydrolase	🟩	🟩	🟩	🟩	⬜
UW551_0003		N-acetyltransferase protein	🟩	🟩	🟩	🟩	🟩
RRSL_02227		D-alanine aminotransferase	🟩	🟩	🟩	🟩	🟩
RRSL_02226		Signal peptide hypothetical protein	🟩	🟩	🟩	🟩	🟩
RRSL_02225		Conserved hypothetical protein	🟩	🟩	🟩	🟩	🟩
RRSL_02224		Cytochrome C peroxidase	🟩	🟩	🟩	🟩	🟩
RRSL_02223		Phosphate-selective porin	🟩	🟩	🟩	🟩	🟩
RRSL_02222		Signal peptide hypothetical protein	🟩	🟩	🟩	🟩	🟩
RRSL_02221		Type III secretion system effector protein with lipase domain	🟩	🟩	🟩	🟩	⬜
RRSL_02220		Transmembrane protein	🟩	🟩	🟩	🟩	⬜
RRSL_02219		L-aspartate dehydrogenase	🟩	🟩	🟩	🟩	🟩
RRSL_02218		Conserved hypothetical protein	🟩	🟩	⬜	⬜	⬜
RRSL_02217		Transmembrane transporter	🟩	🟩	⬜	⬜	🟩
RRSL_02216		Signal peptide hypothetical protein	🟩	🟩	🟩	🟩	🟩
RRSL_02215		Conserved hypothetical protein	🟩	🟩	🟩	🟩	🟩
RRSL_02214		Sterol desaturase	🟩	🟩	🟩	🟩	🟩
RRSL_02213		Type III secretion system effector protein	🟩	🟩	🟩	⬜	🟩
RRSL_02212	*ripTPS*	Type III secretion system effector protein with trehalose-6-phosphate activity	🟩	🟩	🟩	🟩	🟩
RRSL_02211		Hypothetical protein	⬜	🟩	🟩	🟩	🟩
RRSL_02210		Transposase	🟩	⬜	⬜	⬜	⬜
RRSL_02209		Insertion sequence element	⬜	⬜	⬜	⬜	⬜
RRSL_00093		Hypothetical protein	⬜	🟩	⬜	⬜	⬜
RRSL_01728	*vgrG1*	Type VI secretion system Vgr family protein	🟩	🟩	🟩	🟩	🟩
RRSL_01729	*tssL*	Component of the type VI protein secretion system	🟩	🟩	🟩	🟩	🟩
RRSL_01730	*tssK*	Component of the type VI protein secretion system	🟩	🟩	🟩	🟩	🟩
RRSL_01731	*tssJ*	Component of the type VI protein secretion system	🟩	🟩	🟩	🟩	🟩
RRSL_01732		Tetratricopeptide repeat domain protein	🟩	🟩	🟩	🟩	🟩
RRSL_01733	*tssA*	Component of the type VI protein secretion system	🟩	🟩	🟩	🟩	🟩
RRSL_01734	*tssB*	Component of the type VI protein secretion system	🟩	🟩	🟩	🟩	🟩
RRSL_01735	*hcp1*	Type VI secretion system effector, Hcp1 family	🟩	🟩	🟩	🟩	🟩

RRSL_01736		Type VI secretion system lysozyme-related protein					
RRSL_01737	*tssD*	Component of the type VI protein secretion system					
RRSL_01738	*tssE*	Component of the type VI protein secretion system					
RRSL_01739	*tssH*	Type VI secretion ATPase, ClpV1 family					
RRSL_01740	*vgrG2*	Type VI secretion system Vgr family protein					
RRSL_01741		Conserved hypothetical protein					

Table 9. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)		Reference
Srains			
E. Coli			
DH5a	F⁻ *recA lac*ZDM15		Bethesda Research Lab.
E. coli K2013	Km^r		
E.coli (pAGA77)	*E.coli* with pAGA77 Tc^r Sp^r		This Work
R. Solanacearum			
UW551	Wild type strain		(Gabriel et al., 2006)
IPO1609	Wild type strain		(van Elsas et al., 2000)
UW551Δ77 mutant	UW551 with deletion of 77kb region delimited by F1 and F2 (F1-F2::Ω)		This Work
UW551Δ77a mutant	UW551 with deletion of 77kb region delimited by F1 and F3 (F1-F3::Ω)		This Work
UW551Δ77a1 mutant	UW551 with deletion of 77kb region delimited by F1 and A1(F1-A1::Ω)		This Work
UW551Δ77a2 mutant	UW551 with deletion of 77kb region delimited by A1 and F3 (A1-F3::Ω)		This Work
UW551Δ77b mutant	UW551 with deletion of 77kb region delimited by F3 and F2 (F3-F2::Ω)		This Work
UW551Δ*metER* mutant	UW551 with deletion of genes *metE* and *metR* of the 77kb region (*metE-metR*::Ω)		This Work
IPO1609 / Paga77 mutant	IPO1609 with 77kb region of UW551 cloning in pLAFR6		This Work
UW551 omega mutant	Deletion in the intergenic region F3 of the 77kb region of UW551(F3::Ω)		This Work
UW551Δ77 mutant / metER	UW551Δ77 mutant with genes *metE-metR* cloning in pLAFR6		This Work
UW551Δ77a1 mutant / metER	UW551Δ77a mutant with genes *metE-metR* cloning in pLAFR6		This Work
IPO1609 / *metER*	IPO1609 with genes *metE-metR* cloning in pLAFR6		This Work
UW551Δ77 / metER	UW551Δ77a with genes metE-metR cloning in pLAFR3		This Work

Strain or plasmid		Relevant characteristic(s)		Reference
Plasmids				
pGemT		Cloning vector, Amp^r		Promega
pBluescript KS(+)		Cloning vector, Amp^r		Stratagene
pLAFR6		pLAFR1 with trp terminator, Tc^r		(Huynh et al., 1989)
pAGA1		pGemT with F1		This Work
pAGA2		pGemT with F2		This Work
pAGA3		pGemT with F1-F2		This Work
pAGA4		pGemT with F1-F2::Ω		This Work
pAGA5		pGemT with F 3		This Work
pAGA6		pGemT with F1-F3		This Work
pAGA7		pGemT with F1-F3:Ω		This Work
pAGA8		pGemT with F3-F2		This Work
pAGA9		pGemT with F3-F2::Ω		This Work
pAGA10		pGemT with F 3::Ω		This Work
pAGA11		pGemT with A1a		This Work
pAGA12		pGemT with F1-A1a		This Work
pAGA13		pGemT with F1-A1a::Ω		This Work
pAGA14		pGemT with F1-A1b		This Work
pAGA15		pGemT with A1b-F3		This Work
pAGA16		pGemT with A1b-F3::Ω		This Work

Strain or plasmid		Relevant characteristic(s)		Reference
pAGA17		pGemT with *metE*		This Work
pAGA18		pGemT with *metR*		This Work
pAGA19		pGemT with *metE-metR*		This Work
pAGA20		pGemT with *metE-metR*::Ω		This Work
pAGA21		pKS with F1-F3		This Work
pAGA22		pLAFR6::F1-F2		This Work
pAGA77		pLAFR6::F1-F2:: Ω		This Work
pLP57		pLAFR3::metE-metR		This Work

Table 10. List of oligonucleotides used in this study

Name		Sequence: (5' to 3')		Size
F1XbaI		TCTAGACGAACCCCGGGACATCC		23
F1R1		CGACTCCGGAAACAAGGCTT		20
F2R1		GAATTCAAGGGCGCGGGGCATAC		23
F2SacI		GAGCTCGAAACGCTGCATGTACTTG		25
F3RI		GAATTCTCTAGACCTGCCGGGGCTGCGCAATG		32
F3SacI		GAATTCTGCGCGCGCACTTCCTT		23
F3SphI		GCATGCGACCTGCCGGGGCTGCGCAATG		28
A1R1a		GAATTCAGAATGAATGCGCTTTCG		24
A1SacI		GAGCTCCGCCCCATCTCCACG		21
A1XbaI		TCTAGAATGAATGCGCTTTCG		21
A1R1b		CGTTGTCCAGCACCGGATG		19
MetEXba		TCTAGACGCCGGCCACCGGCAGCG		24
MetERI		GAATTCGCCCTGGAATCGTTCTGG		24
MetRR1		GAATTCTGCGGACCCTGATCG		21
MetRSacI		GAGCTCGGCAGCGCGGCCAGGC		22
Ω Up		TGTTACCCGAGAGCTTG		17
Ω Dw		AGTTGGCGAAGTAATCGC		18

CHAPTER IV

DISCUSSION

CHAPTER IV

The genomic comparison performed between two 'race3' strains, UW551 and IPO1609, allowed the identification of a large deletion on the megaplasmid of IPO1609. In this work, we functionally characterized this deletion and provide genetic evidence that this 77kb deletion in strain IPO1609 is responsible for the hypovirulent phenotype of this strain. First, we showed that the creation in strain UW551 of a deletion similar to the one detected in IPO1609 resulted in a mutant strain UW551Δ77 strongly affected in its pathogenicity toward tomato and potato, similarly to the phenotype observed with IPO1609. We then succeeded in cloning the entire 77kb region on a mobilizable pLAFR plasmid and proved that this region is able to restore pathogenicity of strain IPO1609 on tomato and potato plants. During the pathogenicity assays performed in this study, we had no indication about a potential role of this 77kb region in host specificity. In the experimental – *i.e* controlled - conditions used, the behaviour of UW551 and IPO1609 was similar on both potato and tomato hosts. We then continued pathogenicity assays only with tomato since these plants are easier to produce routinely in greenhouse than potato.

The Methionine biosynthesis pathway is essential for full virulence of *R. solanacearum* on plants and belongs to the pathogenicity regulon

We developed various deletion and complementation approaches to identify the molecular determinants from the 77 kb region having a major effect in the pathogenicity of strain UW551. Our work identified a 12kb region as being responsible for most of the phenotype conferred by deletion of the 77kb region. In this 12kb region, two genes *metE* and *metR*, both belonging to the methionine biosynthetic pathway, were shown to contribute significantly to *R. solanacearum* pathogenicity towards plants.

1 **Methionine biosynthesis in *R. solanacearum***

Methionine is essential in all organisms as a building block of proteins and as a component of the universal activated methyl donor *S*-adenosylmethionine (AdoMet).
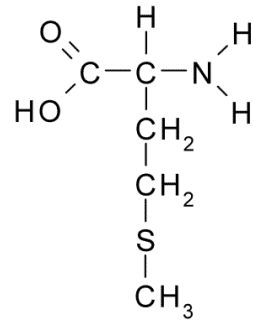
Figure 27. Chemical structure of L-Methionine

Methionine is an essential amino acid classified as nonpolar. It is the only sulfur-containing aminoacid essential for mammals but it is synthesized de novo by plants and most microorganisms.
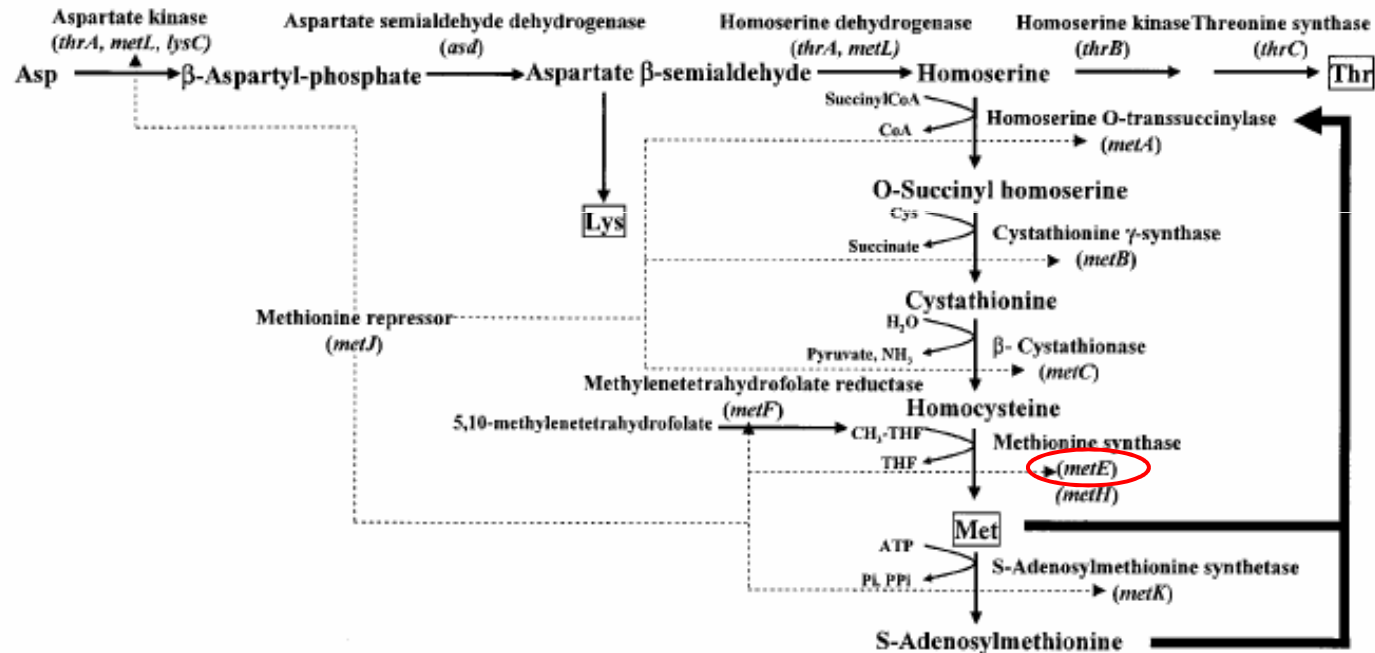
Figure 28. Biosynthesis and regulation of methionine in *Escherichia coli* [from Usuda and Kurahashi, 2005]

Methionine is synthesized via a pathway that uses the amino acid aspartic acid. First, aspartic acid is converted via β-aspartyl-semialdehyde into homoserine and then it converts to O-succinyl homoserine, which reacts with cysteine to produce cystathionine, which is cleaved to yield L-homocysteine. The conversion of L-homocysteine into L-methionine (MET) involved two alternative ways; the first one requires the methionine synthase MetH, enzyme cobalamin dependent, and the other uses MetE, cobalamin independent. Red ring indicates the gene *metE* present in the 77kb region of *R. solanacearum* UW551. The boldface arrows indicate feedback inhibition, and the dotted arrows indicate repression

Methionine is a sulfur-containing amino acid (see Figure 27) which is synthesised from another amino acid, aspartate. The enzymatic pathway for methionine biosynthesis has been well described in *Escherichia coli* and the corresponding genes have been characterized (Figge, 2006 for a review). The first three reactions of methionine production, which transform aspartate into homoserine, are shared with the biosynthetic pathways of several other metabolites, whereas the subsequent ones are specific to methionine biosynthesis (Figure 28).

The terminal step in methionine biosynthesis can be catalyzed by two apparently unrelated proteins, a cobalamin-independent methionine synthase (MetE, EC 2.1.1.14) and a cobalamin-dependent methionine synthase (MetH, EC 2.1.1.13). MetE catalyzes a direct transfer of the methyl group to homocysteine, while MetH uses cobalamin (vitamin B12) as an intermediate methyl carrier (Figge, 2006). Depending on the relative concentration of intracellular methionine, import or export transporters for methionine are involved to maintain a constant level of this amino acid in the bacterial cell.

In *E. coli*, there are two transcriptional regulators of the methionine biosynthetic pathway, *metJ* and *metR*. MetJ is a repressor of methionine biosynthesis enzymes and MetR is a LysR-type transcriptional regulator which contains a helix-turn-helix DNA binding domain. The MetR regulator has a co-activator molecule, homocysteine which is the substrate of the last reaction from the met biosynthetic pathway. In *E. coli*, MetR has been shown to activate the transcription of *metA*, *metF*, *metE* and *metH* genes (Figure 28). Expression of both *metE* and *metH* is dependent on the presence of MetR (Urbanowski et al. 1987; Cai et al. 1989), but differs in response to the presence of its co-activator homocysteine. Homocysteine is required for the full activation of *metE*, but has no effect or tends to repress *metH* expression (Cai et al., 1989; Urbanowski and Stauffer 1989). MetR also auto-regulates its own synthesis and it is repressed by MetJ (Maxon et al., 1989; Cai et al., 1989; Fritsch et al., 2000). The *metR* binding sequence consensus derived from the *E. coli* promoters is TGAannt/anntTCA (Greene 1996).

All the orthologues of the *E. coli* methionine biosynthetic pathway have been identified in the *R. solanacearum* genome (Table 11), with the notable exception of *metJ* which is absent in the genome of all sequenced strains to date. On the other hand, *R. solanacearum* possesses a *metY* gene homologue which is absent in *E. coli* but is involved in the methionine biosynthetic pathway of other bacteria such as

Table 11. Identification of *Ralstonia solanacearum* genes involve in Methionine biosynthesis
Common genes involve in methionine biosynthesis in *E. coli* and *C. glutamicum* were identified in *R. solanacearum* GMI1000 and UW551 genome sequences. *metJ* regulator was only the gene without homology in *R. solanacearum*

Protein function	*Escherichia coli*	*R. solanacearum* GMI1000	*R. solanacearum* UW551
	Gene	Gene	Gene
Homoserine dehydrogenase	*metL*	*rsc1327*	*RRSL_01531*
Homoserine O-acetyltransferase	*metA*	*rsc0027 (metX)*	*RRSL_02960*
Cystathionine- γ- synthase	*metB*	*rsp0781 (metB)*	*RSL_02531*
O-acetylhomoserine sulfhydrylase	(*)	*rsc1562*	*RRSL_02586*
Cystathionine- β-lyase	*metC*	*rsc1639 (metC)*	*RRSL_02586*
Methionine synthetase cobalamin-binding subunit	*metH*	*rsc0295 (metHa)*	*RRSL_01650*
		rsc0294 (metHb)	*RRSL_01651*
Methionine synthetase cobalamin non dependent	*metE*	*rsp0676 (metE)*	*RRSL_02268*
Methylenetetrahydrofolate reductase	*metF*	*rsc0091 (metF)*	*RRSL_04373*
S-adenosylmethionine synthetase	*metK*	*rsc0134 (metK)*	*RRSL_04335*
Transcriptional regulator	*metR*	*rsp0677 (metR)*	*RRSL_02269*
Methionine regulon repressor	*metJ*	-	-

(*) Non homologue in *E. coli* but homologue to *metY* of *Corynebacterium glutamicum*
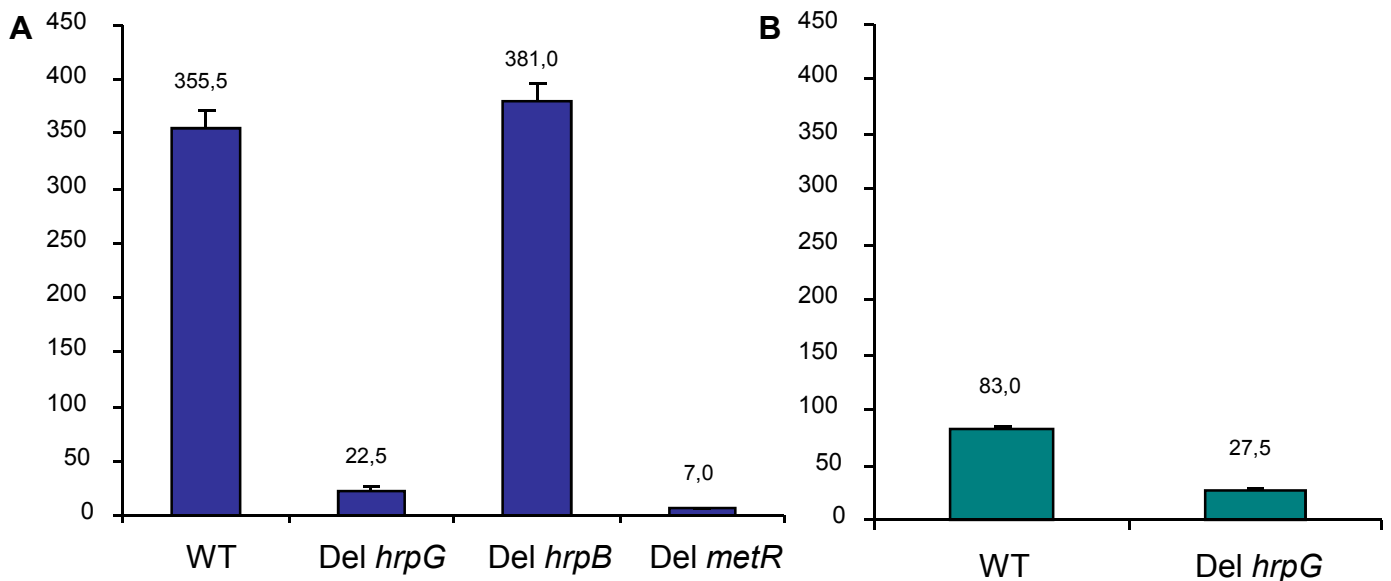
Figure 29. Expression of both *metE* and *metH* is controlled by HrpG *via* MetR in *Ralstonia solanacearum* GMI1000 in minimal medium [from Plener, 2010]

Genomic fusions with *lacZ* reporter gene was generated in *metE* (A) and *metH* (B) genes. These fusions were introduced in different strains: wild type (WT), *hrpG* mutant (Del *hrpG*), hrpB mutant (Del *hrpB*) and *metR* mutant (Del *metR*). Strains were cultivated in minimal medium supplemented with glutamate and β-galactosidase activity of each strain was measured. Results are expressed in Miller units.

(A) Measure of β-galactosidase activity by the reporter fusion in *metE* gene. Reduction of *metE* expression in factor of 10 in the strains muted in *hrpG* and *metR* genes. (B)Measure of β-galactosidase activity by the reporter fusion in *metHab* gene. Reduction of *metHab* operon in factor of 2 in the strains muted in *hrpG* regulator gene.

Corynebacterium glutamicum (Figge, 2006). A minor difference can also be observed for MetH which is encoded by two genes organized in an operon (*metHa* and *MetHb*) in *R. solanacearum* instead of a unique gene in *E. coli*.

2   Transcription of the *metER* genes is under the control of HrpG in strain GMI1000

Concomitant to this work, the work of L. Plener (2010) dedicated to identify the regulatory targets of a master regulator of pathogenicity, HrpG, in *R. solanacearum* GMI1000 led to the discovery that *metR* is a direct target of this transcriptional regulator. Plener further demonstrated that MetR positively regulates the expression of *metE*, and that expression of both *metE* and *metH* was controlled by HrpG *via* MetR (Figure 29). In strain GMI1000, disruption of *metE* or *metR* does not result in auxotrophy for methionine (Plener, 2010), as observed for strains IPO1609 or UW551Δ*metER*. However, a *metE metH* or a *metR metH* double mutant strain displays a Met auxotroph phenotype as they were unable to grow in minimal medium without supplemental Met (Plener, 2010). The fact that the *metR metH* mutant is auxotrophic for Met but not a single *metR* mutant suggests that *metH* expression is only partially controlled by MetR and that expression of *metH* in a *metR* mutant is sufficient to prevent auxotrophy of this strain for methionine (Plener, 2010). Based on the very high level of identity between UW551 and GMI1000 *met* gene-encoded products, we assumed that these regulatory circuits are similarly conserved in both strains, and thus it explains why the IPO1609 strain defective for the *metE metR* genes is not auxotrophic for Met since expression of the chromosomal *metH* gene allows the production of a functional cobalamin-dependent methionine synthase sufficient for growth.

When scored on tomato plants in pathogenicity assays either by the drenching inoculation method or by stem injection, the GMI1000 *metE metH* mutant auxotroph for Met produced no wilting symptoms and remained completely avirulent (Plener, 2010). This result was in agreement with a former study led on *R. solanacearum* strain K60 and showing that mutants auxotrophs for tryptophan, methionine or leucine (but uncharacterized at the molecular level) were avirulent on tomato compared to the wild-type (Coplin et al., 1974). Results obtained for single mutants in the *metE* or *metR* genes show that both corresponding strains were significantly

attenuated in wilting symptom development (Plener, 2010) but no double *metE metR* mutant was engineered in GMI1000 to allow a direct comparison with the IPO1609 or UW551Δ*metER* strains. However, quantification of bacterial loads *in planta* after stem inoculation using GMI1000 carrying single disruption of the *metE*, *metH* or *metR* genes revealed that the *metE* and *metH* mutants were multiplying as efficiently as the wild-type at 3 and 5dpi; only the *metR* mutant appeared to be reduced in growth by two to eight-fold compared to the GMI1000 parent (Plener, 2010). These results are globally similar to those obtained in our study in the UW551 genetic background, although using a different met mutant (Δ*metER*):

First, it established that these different prototrophic *met* mutants are able to multiply efficiently and reach population levels up to 10^8 cfu/g of fresh weight at 5dpi in both the GMI1000 and UW551 backgrounds. The observation that the GMI1000 *metR* regulatory mutant is slightly delayed in growth suggests that the similar delay observed with UW551Δ*metER* strain at 7dpi (Figure 26) may be attributable to the *metR* defect. This more important impact of *metR* on bacterial fitness is probably because it regulates the expression of several genes, i.e *metE* and to a lesser extent *metH* (and putatively other genes).

Second, it supports the hypothesis that attenuated symptoms are not directly linked to a severe growth deficiency in the plant since the GMI1000 *metE* mutant multiplies as efficiently as the wild-type strain at 3 or 5 dpi but notably produces less wilting symptoms.

The finding that the *metR* is under the direct transcriptional control of the pathogenicity regulator HrpG, and that the *metE* and *metH* biosynthetic genes are consequently also expressed in a coordinated manner with a large set of *R. solanacearum* pathogenicity determinants, strongly suggests that this Met metabolic pathway is specifically required during the plant infection process. Interestingly, expression of *hrpG* is specifically induced in presence of plant cells (Brito et al., 1999), suggesting that expression of the Met biosynthetic pathway is also specifically enhanced in the plant. Cobalamin (Vitamin B12) is not synthetized by plants. Accordingly, only the cobalamin-independent methionine synthase activity has been described in higher plants (Ravanel et al., 2004), although cobalamin-dependent enzymes also occur in the photosynthetic protist *Euglena gracilis* (Isegawa et al., 1984). A survey of the *Arabidopsis* genome indicated that there is no plant homolog

of the B$_{12}$-dependent Methionine Synthase found in bacteria, mammals, or the photosynthetic protozoa *E. gracilis.* It is noteworthy that the control of HrpG leads to a very high expression of *metE*, the cobalamin-independent enzyme, rather than *metH*, the cobalamin-dependent methionine synthase. Likewise, in presence of plant cells expression of GMI1000 *metE* is strongly induced and this results in ten-fold more expression than *metH* (Plener, 2010). It is therefore enticing to speculate that *R. solanacearum* mobilizes preferentially *metE* during the infection process to synthesize Met in a cobalamin-deficient environment. In contrast, the ability to synthesize cobalamin, one of the largest known non-polymeric natural products, requires a substantial energetic investment, necessitating the activity of ~30 genes (Roth et al., 1996). This picture of a pathogen possessing two functionally redundant enzymes that are differentially mobilized during its parasitic or saprophytic phases may provide an example of a tight metabolic adaptation of the pathogen to specific conditions encountered in the host.

3   How methionine prototrophy contributes to pathogenicity?

Studying the pathogenicity of mutants auxotrophic for a particular amino acid has supported the hypothesis, originally made by Garber et al. (1956), that nutritional deficiency leads to a loss of virulence of the corresponding mutants, particularly if the host fails to provide an adequate source of the required metabolite in the infection court. As already mentioned, this behavior was also observed for some *R. solanacearum* auxotrophic mutants (Coplin et al., 1974; Boucher et al., 1985). Although we cannot rule out the possibility that the hypovirulent phenotype observed either with the UW551Δ*metER* double mutant strain or the GMI1000 *metR* single mutant strain is due to a partial nutritional deficiency inside the host (since in some conditions the corresponding bacterial populations were reduced compared to the wild-type), some observations mentioned above incite to think that the Met biosynthetic pathway contributes to *R. solanacearum* virulence not only by promoting bacterial growth. The strongest argument supporting this view is that at 5dpi, the UW551Δ*metER* or the GMI1000 *metE* mutants reach population levels similar to their wild-type relatives, whereas significant differences in the rate of symptom development are already visible at this time point (Plener, 2010; and Figure 26). Interestingly, the Coplin et al (1974) study showed that, contrary to the tryptophan

auxotroph mutants, the met auxotroph mutants were able to multiply inside tomato plants after stem-inoculation, and colonized tissues beyond the infection point, thus reinforcing the hypothesis that Met is not a real limiting factor for *R. solanacearum* growth *in planta*.

Methionine is an amino acid implicated in many cellular processes and could serve as a precursor in several secondary metabolic pathways. In that sense, one attracting hypothesis relies on the fact that methionine is a direct precursor for ethylene biosynthesis. Ethylene is also under the transcriptional control of HrpG as was proposed to act as a pathogenicity factor in *R. solanacearum* (Valls et al., 2006). A coordinated *hrpG*-dependent expression of the different enzymes involved in the ethylene biosynthetic pathway could make sense to induce the specific production of this phytohormone once the bacterium enters the plant. It has been suggested that production of ethylene by *R. solanacearum* could complement the action of some T3SS effectors in order to modulate the ethylene-responsive plant defense pathways (Valls et al., 2006). Indeed, it was proven that the phytohormone is produced in sufficient amount by GMI1000 to induce the expression of the Arabidopsis ERF1 and PR4 ethylene-responsive genes (Valls et al., 2006). There is also recent evidence that ethylene contributes to *R. solanacearum*'s fitness in the host in strain competition assays (Macho et al., 2010); however the GMI1000 *efe* mutant defective for the <u>e</u>thylene-<u>f</u>orming <u>e</u>nzyme gene does not appear to be significantly impaired in pathogencity on tomato after soil drenching inoculation (Valls et al., 2006). It will be nevertheless interesting to determine whether ethylene production is altered in the UW551Δ*metE-metR* double mutant strain.

Methionine is also a precursor for the biosynthesis of polyamines, and some enzymes involved in this pathway are also transcriptionally controlled by HrpG, so this pathway could also be impaired in the *metER* mutant. However, the role of polyamines in bacterial virulence or adaptation to plants has not yet been unraveled. Other hypotheses could also be proposed since methionine is also the precursor of SAM (<u>S</u>-<u>a</u>denosyl-<u>m</u>ethionine), which is the major methyl donor in cellular metabolism and a precursor of several other important metabolites. For example, in *R. solanacearum* the last step is the biosynthesis of 3-OH-PAME, en endogenous quorum-sensing molecule, is catalyzed by PhcB, a SAM-dependent

methyltransferase. It could be therefore possible that a *metE* mutant is also altered in its intercellular communication capacities.

Finally, a last hypothesis on the role of methionine biosynthesis genes in bacterial virulence comes from recent observations made in *Pseudomonas aeruginosa*, an important opportunistic human pathogen infecting the pulmonary tract. In this species, deletion of *metR* was shown to lead to deregulation of nine different genes required for swarming motility, resulting in changes in virulence gene expression, antibiotic resistance and likely metabolism (Yeung et al., 2009; Overhage et al., 2008). Thus, in *P. aeruginosa metR* seems to regulate additional genes beyond those involved in methionine biosynthesis. Transcriptomic analyses revealed that among the genes deregulated in a *P. aeruginosa metR* mutant strain were genes of the T3SS and associated effectors (Yeung et al., 2009). Although it is not yet clear whether such deregulation is the result of a direct or indirect effect of the *metR* mutation, this finding reveals that the impact of a *metR* mutation may be wider than previously expected, and should deserve investigation in other bacterial systems.

Methionine auxotrophy in pathogenic bacteria is associated with hypovirulence in most of the cases taken from the literature: in the enteric pathogen *Salmonella enterica* serovar Typhimurium, inactivation of *metC* (encoding cystathionine β-lyase, and responsible for the synthesis of homocysteine, the substrate of *metE* and *metH*) leads to methionine auxotrophy and to reduced virulence in a mouse model (Ejim et al., 2004). In plant pathogens, in addition to *R. solanacearum* (Coplin et al., 1974; Boucher et al., 1985), *met* auxotroph mutants have been described in *Pseudomonas syringae* (Andersen et al., 1998) *and Agrobacterium tumefaciens* (Lippincott et al., 1965). Whereas *A. tumefaciens met* mutants were avirulent, *P. syringae met* auxotrophs were not tested for virulence but were shown to be required for optimum epiphytic fitness (Beattie and Lindow, 1994). These authors showed that low methionine concentrations on leaves could restrict bacterial growth but they also hypothesized that the role of methionine prototrophy in epiphytic fitness is to contribute directly to the ability of the bacteria to tolerate chemical and/or physical stresses in their microenvironment (Andersen et al., 1998).

To our knowledge, there is only one previous report on a methionine biosynthesis gene required for bacterial virulence but not responsible for Met auxotrophy. This work was aimed to identify *in vivo* attenuated mutants after random

mutagenesis of *Brucella melitensis*, the causal agent of Malta fever in humans. During this screen, it was found that a *metH* mutant was strongly affected in its ability to invade and to survive within macrophages and epithelial cells; however it was not auxotrophic for methionine *in vitro* (Lestrate et al., 2000). The authors postulated that *B. melitensis* might use *in vitro* the alternative enzyme, MetE, for methionine biosynthesis and that MetH is only required *in vivo*. Unfortunately, this study was not continued further and this hypothesis could not be experimentally verified. This picture in *B. melitensis* is conceptually similar to the one observed in *R. solanacearum*, with the exception that the involved players are different: in GMI1000, *metH* disruption does not affect virulence but *metE* has a more significant contribution to fitness in the tomato host (Plener, 2010). In *B. melitensis*, it is not yet clear whether this MetE/MetH duality reflects a specific metabolic adaptation to different environments or whether MetH plays a particular role in pathogenesis through an unknown mechanism.

Hypovirulence of strain IPO1609 is determined by multiple genetic traits within the first half of the 77kb deleted region

Deletion of the *metER* genes in UW551 only led to delayed and moderately reduced wilting symptoms as compared to the wild-type strain. The rate of symptoms produced by this UW551Δ*metER* strain was obviously more important than the UW551Δ77 or IPO1609 that carry the deletion of the whole 77kb region. This obviously indicates that the *metER* genes cannot solely account for the hypovirulence phenotype conferred by the 77kb deleted region. Rather surprisingly, complementation of UW551Δ77 or IPO1609 by the pLP157 plasmid carrying the UW551 *metER* genes almost completely restored virulence of these strains. This result favors the view that these *met* genes do have a significant impact in the hypovirulent phenotype of the 77kb deletion and that their overexpression on a replicative plasmid seems to result in enhanced virulence.

Among the various deletion mutants that were generated in the UW551 background, only the UW551Δ77a mutant carrying a 37kb deletion of the left-hand half had a virulence phenotype on plants similar to the UW551Δ77 or IPO1609 strains. Accordingly, deletion of the 40kb right-hand half had no significant impact on pathogenicity of UW551. This clearly establishes that the genetic traits determining hypovirulence of IPO1609 on plants are located within the 37kb corresponding to the
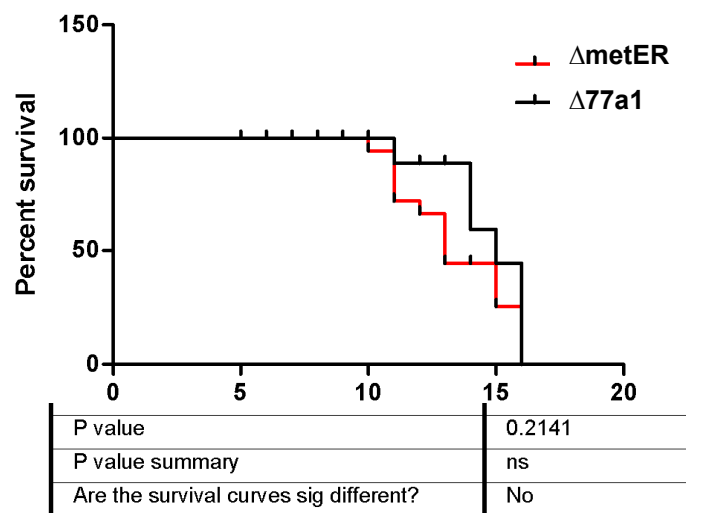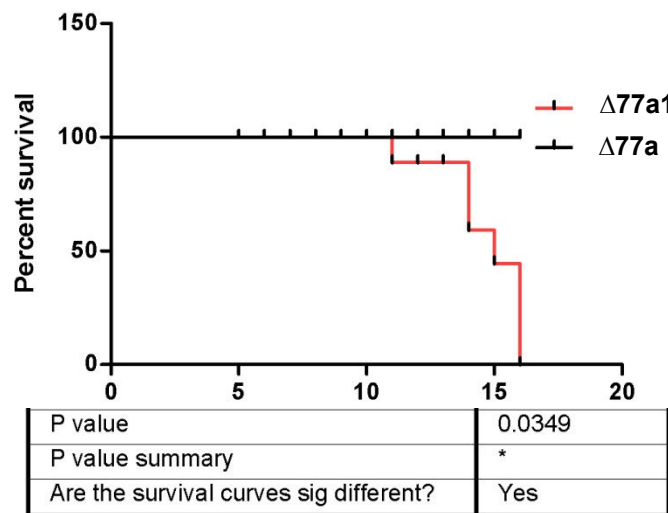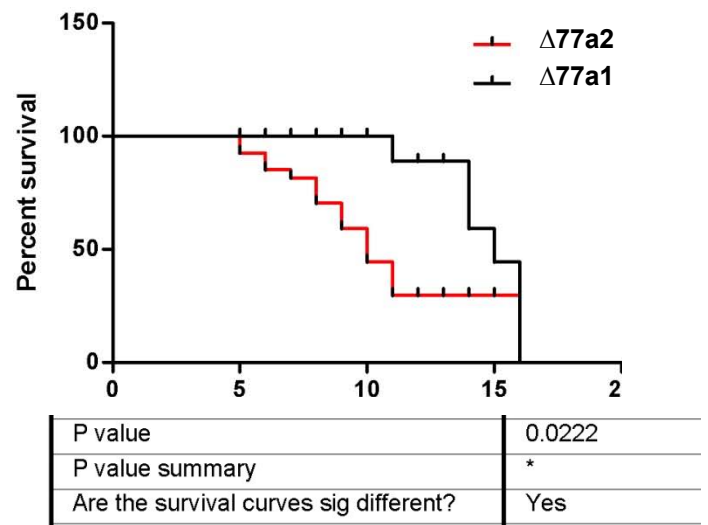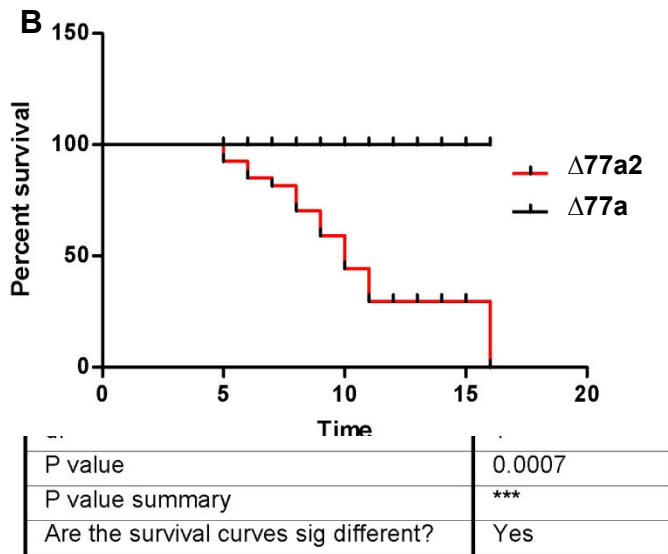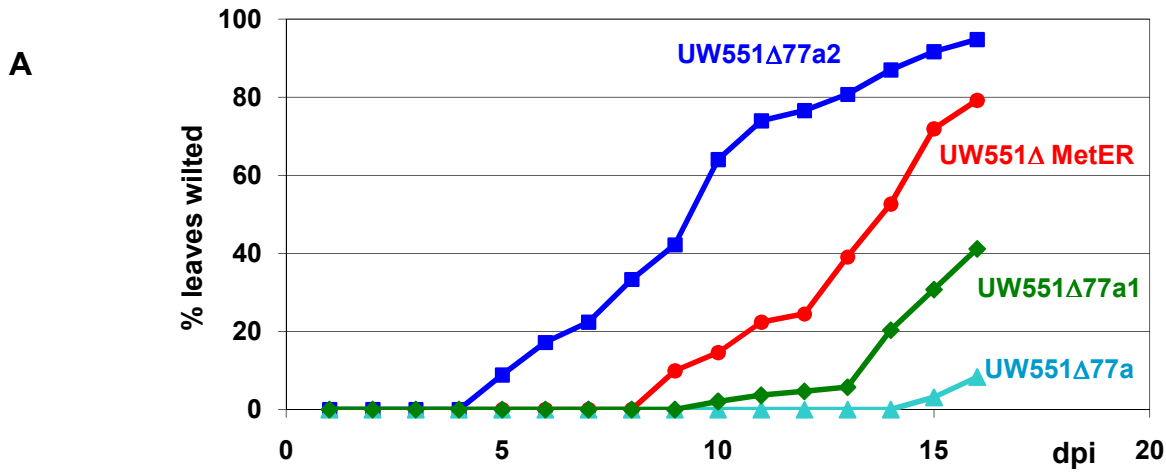
Figure 30. Virulence of different *R. solanacearum* UW551 mutants deleted in the left-hand half region of 77 kb

(A) Percentage of leaves wilted during the time by soaking the soil to a final bacterial population of 1×10^7 CFU/ml per plant. Delayed wilting symptoms were observed after inoculation with UW551 Δ77a and UW551 Δ77a1 UW551mutants UW551Δ*metER* in contrast, the mutant UW551Δ77a2. (B) Comparison of survival curves produced by the treatment of tomato plants with the mutant strains. The statistical analyze was performed using the Log-Rank Test, and the survival curves were considered significantly different with a p-value < 0.05

left-hand half of the deletion. The 'a' region was then subdivided into two parts: a 12kb region from *metE* to RRSL_02276 and a 25kb region from RRSL_02277 to RRSL_02225 (see Figure 22) and the corresponding deletions were engineered into UW551. A comparative analysis of the pathogenicity of these different strains was performed on tomato at low density inoculum (10^7 cfu/ml) and is presented in (Figure 30). Beside the fact that deletion of the *metER* genes has a major impact on pathogenicity, the main conclusions that can be drawn from this comparison are:

- some minor determinants contributing to aggressiveness are located in the a2 25kb region since the strain UW551Δ77a1 produces some wilting symptoms (up to 30% of leaves wilted) whereas UW551Δ77 is scored avirulent in these conditions. This region contains genes of undefined function (including transcriptional regulators, metabolic enzymes and transporters), except for the *hmgABR* genes from the homogentisate pathway that are involved in the degradation of aromatic amino acids.

- wilting symptom production seems also dependent upon additional determinants in the a1 region beside the *metER* genes since the UW551Δ*metER* strain appears more aggressive on tomato plants than the UW551Δ77a1 strain, although statistical differences are at the margin of significance (Figure 30). Apart from the *metER* genes, the 12kb a1 region contains a cluster of 8 genes organized in one (or maybe two) operon(s). Since homologies searches revealed that at least two of these genes are involved in tryptophan metabolism, it was proposed that this locus was involved in the production of an indole-related molecule (Valls et al., 2006). There are three loci in the GMI1000 genome predicted to encode enzymes involved in indole metabolism: a chromosomal locus (RSc2881-RSc2887) predicted to encode the tryptophan biosynthetic pathway, a megaplasmid locus (RSp0693-RSp0698) shown to encode a small diffusible molecule named Hdf and identified as 3-hydroxy-oxindole (Delaspre et al., 2007), and a third locus homologous to the gene cluster present in UW551 region a1. Interestingly, Hdf production is controlled by the T3SS regulator HrpB (Delaspre et al., 2007) and a GMI1000 *hdf* mutant was recently shown to be reduced in fitness in host plants compared to the wild-type (Macho et al., 2010).

The difference in the rate of symptom production between the UW551Δ*metER* and the UW551Δ77a1 strains suggests that the indole/auxin biosynthesis locus adjacent

to the *metER* genes plays a role in pathogenicity. *R. solanacearum* has been shown to produce auxin, *i.e* indole acetic acid (IAA) (Sequeira and Williams, 1964) but this has not yet been established for strain GMI1000. Remarkably, expression of the gene cluster RSp0678-RSp0685, which is homologous to the one in the UW551 a1 region, is controlled by HrpG, similarly to the neighbouring *metER* genes (Valls et al., 2006). It was proposed that this locus could encode an IAA biosynthesis pathway but this has not been experimentally demonstrated.

It is widely recognized that many pathogens may directly or indirectly trigger IAA alterations during initiation of infection and that IAA has a presumed role in creating conditions which are more favorable for multiplication of the pathogen (reviewed in Kazan and Manners, 2009). First, IAA plays a role in lateral root emergence, and also root hair and vascular tissue development. Many soil-borne pathogens such as *R. solanacearum* infect the roots through auxin-rich regions, such as root tips and lateral root initials. Therefore, stimulation of IAA production could be used by the pathogen for entry into the roots and/or movement within the plant. Second, IAA and/or its signaling pathway are also known to modulate plant disease resistance both directly and indirectly (Kazan and Manners, 2009) and pathogens probably exploit this property. For example it is known that IAA produced by *Pseudomonas syringae* pv. *savastanoi* is crucial for the inhibition of plant defences (Robinette and Matthysse, 1990), and in the case of *P. syringae* pv. *tomato* it has been demonstrated that the Type III effector AvrRpt2 increases free IAA levels during infection as a strategy to promote disease susceptibility in host tissue (Chen et al., 2007). The implication of IAA biosynthesis in *R. solanacearum* pathogenicity is not known but the content of IAA has been shown to increase 100-fold in inoculated tobacco plants (Sequeira and Kelman, 1962), although the bacterial or plant origin of this IAA in diseased tissue was not established.

The HrpG-dependent regulation of this gene cluster directing the production of an auxin/indole-related molecule strongly suggests that this locus has a role in *R. solanacearum* pathogenicity or fitness *in planta*. However, a GMI1000ΔRSp0679-RSp0685 mutant is not significantly impaired for pathogenesis on tomato plants following soil-drenching inoculation (M. Valls & S. Genin, unpublished results). It is possible that contribution of this locus to pathogenicity is of secondary importance (as suggested also by the comparative behaviour of the UW551Δ*metER* and UW551Δ77a1 strains on plants) and that our standard pathogenicity assays on whole

plants are not sensitive enough to reveal a minor deficiency. It will therefore more appropriate to use more sensitive methods such as competitive assays in mixed inoculations (Macho et al., 2010) to unravel a possible role of this locus in bacterial fitness *in planta*.

4 Occurrence of genomic deletions in *Ralstonia solanacearum*

R. solanacearum has sometimes been described as a bacterium prone to genetic rearrangements because of its natural competency and its high phenotypic switching versatility. A classical example of such versatility is that under certain growth conditions or prolonged culture on agar plates, *R. solanacearum* spontaneously undergoes a phenotypic conversion, shifting from a mucoid to a nonmucoid colony morphology (Kelman, 1954). It was later found that this phenotypic switching results in fact from various inactivation events targeting a single gene, the central regulator *phcA* (Brumbley et al., 1993; Poussier et al., 2003). Evidence of large deletions in *R. solanacearum* were first documented by Boucher et al. (1986) after isolating Acridine Orange-resistant (Acr[r]) derivatives obtained following growth in presence of the drug. Most Acr[r] mutants were pleiotropic, being characterized by methionine auxotrophy, production of a brown pigment in complete growth medium and avirulence on plants. These Acr[r] mutants were shown to carry large deletions, estimated to 85kb in size or more (Boucher et al., 1986) and further studies have shown the deleted region comprised the *hrp* cluster, thus explaining the avirulent phenotype (Boucher et al., 1988). Methionine auxotrophy of Acr[r] mutants is probably due to the deletion of the *metB* gene, which is distant from ~100kb of the *hrp* gene cluster in GMI1000. It is probable that the deleted region contains a gene conferring sensitivity to Acridine Orange but the mechanism generating these deletions remains unexplained, in particular whether the drug only acts as a selective agent or if it is involved in the induction of the deletion event.

Recently, a comparative genomic study led on two race 3 biovar 2 strains identified in strain KZR-5 (isolated in The Netherlands) a deletion of a 17.6-Kb region, denoted as a putative genomic island PGI-1 (Stevens and van Elsas, 2010). These authors used a suppressive subtractive hybridization approach using tropical potato strain 715 as the comparator. Comparison of the PGI-1 region with similar ones in other *R. solanacearum* sequenced strains provided support for the notion that PGI-1

is a genetically flexible region since it was (1) consistently present in race 3 strains IPO1609 and UW551 and, by inference, 715, and (2) partially and differentially present across the other strains, and it was absent from strain KZR-5. This observation led the authors to hypothesize that the region is a genomic island, as the PGI-1 region was flanked by IS elements in strains 715, IPO1609 and UW551. There is no overlap between the PGI-1 region and the 77kb region deleted in IPO1609. The PGI-1 region contained genes encoding a (p)ppGpp synthetase, a transporter protein, a transcriptional regulator, a cellobiohydrolase, a site-specific integrase/recombinase, a phage-related protein and seven hypothetical proteins. As yet, no phenotype could be assigned to the loss of PGI-1 (Stevens and van Elsas, 2010). Deletion of a 33kb region (RSp0128–RSp0154) flanked by IS elements was also recently reported to occur in strain GMI1000 after serial passage experiments *in planta* but again no phenotype could be associated to this deletion (Marchetti et al., 2010).

5   Biological significance of the 77kb deletion in *R. solanacearum?*

There is little variation at the nucleotide level between the race3 biovar 2 strains IPO1609 and UW551 genomes. This might corroborate the presumed recent spread of R3bv2 strains from a single source. Alternatively, strong selection for a particular genetic make-up may have been required for efficient plant invasion (with the plant acting as the ecological/evolutionary bottleneck). However, as these genome data are based on just two strains, we ignore the true extent of genome diversity in R3bv2. As far as we know, this 77kb deletion was never detected in any other *R. solanacearum* strains (out of 45 strains tested by Comparative Hybridization Studies) or variants, but detection approaches need to be carried out on a larger scale to determine whether this deletion (or rearrangements within the 77kb region) can be found in natural *R. solanacearum* isolates and to estimate how frequent and how widespread among taxonomical groups is its distribution if any.

A comparative analysis of gene content from this 77kb region reveals that it appears widely conserved in the species (Table 8). This observation suggests that the set of genes missing in IPO1609 and determining hypovirulence may not be specific to

R3bv2 strains (and this is established for the metER and indole/auxin biosynthesis genes). We do not have indication that the contribution of these determinants to pathogenicity is variable from one strain/host interaction to another. For example, the impact of a similar deletion on pathogenicity of strain GMI1000 has not been evaluated. However, since disruption of the GMI1000 *metR* gene leads to reduced wilting symptoms, it seems probable that an efficient methionine biosynthetic pathway is required for full pathogenicity among strains of the *R. solanacearum* 'species complex', but it will be interesting to determine if this contribution varies depending on the host plant.

6   How and when the 77kb deletion appeared in strain IPO1609?

Strain IPO1609 was isolated in 1995 from potato in the Netherlands by the Dutch plant protection service. We obtained the strain in 2001 from Dr D. van Elsas which first published the characteristics of this R3bv2 strain, and at the time, IPO1609 aggressiveness consistently resulted in complete wilting of tomato plants in up to two weeks (van Elsas et al., 2000). Recently Stevens et al. (2010) in the same laboratory reported a reduced virulence of IPO1609 on tomato plants, revealed as a slow wilt and symptoms less severe, using high inoculum concentrations, suggesting that the isolate used in this study also contained the deletion. In our hands, IPO1609 was always scored as hypoagressive both on tomato and potato plants under high inoculum concentrations (10^8 cfu/ml), and also following the stem-inoculation method which generally results in rapid symptom production with wild-type strains. Although we do not have clue about when this rearrangement took place or whether a sample of the original isolate still exists at the Dutch plant protection service, it seems very probable that the deletion occurred under laboratory conditions after its isolation. The mechanism that produced the deletion also remains obscure; there are no mobile genetic elements flanking the 77kb region in any of the *R. solanacearum* sequenced strains and no clear evidence of a recombinase-mediated event. Only a six nucleotide direct repeat was identified on each left-side of the DNA break, which could be indicative of an active excision mechanism.

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CONCLUSION AND PERSPECTIVES

Conclusion and Perspectives

R. solanacearum are tropical bacteria adapted to warmer climates, with the exception of a clonal group belonging to phylotype II, sequevar 1 of the *R. solanacearum* species complex and historically known as Race 3 biovar 2 (R3bv2). These R3bv2 strains causes brown rot of potato and bacterial wilt of tomato in tropical highlands and some temperate zones (Denny, 2006). Because of its virulence at relatively cool temperatures, R3bv2 is a quarantine pest in Europe and Canada, and a Select Agent pathogen in the USA (Lambert, 2002).

Much of what is known about *R. solanacearum* comes from studies caused by tropical or warm-temperate strains such as the reference strains K60 and GMI1000. Only very few genetic and molecular studies have been led on R3bv2 *R. solanacearum* strains (Colburn-Clifford et al., 2010; Colburn-Clifford and Allen, 2010). In 2006, the genome sequencing of strain UW551 opened the way to the analysis of the repertoire of candidate pathogenicity factors in this strain (Gabriel et al., 2006). R3bv2 probably originated in the Andes with potato, and isolates from around the world are nearly genetically and phenotypically identical, suggesting that it was distributed from South America in potato tubers (Milling et al., 2009), which probably explains why this group of strains has unique physiological and biochemical properties. For example, these strains differ from all other *R. solanacearum* strains in their adaptation to cold temperatures: R3bv2 cells remain viable up to a year in field soils in the Netherlands at 12°C (Van Elsas et al., 2000). Given the strong relatedness of R3bv2 strains, the aim of this work was to understand the obvious difference in pathogenicity trait between strain UW551 and strain IPO1609 which was recently sequenced in our laboratory (Guidot et al., 2009). Global genomic comparison approaches were limited by the fact that both UW551 and IPO1609 genome sequences are not assembled. However, we were able to identify a region on the megaplasmid replicon that appeared to be specifically absent from strain IPO1609. In this study, we proved that IPO1609 indeed carries a 77kb deletion and we provide genetic evidence that absence of this region is responsible for the hypovirulent phenotype of the strain.

We then focused our study on the identification of the genetic determinants present in the deleted region and responsible for this phenotype since this 77kb

region did not contain genes already reported to play a role in *R. solanacearum* pathogenicity. The results presented in this work indicate that the hypovirulent phenotype of strain IPO1609 involves multiple determinants located within the first 37kb half of the deleted region. We identified herein two loci having a major contribution to IPO1609 virulence: the *metER* genes and a gene cluster putatively involved in the biosynthesis of an indole-related molecule. Comparison of the behaviour of the various deletion mutants on plants also suggests that the 25kb region 'a2' contains one or multiple genes contributing in a more discrete manner to this trait. A transposon mutagenesis of this region could be performed to tentatively identify the(se) determinant(s). Alternatively, functional complementation approaches of UW551Δ77 as a recipient strain using specific sub-regions of pAGA77 could be undertaken. However, since the contribution of such determinant(s) appears relatively minor in pathogenicity assays, it may be difficult to identify mutations or complementing DNA fragments that are predicted to lead to incremental changes in such assays.

Among the two loci identified within the a1 region that impacted wilting symptom production, our data indicate that the putative indole/auxin biosynthetic cluster may have a significant impact on virulence although no mutant directly targeting this locus was tested in this work. The function of this gene cluster remains hypothetical and a direct outcome of this work will be to investigate the function of this locus, especially in determining if it is involved in the biosynthesis of auxin (a phytohormone known to be produced by some *R. solanacearum* strains) or another indole-derived molecule. Although a deletion mutant in GMI1000 was not significantly impaired in pathogenicity, it will be interesting to evaluate the behaviour of this mutant strain on other hosts than tomato and by using more sensitive techniques than pathogenicity assays such as the determination of the *in vivo* competitive index after mixed infection with the wild-type strain (Macho et al., 2010).

The locus that explained most of the hypovirulent phenotype on plants of strain IPO1609 is the *metER* operon. Although some *R. solanacearum* Met auxotrophic mutants were known to be avirulent (Coplin et al., 1974), the Δ*metER* mutant strain displays an original phenotype since it is altered in pathogenicity but is not auxotrophic for methionine, and, consequently, is not (or weakly) affected for growth *in planta*. As soon as it enters in the host root tissue, *R. solanacearum* has to face hostile environmental conditions due to plant defense reactions and to limited

nutritional resources in the plant apoplasm. In spite of these hostile conditions, the bacterium has to deploy energy to multiply efficiently and especially to set up the necessary elements (such as the T3SS) for the success of the infectious process. This implies that the pathogen has to be metabolically proficient and to rely on a regulation system to coordinate the expression of its various pathogenicity determinants according to the environmental stimuli. A key regulator of this regulatory system is HrpG, which directly or indirectly controls the transcriptional induction of more than 350 genes in presence of plant cells, including those directing the synthesis of the T3SS and effector substrates (Valls et al., 2006). In a separate study led in the laboratory, it was established that 1/ the *metER* genes belong to the HrpG regulon, 2/ the *metR* regulatory gene is a direct target of HrpG, 3/ expression of *metE* is strongly and specifically induced *in planta* in a *hrpG* and *metR*-dependent manner, and 4/ the *metE* and *metR* genes are each required for full pathogenicity of strain GMI1000 (Plener, 2010). Altogether, these studies provide the first direct link between metabolism and pathogenicity in *R. solanacearum*, revealing that methionine metabolism plays a crucial role during the infectious process, and that this role goes probably beyond a direct contribution to efficient bacterial growth *in vivo*. Moreover, it shows that this trait is important for pathogenicity of both R3bv2 or tropical strains. Future experiments aimed at investigating for example whether ethylene synthesis is impaired in the *metER* mutant or if global protein synthesis is significantly reduced will possibly bring clues about the contribution of these genes to pathogenesis. The finding that the pathogen preferentially induces *metE* expression rather than *metH* in the plant also provides suspicion for a probable metabolic adaptation to physiological host conditions since this induction of *metE*, the B12-independent methionine synthase, occurs in an environment in which B12, the required co-factor for MetH, is absent.

Finally, it is noteworthy that the two aforementioned loci that were identified in the a1 region are both under the control of the HrpG regulator. Global transcriptomic analyses revealed that in addition to pathogenicity determinants already characterized HrpG controls several metabolic enzymes or transmembrane transporters of unknown function, and many hypothetical proteins (Valls et al., 2006; Genin, 2010). Implication in pathogenicity/host adaptation of these determinants has not yet been demonstrated but they are presumably 'guilty by association' on the basis of apparent co-regulation. The involvement of the *metER* and RRSL_02270-

RRSL_02276 genes in wilting symptom production also reinforces this view. Several approaches are actually undertaken in the laboratory in order to functionally characterize the large set of *hrpG* targets that may determine the success of the infectious path: 1/ the HrpG regulon will be soon more accurately defined following a high-depth RNA sequencing approach led on strain GMI1000 since exploitable transcriptomic data are still not available for a certain number of genes, 2/ a random transposon library has been generated in GMI1000 and each insertion border has been sequenced, so the characterization of any non-essential genes will be facilitated, in particular for screening the set of HrpG regulatory targets, and 3/ each of the ~20 transcription regulators that are controlled by HrpG will be disrupted in order to identify the downstream targets and therefore provide a better picture of the regulatory network with intermediate regulatory nodes.

The characterization of the 77kb deletion in strain IPO1609 did not provide clues about the significance of this event in the biology of this particular strain. Seven *R. solanacearum* genomic sequences are actually available but one can undoubtedly predict that this number will significantly increase in the future years, and this will better cover the biodiversity of the strains composing this 'species complex' but also refine our current vision of infraspecific variability. At that time, it will therefore be interesting to investigate whether the 77kb deletion or related genomic alterations in the same region can be found in other strains, and more generally, to establish an exhaustive list of these deletions/rearrangements in this group of highly related R3bv2 strains. Such approaches could in some cases unravel specific traits governing adaptation of these strains, as recently revealed in another study (Stevens and van Elsas, 2010) but will also provide important markers for a more mechanistic understanding of the evolutionary dynamics of these strains.

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AUTHOR: Adriana GONZALEZ

TITRE: Functional characterization of a large genomic deletion resulting in loss of virulence in */Ralstonia solanacearum/* race3 biovar2 strains

PhD SUPERVISOR: Stephane GENIN

ABSTRACT

Ralstonia solanacearum is one of the most devastating plant pathogen worldwide. Through a comparative genomic analysis between *R. solanacearum* 'race3 biovar 2' strains, the causal agent of potato brown rot, we identified a 77kb region which is absent in the hypovirulent strain IPO1609. We proved that IPO1609 indeed carries a 77kb genomic deletion and we provide genetic evidence that presence of this deletion is responsible for almost complete loss of pathogenicity of this strain. We identified two loci having a major contribution to IPO1609 pathogenesis: the methionine biosynthesis genes *metER* and a gene cluster putatively involved in the biosynthesis of the phytohormone auxin. Expression of *metER* is controlled by the pathogencity regulator HrpG, and the finding that a *metER* mutant is not auxotrophic but is significantly reduced in virulence provides the first direct link between methionine metabolism and pathogenicity in *R. solanacearum*.

KEYWORDS: *Ralstonia solanacearum*, race3 biovar2, pathogenicity, genomic deletion, methionine, auxin.

DISCIPLINE: Microbiology

LABORATORY: Laboratoire des Interactions Plantes Micro-organismes

UMR CNRS-INRA 2594/441

24 Chemin de Borde-Rouge BP52326

31326 Castanet Tolosan cedex, France

AUTEUR: Adriana GONZALEZ

TITRE: Caractérisation fonctionnelle d'une délétion génomique entraînant une perte de virulence chez les souches 'race3 biovar2' de la bactérie phytopathogène *Ralstonia solanacearum*

DIRECTEUR DE THÈSE: Stephane GENIN

RÉSUMÉ

Les souches de *Ralstonia solanacearum* dites 'race 3 biovar 2' sont les agents responsables de la maladie de la pourriture noire de la pomme de terre, une maladie dévastatrice dans les régions intertropicales et émergente en Europe. Par une analyse de génomique comparative, nous avons identifié chez la souche IPO1609 une délétion génomique de 77 kilobases entrainant une perte quasi-complète de virulence chez la souche IPO1609. La caractérisation fonctionnelle de cette région deletée a permis d'identifier deux loci majeurs contribuant à la virulence de la souche IPO1609: deux gènes de la voie de biosynthèse de la méthionine, *metER*, et des gènes vraisemblablement impliqués dans la biosynthèse d'auxine. L'expression des gènes *metER* est dépendante du régulateur central de la pathogénie HrpG. Un mutant *metER* n'est pas auxotrophe mais est significativement atténué en virulence, démontrant ainsi un premier lien direct entre le métabolisme de la méthionine et la pathogénie de *R. solanacearum*.

MOTS-CLÉS: *Ralstonia solanacearum*, race3 biovar2, pouvoir pathogene, deletion genomique, méthionine, auxine.

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LABORATOIRE: Laboratoire des Interactions Plantes Micro-organismes

UMR CNRS-INRA 2594/441

24 Chemin de Borde-Rouge BP52326

31326 Castanet Tolosan cedex, France