Adaptation of Ralstonia solanacearum biovar 2 to temperate climates

Stevens, Patricia

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Patricia Stevens 2010

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Centre for Ecological and Evolutionairy studies
University of Groningen, PO BOX 14
9750 AA Haren, The Netherlands

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Adaptation of *Ralstonia solanacearum* biovar 2 to temperate climates

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Kiek noar die zulf!

Woarom kikst noar mie?
Omdat ik aans bin as doe?
Omdat ik aans denk as doe?
Omdat ik aans dou as doe?
Mag dat den nait?
Mout ik den aaltied doun
wat n aander dut?
wat n aander denkt?
wat n aander goud vindt?
Den bin ik miezulf nait meer
mor n aander!

Auteur:
Nies Sap-Akkerman

Voor mama

CONTENTS

List of abbreviations

3-OH-PAME - 3-OH palmitic acid methyl ester

ACUR - Alternative Codon Usage Region

AFLP - Amplified Fragment Length Polymorphism

CFU - Colony Forming Unit

CWDE - Cell-Wall-Degrading Enzyme

EPS - Extracellular Polysaccharide

GEI - Genomic Island

HGT - Horizontal Gene Transfer

HR - Hypersensitive Response

Hrp - Hypersensitive response and pathogenicity

IS - Insertion Sequence

ITS - Intergenic Spacer

MLST - Multi Locus Sequence Typing

ORF- Open Reading Frame

PAI - Pathogenicity Island

PC - Phenotypic Conversion

PFGE - Pulsed Field Gel Electrophoresis

PIP- Plant-Inducible Promoter

Rep PCR - Repetitive element polymorphism PCR

REP sequences - Repetitive Extragenic Palindromic sequences

RFLP - Restriction Fragment Length Polymorphism

Rhs element - Recombinational hot spot element

T2SS - Type II Secretion System

T3SS - Type III Secretion System

VBNC - viable-but non-culturable

Vgr- Valine-glycine dipeptide repetition

Chapter I

General introduction

Ralstonia solanacearum biovar 2 behavior and genomics

General introduction

Bacterial wilt, caused by members of the *Ralstonia solanacearum* species complex, is a key emergent disease in non-tropical regions of the world. *R. solanacearum* is distributed in many habitats all over the world and has an unusually broad host range (Hayward 1991, Denny 2006). It can infect over 200 plant species representing over 50 botanical families (Hayward 1991). Due to its global distribution, adaptive potential and large host range, *R. solanacearum* has turned into a model system to study plant-microbe interactions, pathogenicity determinants and pathogen ecological behavior. Given the fact that *R. solanacearum* is a soil-borne pathogen and that resistance of the host is limited, bacterial wilt is very difficult to control (Hayward 1991, Saddler 2005). Historically, this disease has occurred mainly in tropical and warm regions of the world. However, one subgroup, i.e biovar (bv) 2, appears to be relatively adapted to lower temperatures. The strains belonging to this subgroup are also known as potato strains, as they often cause disease in potato. These *R. solanacearum* strains, hereafter denoted as bv2, may have originated in the highland tropics of the Andes and grow optimally at lower temperatures than other *R. solanacearum* strains. They probably have spread incidentally from their regions of origin to different regions of the world. They have been isolated from several disparate regions (Figure 1), most of which are or have been used for potato cropping.

R. solanacearum bv2 has thus been detected in the highlands of the tropics as well as in several (sub) tropical and some temperate areas throughout the world. It is notably sparse in North America. In the European Union, the pathogen has a quarantine status (Anonymous 2000) and it is on the list of potential bioterrorism agents in the USA (Lambert 2002). In the Netherlands, outbreaks of bacterial wilt in potato production fields have been reported in the mid 1990-ies (Janse 1998). The outbreaks with greatest impacts occurred between 1995 and 1998. Currently, all seed potato lots are tested every year, which has resulted in a reduction of potato brown rot cases to less than <0.01% (Breukers et al. 2005).

The control strategies used have prevented direct yield losses (infected crops/fields) as well as indirect losses that result from restrictions for future production on infested fields (Elphinstone 2005).

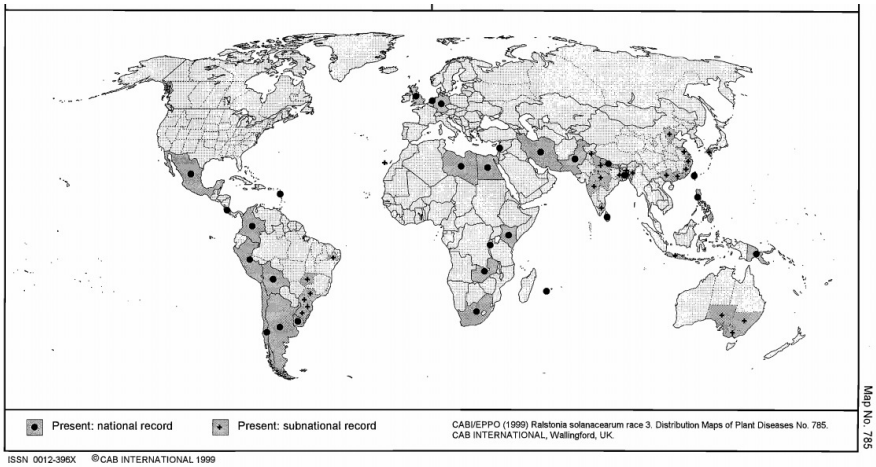

Figure 1. Distribution map of *R. solanacearum* bv2 that causes brown rot of potato. Records are of confirmed or assumed bv2 strains. The map is of 1999 and is based on data from the CAB abstracts database and plant quarantine information compiled by EPPO.

Early studies on the survival of *R. solanacearum* bv2 in field soils, ditches and canals have been published by van Elsas et al. (2000, 2001). From these studies, the soil- and water-borne nature of *R. solanacearum* bv2 in the Netherlands and the extent of its persistence in these open environments, have become very clear. The occurrence of *R. solanacearum* bv2 in infested field soils and surface waters clearly poses a constant threat to local potato production areas. *R. solanacearum* can persist in high numbers in sediments in surface waters (van Elsas et al 2001) and from there the organism may migrate to other sites via local waterways. Moreover, *R. solanacearum* bv2 has not only been found in association with potato, but also with bittersweet (*Solanum dulcamara*) plants (Elphinstone et al. 1998; Janse 1998). These bittersweet plants are believed to provide shelter sites (refuges) during winter periods, from which the bacteria may be released into the bulk water when temperatures rise again in spring/summer. A depiction of a typical Dutch local habitat that may still be posing a threat to potato production almost 15 years after the outbreaks in the mid nine-ties is shown in Figure 2.

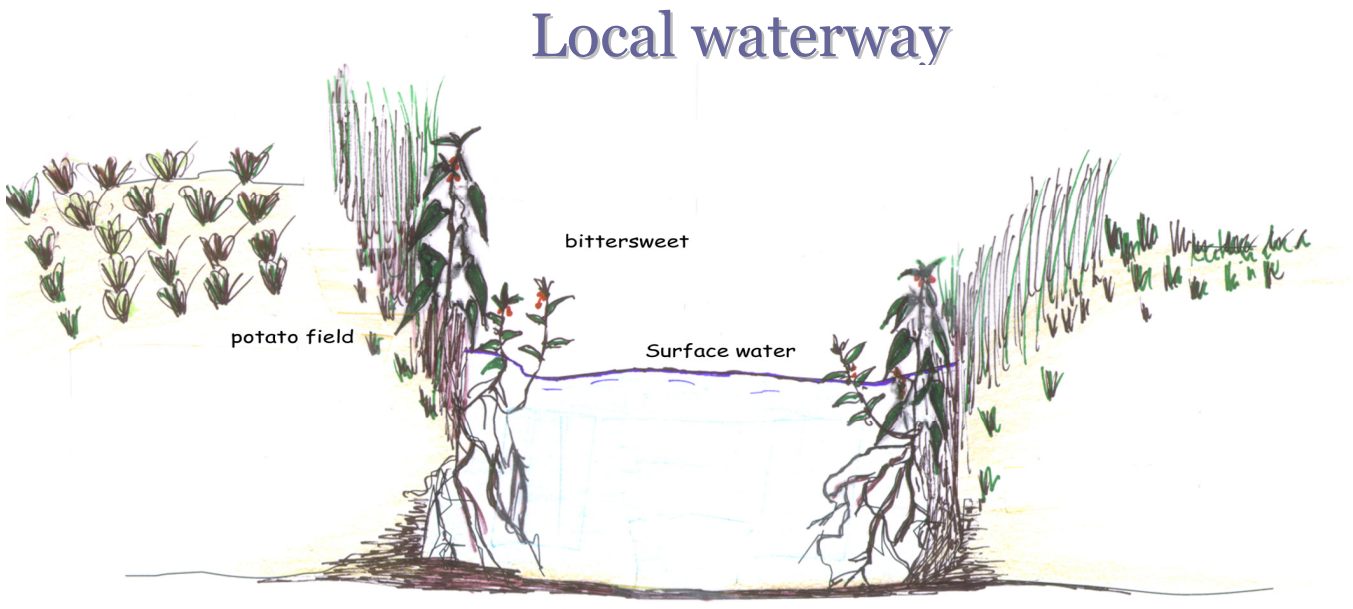

Figure 2. A sketch of a typical Dutch local waterway as it is found in the Northern part of the Netherlands. Potato production fields can be found in close vicinity of waterways where the potato brown rot bacterium, *R. solanacearum* bv2, is present. Bittersweet plants grow with their roots and lower stem parts in the water/sediment. These plants, and potentially other host(s), may provide shelter sites for the bacterium and to contribute significantly to its survival and persistence, especially during stressful conditions such as prolonged low temperatures. When temperature rises in spring and summer, bacterial cells may be shed from the host plants into the surrounding sediment and water, leading to increased population sizes. Farmers that produce potato in these so called "high-risk areas" need to follow strict regulations in order to prevent local losses and, more importantly, spread to other potato lots and production fields.

Also, animals might be responsible for the (re-)introduction of *R. solanacearum* cells into local waterways. Birds, for instance, that feed on the potato production fields or nearby infected

soils, can simply transfer contaminated soil into the water providing new opportunities for the bacteria to colonize additional ecological niches. Also, latently-infected weeds, such as black nightshade (*Solanum nigrum*), growing in or near agricultural fields, can constitute sources of infection (Álvarez et al. 2008b, van Overbeek, pers.comm.), which may further propagate and disseminate the organism. In particular, bittersweet plants growing at riverbanks play important roles in the accumulation of the pathogen and infestation of surface waters. For example, in Sweden, *R. solanacearum* was eradicated from local waterways by removal of infested bittersweet plants for 5 years up to a 30 km distance from the initial source (Persson 1998), but follow-up reports on this strategy are lacking. However, the initial success indeed strengthens the contention that bittersweet plants are key catalysts of the persistence of the organism in colder regions. The relevance of understanding and controlling the ecology and spread of the bacterium has become increasingly clear when *R. solanacearum* bv2 was, unexpectedly, found in geranium (*Pelargonium x hortorum*) imported into the USA. In fact, the isolates obtained from these plants were able to cause bacterial wilt in potato (Williamson et al. 2002). This finding had serious consequences for the production of ornamental plant cuttings produced in areas where *R. solanacearum* bv2 is endemic. Thus, solanaceous plants are not the only hosts for *R. solanacearum* bv2 and transfers from ornamental or wild plant species to potato should be considered to present realistic threats (Álvarez et al 2008b). For instance, a common weed such as stinging nettle (*Urtica dioica*) could also serve as a host, although no infested plants were found in the open environment (Wenneker et al.1999). With the example of imported contaminated geranium cuttings into the US, it is clear that bacterial wilt can not only cause significant losses on a local scale, but also plays a significant (negative) role in worldwide agriculture.

By and large, it is clear that *R. solanacearum* bv2 has a variety of natural refuges in terrestrial and aquatic habitats, in which it survives or even propagates. It is thus important that we enhance our understanding of the survival and adaptation of the organism and develop strategies that can mitigate its chances of causing disease in susceptible plants.

The *Ralstonia solanacearum* species complex

R. solanacearum (Smith 1896) Yabuuchi et al. 1995 (formerly called *Pseudomonas solanacearum*) is a Gram-negative, rod-shaped, aerobic bacterium that belongs to the β-proteobacteria. The species *R. solanacearum* is highly polymorphic, as it is composed of strains with varying biochemical properties which can infest a large spectrum of possible plant hosts. The high genetic as well as phenotypic variation between strains has incited investigators to define *R. solanacearum* as a species complex (Gillings & Fahy 1994, Fegan & Prior 2005). Based on DNA:DNA hybridizations, *R. solanacearum* is closely related to *R. pickettii* (Ralston et al. 1973), *R. syzygii* (Roberts et al. 1990) and the so-called blood disease bacterium (BDB - also known as *P. celebensis* or *R. solanacearum* race 2). *R. pickettii* is an uncommon plant pathogen of relatively low virulence which differs from *R. solanacearum* in pathogenesis and ecology, although there are also similarities in their phenotypic properties (Hayward 1991). *R. syzygii* causes the so-called Sumatra disease on clove (*Syzygium aromaticum*) and is, based on DNA:DNA hybridization and homology, related to *R. solanacearum*. However, it is clearly distinct in cultivation and physiological properties (Hayward

1991, Villa et al. 2005). The BDB appears to be solely restricted to banana and so far has only been found in Indonesia and the surrounding islands (Supriadi 2005).

All *R. solanacearum* strains have traditionally been classified into five races (loosely based on host range) and six biovars (based on the ability to acidify a panel of 5 to 8 carbohydrate substrates (Buddenhagen et al. 1962). Restriction fragment length polymorphism (RFLP) analysis (Cook et al. 1989; Cook & Sequira 1994) showed that the species can be divided in two major divisions, i.e. division 1, primarily encompassing isolates from Asia (biovars 3, 4 and 5), and division 2, which contains strains primarily isolated from the Americas (biovars 1, 2 and N2). Using 16S rRNA gene sequence analysis, a further separation within division 2 was found to exist, and this comprised strains from Indonesia and the closely-related BDB and *R. syzygii* (Taghavi et al. 1996). In addition, phylogenetic analysis of the endoglucanase and *hrpB* genes revealed a fourth group of strains, which all originated from Africa (Poussier et al. 1999, 2000). The clear separation of all *R. solanacearum* strains into two main clusters indicated an ancient divergence, after which the different populations evolved further, resulting in numerous different types (or groups) of strains showing different phenotypes with respect to their host ranges and preferred ecological niches.

Recently, the phenotypic and genotypic variation of the species was captured in an all-encompassing phylotyping scheme, defining phylotypes I through IV. This novel scheme correlates with the geographical origin of strains, rather than with the phenotypic properties that determine biovars (or races) (Fegan and Prior, 2005). In the scheme, the clustering of strains was based on the sequences of four marker genes, i.e. the 16S-23S rRNA gene intergenic spacer (ITS) region, *mutS*, *egl* and hrpB sequences as well as amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP) data (Fegan et al. 1998, Poussier et al. 2000, Fegan and Prior 2005). Based on the 16S-23S ITS region, a single multiplex PCR to determine phylotype was developed (Fegan and Prior 2005). Thus, phylotypes I and II correspond to divisions 1 and 2, respectively (Cook & Sequira 1994). Phylotype III contained strains from Africa and islands in the Indian Ocean and phylotype IV those from Indonesia, Japan and Australia (it also included the BDB bacterium and *R. syzygii)*. Further discrimination of strains within each phylotype was obtained from the sequence of the *egl* gene (defining so-called sequevars), as this region showed sufficient diversity. Finally, clonal lines within a sequevar may be identified using genomic fingerprinting methods such pulsed field gel electrophoresis (PFGE), AFLP or repetitive element polymorphism (rep)-PCR (Fegan & Prior 2005).

The robustness of the phylotyping scheme was recently confirmed by Castillo & Greenberg (2007), who applied multilocus sequence typing (MLST) on 58 strains representing the four phylotypes. These authors also showed a further subdivision of phylotype II into two subgroups, denoted IIa and IIb. *R. solanacearum* bv2 strains all clustered in phylotype IIa, which was actually exclusively composed of bv2 strains. As evidenced from these and other studies, the diversity of *R. solanacearum* bv2 strains has been thought of to be low compared to that in the other clusters in the species complex (van der Wolf et al. 1998, Poussier et al. 1999, 2000, Castillo & Greenberg 2007). The explanation sought was that, because *R. solanacearum* bv2 is of tropical origin and is supposedly non-endemic in Europe, the isolates found in European agricultural systems all may have originated from similar ancestral sources, which are near-clonal.

The core genome, which determines the basic and essential factors that define the species, and the distribution of variable (accessory) genes among different members of the *R. solanacearum*

species complex was recently studied by Guidot et al. (2007), who used comparative genomic hybridization on a large set of strains representing all four phylotypes. Hierarchical clustering based on the distribution of the variable genes was found to be consistent with phylotype classification, indicating that the variable gene content largely determines the actual genotype – and derived phenotype - of strains and thus the classification within a given phylotype. A large pool of variable genes appears to be present within the *R. solanacearum* species complex. The core genome was suggested to cover approximately 53% of the total genome of the bv3 strain GMI1000 (Guidot et al. 2007). This strengthens the notion that the *R. solanacearum* species complex is highly diverse and versatile. This has, over long-term evolution, resulted in phylotype-/strain-specific properties (variable genes), while retaining the basic core functions necessary for cell metabolism, interaction with plants and the induction of wilt in susceptible host plants. Thus, many basic functions may have remained conserved between the different members of the complex species. The relatively low number of genes that make up the *R. solanacearum* core genome correlates with the observation that DNA:DNA hybridization values between strains can be low, even below 70% (Palleroni & Doudoroff 1971). This is at the border of what makes a species coherent. Given this observation, I predict that it may not take very long before a proposal to split the *R. solanacearum* species complex into two or more species will emerge.

Bacterial genomes and evolution

The composition of bacterial genomes can change rapidly and dramatically through different genetic processes. These include the frequent occurrence of mutations (insertions, deletions and point mutations), recombinations/gene silencing events, gene duplications and horizontal gene transfer (HGT). Such changes may take place under either neutral or diversifying selection, thereby increasing diversity, or under directional selection (selective sweep mechanisms), which leads to fixation of particular changes within the population. Moreover, the level of selection may differ between the organism's flexible and core genes, depending on the final function of the gene product. Furthermore, the asymmetric manner by which bacterial chromosomes replicate (with a leading and a lagging strand), may have incurred different mutational rates for the two strands (Lobry & Sueoka 2002). In fact, in many bacterial genomes, genes near the terminus of replication tend to have a lower G+C content and show higher rates of evolution (Daubin & Perriere 2003). The replication terminus of the chromosome of strain GMI1000 indeed showed a lower average G+C content compared to the replication origin (Salanoubat et al. 2002). In addition, it has become very clear that HGT coupled to recombination plays a crucial role in shaping bacterial genomes (Gogarten & Townsend 2005, Darling et al. 2008). Genome reduction (loss of genes or islands) may also contribute to increased fitness, which is most evident in organisms that are host-dependent. Free-living organisms, however, can also be subjected to genome reductions, especially if they underwent a recent change of niche (Thomson et al. 2003). In recent years, the rapidly increasing availability of complete genome sequences, combined with comparative genomics, has revealed an unexpected degree of diversity among prokaryotic genomes, even within species. This has dramatically changed our view of the genome evolution of prokaryotes, highlighting HGT as a major driver in it.

Horizontal gene transfer (HGT)

The acquisition of new genes via HGT can be mediated by three mechanisms: conjugation, transduction and transformation. Conjugation involves the transfer of DNA directly from one organism to another one through cell-to-cell contact. In transduction, the DNA is transferred by the action of bacteriophages. Transformation is a process by which a competent cell takes up external DNA and incorporates this into its genome. With the increasing numbers of bacterial and archaeal genomes available, the major role of HGT in bacterial evolution has been firmly established. The finding of many genes that enhance bacterial fitness, on so-called "genomic islands" (GEIs), pays tribute to such past HGT events. A major asset of HGT is that a large number of genes (which may confer complete adaptive traits) can be transmitted all-at-once to the genome of a recipient organism. This may enhance the recipient's fitness in a specific ecological niche, leading to "quantum leaps" in evolution. GEIs often carry genes that enhance the virulence of pathogens, and are thus known as pathogenicity islands (PAIs). These PAIs are often absent in closely-related non-pathogenic bacteria. Some GEIs exhibit features of integrative and conjugative elements, which are transferred via conjugation and integrated into the host genome by site-specific recombination (Burrus et al. 2002). In general, GEIs may have evolved from lysogenic bacteriophages and self-replicating plasmids by site-specific chromosomal integration, followed by rearrangements and/or losses of the genes necessary for mobilization, thereby facilitating fixation in the chromosome. GEIs may further evolve by acquisition of extra insertion sequence elements or transposons. When relatively recently acquired, GEIs often have G+C contents that differ from that of the genome backbone. They are often associated with tRNA genes and flanked by repeat sequences. In addition, they frequently carry so-called "mobility" genes that encode integrases or transposases (Hacker & Kaper 2000). GEIs can, next to pathogenicity traits, carry a range of accessory functions, notably resistances to antibiotics or heavy metals, the capacity to degrade xenobiotic compounds, to fix nitrogen or to efficiently acquire iron. Depending on the functions they encode, GEIs may be called pathogenicity, symbiosis, saprophytic, fitness, metabolic or resistance islands (Hacker & Carniel 2001). The occurrence of GEIs in pathogenic as well as in non-pathogenic strains suggests that the genes they carry are likely to be involved in general adaptation, fitness and competition.

Sites in the environment where HGT is thought to occur at elevated frequencies are those where large numbers of diverse bacterial species are present as a result of active growth and consequent cell-to-cell contacts (van Elsas & Bailey 2002). In soil, these sites, also called HGT hotspots, include relatively nutrient-rich regions like the plant rhizosphere and phyllosphere, as well as soil surfaces such as clay complexes. Water flow caused by the presence of plant roots may also enhance bacterial movement and growth, thereby increasing the chances of cell-to-cell contact. In aquatic habitats, the distribution of bacterial populations is not evenly distributed as well. The bulk water is often poor in nutrients and, consequently, may contain few bacterial cells. On the other hand, the presence of nutrient-enriched sediment particles, stone surfaces or other (suspended) particles supports growth of bacterial communities that occur mainly in biofilms and microcolonies (van Elsas et al. 2000a). Because bacterial cells form such structured communities (next to being free-living in the bulk water phase), aquatic habitats also appear to provide important sites for cell-to-cell contacts (Hill et al. 1994, van Elsas et al. 2000a).

In addition, bacterial characteristics (e.g physiological status, existing regulatory networks) also influence the local rates of HGT, and so do local biotic as well as abiotic factors. Given the fact

that these factors all contribute to the constantly changing local environment and that the microbial communities also may vary in space and time, it is impossible to predict to what extent HGT actually occurs in the natural environment.

R. solanacearum can become naturally competent and thus integrate external DNA into its genome (Bertolla et al.1999, Guidot et al. 2009a). However, the conditions required in the open environment for *R. solanacearum* to induce competence have not yet been studied. Moreover, mechanisms controlling the uptake and integration of DNA into the host genome may be inhibited by mismatch repair proteins (Mercier et al. 2007). Nevertheless, HGT appears to have been a major factor in shaping the genome of *R. solanacearum* over evolutionary time, as the genome contains multiple islands, such as, for instance, phage remnants next to insertion sequence (IS) elements (Salanoubat et al. 2002). As the organism inhabits plants, soil and aquatic HGT hot spots, transfers may predominantly have occurred in such sites.

Insertion sequence (IS) elements

Transposable elements, i.e IS elements and transposons, are widespread in virtually all organisms. They are important in genome flexibility and participate in a range of genetic events, including genome rearrangements, insertions, deletions and duplications. Many IS elements can activate the expression of neighboring genes (Mahillon & Chandler 1998). IS elements consist, in their simplest forms, of a transposase/recombinase required for transposition. At their ends, they usually carry inverted repeat sequences of 10-40 bp, which are required for transposition. Most IS elements generate short duplications of fixed sizes (between 2-13 bp) upon insertion (Argos et al. 1986). The transposases of many IS families harbor a conserved amino acid motif, DD-E, as part of the catalytic site (Mahillon & Chandler, 1998). The mechanisms involved in transposition have been studied in depth. Generally, the process can be divided into several defined steps, which include (1) binding of the recombinase to the repeat ends in the DNA, (2) elaboration of a synaptic complex involving the recombinase, possibly accessory proteins and both transposon ends, (3) cleavage and strand transfer of the transposon ends into the target and (4) processing of the strand transfer complex to a final product.

Based on the structures and mechanisms of transposition, all IS elements have been classified into, at least, 19 families (Mahillon & Chandler, 1998). The IS*3* and IS*5* families are the most abundant in prokaryotic genomes and will be discussed briefly.

IS3 family - The members of the IS*3* family are very similar in many aspects, covering a large group of IS elements. They have been found in more than 40 Gram-negative as well as Gram-positive bacterial species. They generally have two partially overlapping reading frames, *orfA* and *orfB*. The OrfB protein is similar to retroviral integrases, as they carry a DD-E motif which is responsible for catalytic activity (Fayet et al. 1990). The target recognition capacity is usually located in the OrfA protein, which is thought to provide sequence-specific binding to the DNA (Rousseau et al. 2004). Sometimes, a fusion protein, OrfAB, is generated by programmed translational frameshifting (usually a -1 frameshift), which might promote cleavage and integration of the element.

IS5 family - The IS*5* family represents a relatively heterogeneous group of IS elements and even includes sequences from members of the *Archaea*. The majority of the elements of this family carry a single ORF, with lengths ranging from 850 to 1643 bp. The main feature defining this group is the similarity of their putative transposases (Rezsohazy et al. 1993).

The selection of target sites by transposable elements is generally poorly understood. In fact, most IS elements display little target site selectivity. However, a striking preference for short repetitive extragenic palindromic (REP) sequences, which are widely distributed in bacterial genomes, has been shown for particular IS elements of *Pseudomonas syringae*, *P. putida* and *Sinorhizobium meliloti* (Tobes & Pareja 2006). In these cases (involving IS*3*, IS*4* and IS*110* family IS elements), there was a strict association ratio with specific REP sequences, which clearly demonstrated site-specific selection to be involved in the respective transposition events.

Recombinational hot spot (Rhs) elements

Rhs (recombinational hot spot) elements represent large sequence repetitions in *E. coli* which may serve as hot spots for recombinational or integrative events. They comprise three subfamilies (Wang et al. 1998). The genomes of some *Escherichia coli* strains contain up to eight Rhs elements, but these elements may be absent from other strains (Hill et al.1995). The conditions that spur *Rhs* gene expression are still unclear. However, the Rhs elements clearly provide advantages in some specific ecological niches, for instance by providing reservoirs of sequence repetition and, consequently, opportunities for genome rearrangements (Lin et al. 1984, Hill 1999).

Rhs elements contain 3.7-Kb conserved *Rhs* core sequences, small (0.4-0.6Kb) variable downstream core extensions (dsORF) and core extensions encoding a unique ORF. Interestingly, *Rhs* core sequences have average G+C contents of 62%, while those of core extensions and their linked ORFs may be as low as <40% (for comparison, the *E. coli* genome G+C % is ~51%). Moreover, the genetic composition of Rhs elements and the abrupt changes in G+C content from the core to the core extension are conserved in several species beyond *E. coli* (Hill 1999). Another component of some Rhs elements is the region called Vgr (stands for a valine-glycine dipeptide repetition). Together with the Rhs core and the dsORF, this element is expressed from a single promoter (Hill 1999). The recombination frequency between different *Rhs* elements can be high (e.g. 2×10^{-4}), especially when two cores belong to the same subfamily (Lin et al. 1984). Sequences homologous to the Rhs cores of *E. coli* have been found to exist in the genomes of diverse species, including *R. solanacearum* (specifically, in strain GMI1000), indicating their importance in the shaping of these genomes.

The *Ralstonia solanacearum* genome

The broad-host-range *R. solanacearum* bv3 strain GMI1000 was one of the first phytopathogenic bacteria to have its complete genome sequenced (Salanoubat et al. 2002). In addition, the draft genome sequences of two *R. solanacearum* bv2 strains 1609 and UW551, as well as that of the banana bv1 strain Molk2, have been determined. Comparison of the different *R. solanacearum* genome sequences has already increased our knowledge about the genetics underlying the phenotypic differences between strains. For instance, a few protein-encoding genes appear to be present in the GMI1000 genome, that are lacking in UW551. Some of these probably explain the

difference in the ability to use three sugar alcohols as sole carbon sources (Gabriel et al. 2006). The overall gene organization on the genome was found to be 71% syntenic between these strains. Moreover, the gene clusters encoding type II secretion, flagellar assembly, type IV pili and extracellular polysaccharide (EPS) turned out to be conserved and organized similarly in GMI1000 and UW551 (Gabriel et al. 2006, Guidot et al. 2007). Both strains were found to possess the capacity to encode multiple (type IV) pili/fimbrae-coding systems and a large number of predicted adhesion functions. The latter showed similarity to haemagglutinin-type proteins, which serve as adhesins in other bacterial pathogens (Salanoubat et al. 2002). A subset, i.e. 402, of the 4,454 protein-encoding ORFs (<10%) of strain UW551 was absent, or divergent, in strain GMI1000.

The 5.8-Mb *R. solanacearum* bv3 genome was found to be organized into two circular replicons of 3.7 Mb and 2.1 Mb, respectively. The larger replicon, also denoted the chromosome, shows characteristics of a chromosomal origin of replication and encodes all the basic mechanisms required for cell functioning. The smaller replicon, showing features of plasmid-borne *ori* loci, is often referred to as the megaplasmid (Salanoubat et al. 2002). It carries the *hrp*(hypersensitive response and pathogenicity) genes, flagellum biosynthesis genes and most genes of the exopolysaccaride pathway, which suggests it has a significant function in the overall fitness of the bacterium, especially during pathogenesis. The average G+C content of the genome was 67%, with variations ranging from 50 to 70%. Approximately 7% of the genome showed a biased G+C composition, yielding some alternative codon usage regions (ACURs), which are dispersed throughout the genome within regions of standard composition. Approximately half of the ACURs encoded genetic elements that can be mobile (prophages, complete or truncated IS elements or tRNAs) directly within the ACUR or within 1-Kb flanking regions. Based on these features, it is likely that these ACURs have been acquired through HGT. Because the megaplasmid was found to carry several duplications of essential housekeeping genes, such as one copy of an rRNA gene, and because there was no bias in average base composition, it is generally assumed that the two replicons have co-evolved over a long time. This is also evident from the composition of the *hrp* gene cluster and associated effectors. The *hrp* gene locus is believed to represent an ancestral type III secretion system (T3SS), because the region has a G+C content similar to the rest of the genome and no evidence for DNA mobility was found in the flanking regions (30 Kb). This is in contrast to the *hrp* gene cluster found in other plant pathogens such as *P. syringae* (Alfano et al. 2000). Expectedly, the genes absolutely required for T3SS are widely conserved within the species (Guidot et al. 2007, Castillo & Greenberg 2007). On the other hand, genes encoding T3SS-dependent effectors were scattered throughout the genome and many of them reside within ACURs. Five of these ACURs had typical features of PAIs. They possess DNA sequences that indicate gene mobility (IS elements) or recombination events (Rhs and Vgr elements). Since Rhs and Vgr elements are recombinational hotspots in *E. coli* (Wang et al. 1998, Hill 1998), this strongly indicates *R. solanacearum* may have acquired new effector genes through HGT. Hence, the involvement of IS elements and recombination events in genome flexibility and evolution of *R. solanacearum* might be of great importance. The genome contains many complete and truncated copies of 17 distinct IS elements that belong to seven families. Eighty one complete IS elements were present, which belonged to seven different families (Table 1). Other IS families found so far in *R. solanacearum* are IS*L3*, IS*4*, IS*21*, IS*256*, IS*630* and IS*701*. Similar to the situation in other prokaryotic genomes, elements of the IS3 and IS5 families were found to be most abundant

in the *R. solanacearum* GMI1000 genome (23 and 26 copies respectively, Table 1). It is unknown to what extent target site specificity plays a role for the abundance and movement of the IS elements known to exist in *R. solanacearum*.

Table 1. IS elements identified in *R. solanacearum* bv3 strain GMI1000 and bv2 strain ACH058. Where known, the IS copy number in both strains is indicated.

			copy number		
family	IS element	synonym	GMI1000	ACH058	reference
IS3	ISRso10		6		Salanoubat et al., 2002
IS3	ISRso11		4		Salanoubat et al., 2002
IS3	ISRso12		6		Salanoubat et al., 2002
IS3	ISRso14		4		Salanoubat et al., 2002
IS3	ISRso16		3		Salanoubat et al., 2002
IS3	ISRso20 [1]		?		Gabriel et al., 2006
IS3	ISRso8		4		Salanoubat et al., 2002
IS4	ISRso13		7		Salanoubat et al., 2002
IS5	ISRso1	ISRso4	10	6	Salanoubat et al., 2002, Jeong & Timmis, 2000
IS5	ISRso18		1		Salanoubat et al., 2002
IS5	ISRso2	IS1421	7	12	Salanoubat et al., 2002, Jeong & Timmis, 2000
IS5	ISRso3	IS1021	2	>40	Salanoubat et al., 2002, Jeong & Timmis, 2000
IS5	ISRso9		6		Salanoubat et al., 2002
IS21	ISRso19 [2]		?		Lee & Khor, 2001
IS21	ISRso6		4		Salanoubat et al., 2002
IS256	ISRso7		1		Salanoubat et al., 2002
IS630	ISRso5		6		Salanoubat et al., 2002
IS701	ISRso17		8		Salanoubat et al., 2002
ISL3	ISRso15		2		Salanoubat et al., 2002

[1] IS element was identified in strain bv2 strain UW551

[2] IS element is similar to IS*Rso6* and found in a race 2 strain (biovar unknown)

The number and distribution of ACURs and the complete repertoire of IS elements in other strains than GMI1000 is not known. However, it is likely that the genomes of other strains also possess a significant number of ACURs and IS elements. Also indicative of the high potential for evolution was the presence in the *R. solanacearum* genome of a 31-Kb tandem duplication and a conjugative transposon related to the 55-Kb transposon Tn*4371* from *R. metallidurans* (Salanoubat et al. 2002). This transposon encodes *tra* and *trb* genes for conjugation as well as an integrase and several loci that encode Rhs-and Vgr-related elements. Assessment of the gene distribution of the GMI1000 genome content in 18 strains that represent the diversity of the *R. solanacearum* species complex revealed that a large proportion of the variable gene content is organized in a total of 48 GEIs that are dispersed over the two replicons. There was a relative overrepresentation of the variable genes on the megaplasmid (63% of the total). Similarly, genes that make up the core genome were found to be overrepresented (77%) on the chromosome.

A large proportion of the variable gene content of the *R. solanacearum* genome lies in the distribution of elements of external origin (denoted as "class V"), genes encoding hypothetical proteins (class VI), ACURs, effectors and the HrpB/HrpG regulon (Figure 3, upper panel). The

variable gene content includes almost all ACURs (94%) and elements of external origin (95%). Interestingly, the G+C content of both variable and core genes showed a similar distribution, except that variable genes are overrepresented among the low G+C value regions (Figure 3, lower panel). Genes that fall into the so-called functional categories I-IV (small and macromolecular metabolism, structural elements and cell processes, Salanoubat el al. 2002), as defined for the genome sequence of GMI1000, were represented by a relatively larger proportion of core genes, although approximately 30% of these genes was also found to belong to the variable genome. These genes do not differ significantly from core genes in terms of base composition. They could thus represent ancestral genes that were simultaneously lost by deletion during evolution. Because this analysis made use of the known genes of only one representative strain, the variable gene content of the species is likely to be much higher. However, analysis of the genes that were present in all 18 strains tested indicated that the estimated core genome consists of 2,690 genes (53% of the total). Most of these genes (2,515) were shared with *R. syzygii* (Guidot et al. 2007). An interesting and unexpected result was the clear correlation between the phylogeny of the species and the distribution of ACURs (Guidot et al. 2007). This was unexpected because the genes encoded by ACURs are likely to be acquired through HGT and consequently expected to de-structure populations. In contrast, the lack of congruence in the distribution of prophages and IS elements was an indication that these genetic elements have been, and are still, active and may still be spreading within populations.

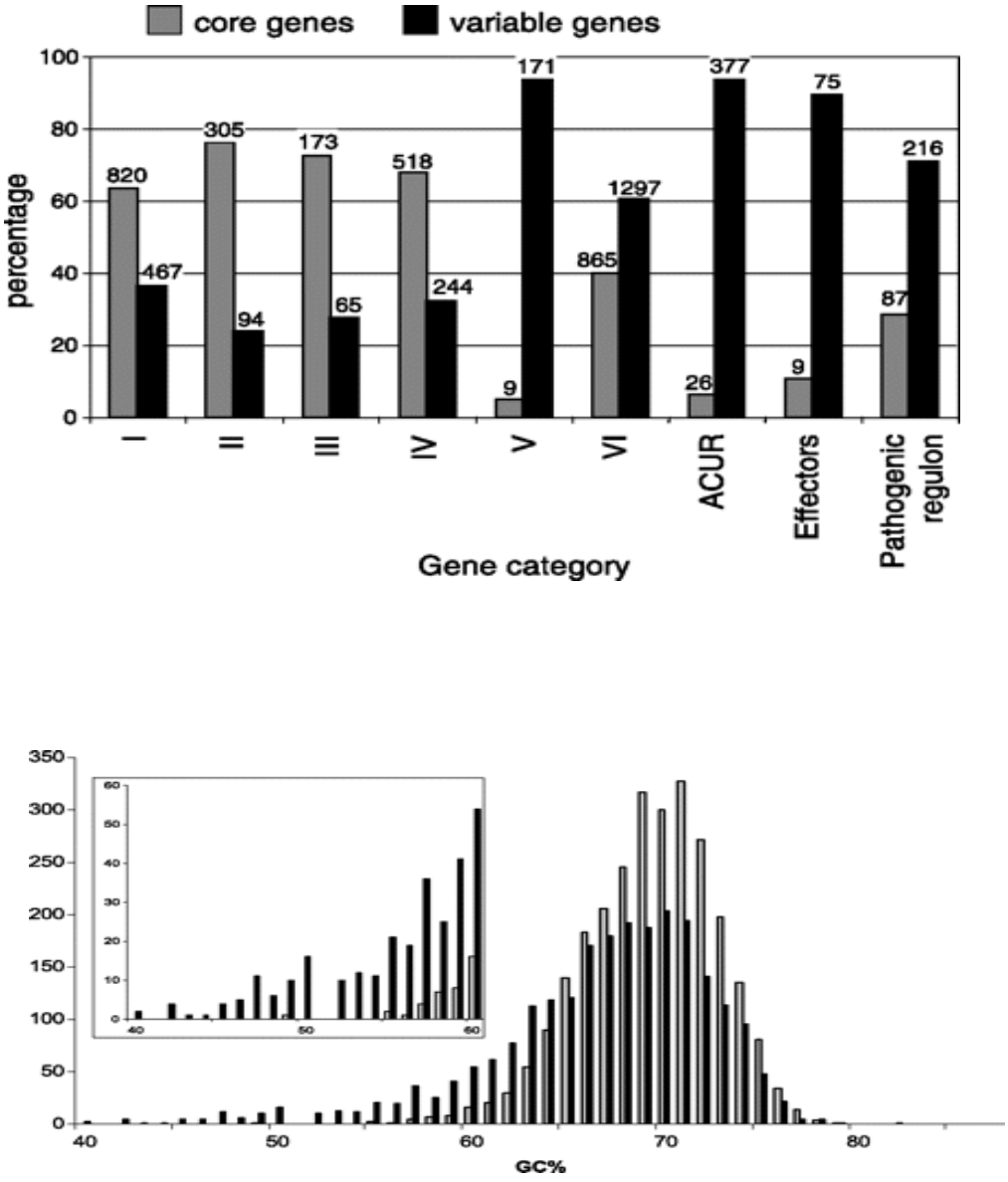

Figure 3. Distribution of core and variable genes within the *R. solanacearum* species complex. Adapted from Guidot et al. (2007). Upper panel: The distribution of core and variable genes within different functional groups according to the annotation of the genome of *R. solanacearum* bv3 strain GMI1000. Class I: small molecule metabolism; Class II: macromolecule metabolism; Class III: structural elements; class IV:cell processes; Class V:elements of external origin, Class VI: miscellaneous. Lower panel: the distribution of the core genes (gray bars) and variable genes (black bars) according to the G+C content of the corresponding sequences. The inset is a magnification of the histogram for low G+C content genes.

The life cycle of *R. solanacearum*

R. solanacearum has a so-called biphasic life cycle, which is characterized by (i) life in open systems such as soil, sediment and water, usually at relatively low cell densities and (ii) life in association with plants, in which a highly-evolved interaction takes place that may lead to wilting (and death) of susceptible plants. The latter results from the massive populations of *R. solanacearum* that build up inside the plant (often amounting to 10^8 to 10^{10} CFU/g tissue), and cause clogging of the vascular tissue as a result of their massive production of EPSI. The virulence phase is accompanied by the expression of virulence factors which are under quorum sensing control and thus are expressed only at high cell densities.

The organism's life cycle is regulated by the expression of *phcA*, a global regulator of virulence, and, broadly speaking, the determinant of either virulence or saprophytism. The natural lifestyle of *R. solanacearum* can therefore be considered as a "cycle", or oscillation, in which cells alternate between saprophytism and virulence. This correlates with low or high cell density phenotypes (Denny 2005).

Persistence in the open (soil, sediment or water) environment

Using combinations of immunofluorescent cell staining, cultivation and methods that assess the viability of cells, it has become clear that *R. solanacearum* bv2 is able to survive for considerable periods of time in open soil, sediment and water environments (van Elsas et al. 2000b, 2001). Whereas survival at 20°C was surprisingly extended, even without added nutrients, that at low temperature (4°C) was limited. As a striking phenomenon was that, when exposed to low temperature, part of the population of different bv2 strains was found to enter a so-called viable-but non-culturable (VBNC, now called dormant) state (van Elsas et al. 2000b, Grey & Steck 2001, van Overbeek et al. 2004, Álvarez et al. 2007). As outlined below (page 26), the mechanism that induced the VBNC form in *R. solanacearum* bv2 were thus low temperature, whereas the presence of a heavy metal (Cu) also appeared to induce this state (Grey & Steck 2001).

In the Netherlands, where no severe cases of brown rot have occurred in the last decade, the life of the extant *R. solanacearum* bv2 populations is likely to be characterized by their persistence in bulk water or sediment and their survival in possible refuge sites offered by bittersweet. Whereas organismal fate in bulk water/sediment is reined by local abiotic conditions and resources, it is unknown what drives the fate in bittersweet refuges. However, it is likely that, faced with the vagaries of these environments, *R. solanacearum* bv2 has evolved specific cellular systems that allow it to cope with these stressful environmental conditions.

Monitoring of the population dynamics of *R. solanacearum* bv2 populations after their release from diseased potato plants in a Dutch field showed that, although population densities decreased progressively over months after disease outbreak, the pathogen remained detectable as CFUs at various sites to about 9 months after the outbreak (van Elsas and co-workers, unpublished results). The exact survival mechanisms and contribution of potential alternative hosts, such as *S. nigrum*, are still poorly understood, but it has been suggested that, in these cases, potato volunteers played a key role (van Elsas et al. 2005b). The ability of *R .solanacearum* bv2 to persist in potato volunteers as well as to colonize diverse non-host and tolerant plant species

(Álvarez et al. 2008b) may result in these plant species serving as refuges or reservoirs that can greatly enhance the persistence of *R. solanacearum* bv2 in soil systems.

Concerning survival in the absence of host plants, the majority of natural soil and water systems are restricted by the levels of carbonaceous nutrients necessary for bacterial growth. Consequently, non-growth, or growth at extremely low rate, may ensue in the bacterial populations that dwell in these environments. Alternatively, hot spots such as the plant rhizosphere, decaying material of the mycosphere, as well as nutrient-rich sediments, may constitute habitats that offer growth possibilities to the bacterial inhabitants by virtue of their elevated levels of nutrients and other compounds.

Strikingly, however, *R. solanacearum* has been shown to survive or even grow under conditions of nutrient scarcity. For instance, following introduction into pure water, populations of some *R. solanacearum* strains have been found to survive or even multiply for several generations (Wakimoto et al. 1982, van Elsas et al. 2001). This indicated that these cells either used stored energy sources very efficiently or turned out to be excellent scavengers of gaseous or solid-bound nutrients that occur at very low concentrations in the system. This surprising capacity of these *R. solanacearum* bv2 strains to deal with nutrient scarcity has not been investigated to a sufficient extent, but it may relate to the mode of this organism to perceive and respond to diverse nutrient levels.

In nutrient-limited environments such as environmental waters, but also soil pores or plant xylem environments, *R solanacearum* bv2 may survive and even thrive and proliferate if it (1) handles nutrient scarcity in an efficient way, and (2) is successful in the acquisition of nutrients once these become available. In the latter process, it is likely the organism has to compete with other species that are locally present. Since *R. solanacearum* bv2 is often found in Dutch aquatic ecosystems where bittersweet plants are present (Janse 1998, Wenneker et al. 1999, van Elsas et al. 2001), it is likely the bittersweet/water interface provides a preferred niche. The bacterium may then persist in freshwater environments for long periods of time provided it can withstand the stresses that occur in these habitats (van Elsas et al. 2001). Next to nutrient availability, key factors that have been found to influence the *R. solanacearum* bv2 population sizes in the open (water) environments were (1) temperature, (2) soil moisture content, (3) the presence of indigenous organisms and (4) the presence of sediment in water (van Elsas et al., 2001, Alvarez et al. 2007).

In microcosm experiments, we followed the release of *R. solanacearum* bv2 strain 1609 cells from bittersweet plants that were kept with their roots in ditch water following spiking of the latter with 10^4 CFU/ml of cells. The ditch water compartment was repeatedly refreshed and the release of *R. solanacearum* cells into each water batch was measured. The results (Figure 4; five batches of ditch water shown) clearly showed that large numbers of cells are consistently released from the bittersweet roots into the surrounding water at temperatures of 18°C as well as 24°C. No significant effect of temperature was observed ($P>0.05$), although the numbers shed at 24°C were consistently higher than those shed at 18°C.

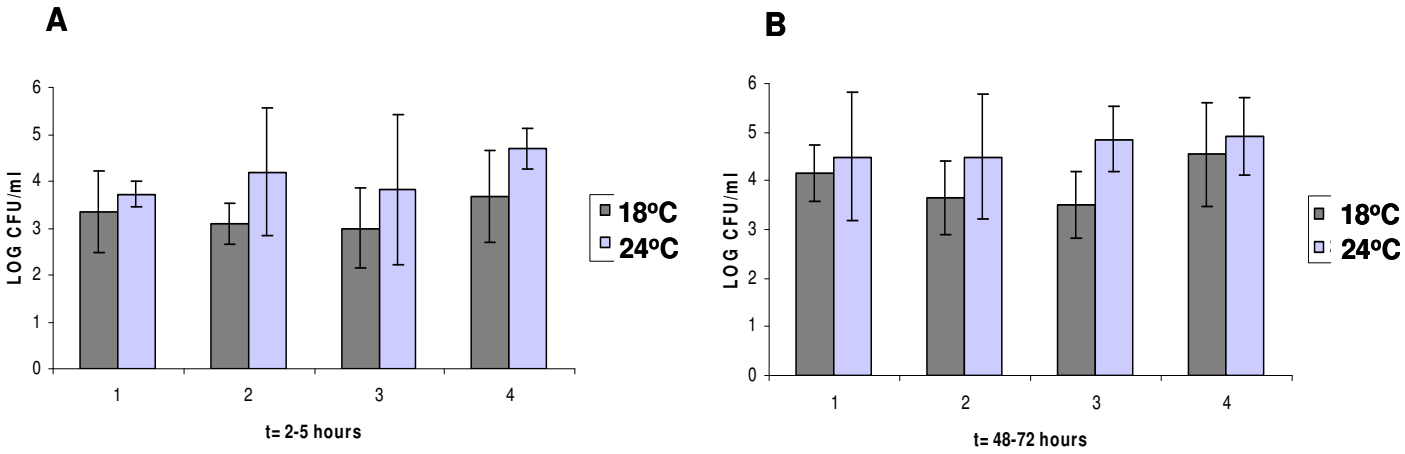

Figure 4. The numbers of *R. solanacearum* bv2 strain 1609 CFU/ml in ditch water after serial transfers of *R. solanacearum*-infested bittersweet plants to novel ditch water batches (*R .solanacearum* absent) determined at 18ºC and 24ºC. Plants were initially placed in ditch water containing 10^4 CFU/ml *R. solanacearum* cells. After four days, the plants were transferred to fresh ditch water not containing *R. solanacearum*. The number of CFU/ml after 2-5 hours (A) and after 48-72 hours (B) is shown. The graph shows the average of five plants (4 replicates) at 18 and 24ºC.

A similar result was observed when *R. solanacearum* bv2 infected geranium cuttings (10^8-10^9 CFU/g tissue) were placed in soil/water systems (Swanson et al. 2005). The number of *R. solanacearum* bv2 cells in the run-off water of both latently-infected and symptomatic plants was between 10^5 and 10^6 CFU/ml. After surviving or multiplying in these plant refuge sites, cells can be released and re-enter the surrounding soil, water or sediment and repeat the cycle (Figure 5)

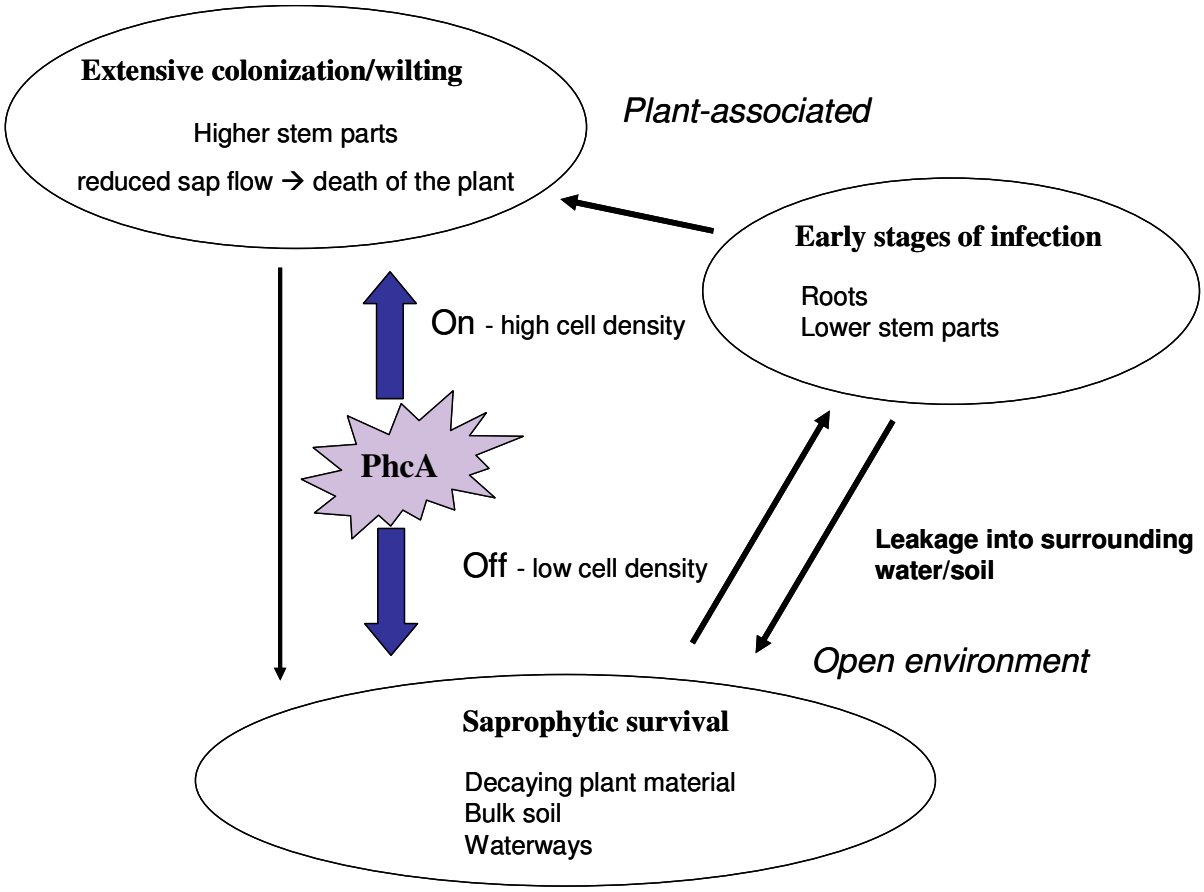

Figure 5. A model showing the different life stages of *R. solanacearum.* Cells can be free-living and occur at low population sizes due to low nutrient availability, competing organisms and stressful environmental conditions (such as unfavourable pH, oxygen, temperature and/or soil moisture). Cells may be attracted to the rhizosperes of potential host plants and start colonizing the roots and lower parts of susceptible, tolerant or resistant plants. From there, they may further colonize the upper parts of the plant and cause disease symptoms due to the activity of the global virulence regulator, PhcA, which is activated in a quorum sensing- dependent manner and represses and activates many genes directly or indirectly. Alternatively, cells may return from latently-infected or less susceptible plants into soil or water systems.

Starvation and physiological responses

Studies on the long-term population dynamics of *R. solanacearum* bv2 in environmental water microcosms have revealed diverse survival mechanisms that affect the organism's physiology. One of them was a classical starvation response which consisted of maintaining populations in a non-growing but culturable state as described for other bacterial species (Morita 1987). Although not extensively investigated in *R. solanacearum*, this survival modus/state differs from active growth by the reduced respiration rate and low endogenous metabolism. It may also be characterized by reductions in cell size leading to increased cell numbers (reductive cell division) and changes to increased cell surface/volume ratio. These morphological adaptations may ensure a better capture of the scarce nutrients that are available (Álvarez et al. 2008a). Under nutrient limitation, part of the population has been speculated to undergo a transition into the VBNC state (see below). Furthermore, stressed *R. solanacearum* populations were found to aggregate and produce filamentous cells (Álvarez et al. 2008a). This latter response can also be considered to

represent a survival mechanism, since cell aggregation enables the utilization of the content of lysing cells and protection from environmental hazards (Blat & Eisenbach 1995). Moreover, *R. solanacearum* bv2 cells which were kept under prolonged starvation conditions appeared to retain virulence on susceptible host plants, although the rate of infection might become reduced (van Overbeek et al. 2004, Caruso et al. 2005, Hong et al. 2005, Álvarez et al. 2007). Overall, and for reasons explained earlier, *R. solanacearum* bv2 appears to possess a remarkable capacity to adapt its cellular status to prolonged nutrient scarcity by a starvation response, in the meantime retaining pathogenicity.

The viable-but-non-culturable (VBNC) state

A factor of great phytosanitary concern is the potential occurrence of VBNC, or dormant, *R. solanacearum* bv2 cells in agricultural fields as well as in surface water. VBNC cells can occur in a wide range of Gram-negative bacteria, including plant and animal pathogens, saprophytic and symbiotic bacteria (Oliver 2000). The VBNC state can be circumscribed as the phenomenon that bacterial cells are not (easily) culturable as CFUs on agar plates. However, these cells may be fully viable using a range of metabolic criteria (Xu et al. 1982, Roszak & Colwell 1987). It has been postulated that, when *R. solanacearum* bv2 cells are exposed to environmental stresses, they may become "injured" and appear as non-culturable cells, which in fact are cells on their way to cell death (van Elsas et al. 2005a).

Factors found to induce the nonculturable state in *R. solanacearum* bv2 have been suggested to be the presence of elevated levels of copper (Grey and Steck 2001), prolonged persistence under oligotrophic conditions (Álvarez et al. 2007), colonization of plants (Grey & Steck 2001), and low temperature (Van Elsas et al. 2001, van Overbeek et al. 2004). However, the observed effects may be strain-dependent. For instance, *R. solanacearum* bv1 strain AW1 appeared sensitive to copper at concentrations of 0.05mM, leading to conversion to VBNC forms within 18 days. On the other hand, *R. solanacearum* bv2 strain 1609 was able to cope with copper concentrations of up to 25 mM for 3 weeks (van Elsas et al. 2005a). In addition, strain AW1 turned VBNC *in planta* (Grey and Steck 2001), while strain 1609 appeared to resuscitate from its VBNC state by inoculation and recovery from tomato plants (van Overbeek et al. 2004, van Elsas et al. 2005a). Thus, the effect of different stressors may differ between different *R. solanacearum* strains.

There is much debate about the actual physiological status of VBNC cells. It has been indicated that VBNC cells are able to convert to fully culturable forms under different conditions, such as passage through host organisms or relief of (low temperature) stress (Colwell et al. 1985, Oliver 1995, Whitesides & Oliver 1997, Oliver 2000, van Elsas et al. 2005a). However, it has been proven to be extremely difficult to show that real "resuscitation" occurs instead of re-growth of just a few cells present that were still culturable. In *R. solanacearum*, induction of VBNC cells may be related to the occurrence of oxidative stress, as addition of catalase to VBNC populations leads to a temporary increase in culturability (van Overbeek et al. 2004). However, other as-yet-undefined factors are likely to play a role as well. It has been postulated that the VBNC state in *R. solanacearum* bv2 might be linked to the process of phenotypic conversion (PC), but the molecular and physiological mechanisms and the exact ecological relevance underlying both conversions are still poorly understood (van Elsas et al. 2005a, Poussier et al. 2005).

Because VBNC cells are not detectable with techniques that require cultivation steps, these cells escape attention in screenings of soil and water samples when relying on cultivation-based

methods. Thus, the occurrence and understanding of the induction of the VBNC state of *R. solanacearum* bv2 in its natural environment is of great importance for pathogen control strategies.

Stress/stringent response

Several physiological changes can occur in non-differentiating bacteria in response to stress. Basically, the cellular stress response can be seen as a defense reaction imposed on cells by environmental force(s) on macromolecules, which is sensed as deformation or damage to DNA, proteins or other essential macromolecules (Kültz 2003). This results in alterations in cell cycle control, protein chaperoning and repair, DNA stabilization and repair and removal of damaged proteins (Kültz 2003). Consequently, many proteins that are induced upon stress function in sensing, repairing and minimizing DNA and protein damage. In addition, others are involved in energy metabolism and cellular redox regulation.

The RNA polymerase subunit, RpoS, is the prime regulator of stationary-phase- induced genes whose levels are controlled by proteolysis. It is required for reductive cell division (Lange & Hengge-Aronis 1991) and was found to be a master regulator of the general stress response in *E. coli* and other bacteria (Hengge-Aronis 2002). In line with this, it may not be surprising that *E. coli* strains with elevated levels of RpoS show enhanced resistance to stress (Weber et al. 2005). Regulatory mechanisms that control the level of RpoS occur in response to stresses such as near-UV irradiation, acid, temperature or osmotic shock, oxidative stress and nutrient deprivation. Although many key regulatory entities have been identified, the precise mechanisms by which they signal *rpoS* transcription, translation or proteolysis, have remained largely uncharacterized.

When bacterial cells are starved for nutrients, the cell physiology may be altered in two fundamental ways. First, *de novo* protein synthesis makes the cells into more efficient scavengers of scarce nutrients. Secondly, when starvation prolongs, it may convert them to phenotypes with enhanced stress resistance. In *E. coli* and many other bacteria, starvation, in particular for essential amino acids, rapidly incites the accumulation of a small signal molecule, a hyperphosphorylated guanosine nucleotide, (p)ppGpp, which acts as a nutritional "alarmone". The role of RpoS is complementary to that of the alarmone, as they appear to co-operate. Expression of RpoS-controlled genes is partly dependent on (p)ppGpp. In *E. coli*, the functioning of two homologous enzymes, i.e the RelA and SpoT proteins, mediates the synthesis and degradation of (p)pGpp. The activity of these enzymes leads to elevated levels of (p)ppGpp in the cell in response to amino acid and/or carbon starvation. They are thus necessary for balancing the cell's nutritional capability and survival under stress conditions (Cashel et al. 1996). RelA is known to be required for the synthesis of (p)ppGpp, while SpoT has bifunctional activity, as it is involved in the synthesis as well as degradation of (p)ppGpp. Unlike Gram-negative bacteria such as *E. coli*, most bacteria harbor a single RelA/SpoT-like enzyme (named Rel, RelA) that functions in both the degradation as well as the synthesis of (p)ppGpp ((Mittenhuber 2001). Recently, additional (p)ppGpp synthetases have been identified in *Bacillus subtilis* (Nanamiya et al. 2008) and *Streptococcus mutans* (Lemos et al. 2007), but their exact function is still unknown. The role of RelA and/or SpoT and the regulation of cellular (p)ppGpp levels under a variety of conditions have been described in diverse organisms such as *Rhizobium etli* (Calderón-Flores et al. 2005), *Vibrio cholerae* (Das & Bhadra 2008), *Bacillus subtilis* (Wendrich & Marahiel 1997, Nanamiya et al. 2008) and *Yersinia pestis* (Sun et al. 2009). From these and other publications, it has become clear that (p)ppGpp acts as a global regulator during adaptation to a variety of environmental conditions, including

interactions with eukaryotic hosts (pathogenesis, symbiosis), survival at low temperatures and bacterial multicellular behavior (Braeken et al. 2006; Spira et al. 2008).

In addition to increasing the capacity for nutrient scavenging, it is known that bacterial cells exposed to one kind of stress often show enhanced resistance to other stresses (cross tolerance and stress hardening) (Jenkins et al. 1988, 1990, van Overbeek et al. 1995). The basis for this cross-tolerance probably lies in the fact that common stress proteins are induced, which is often involved in general aspects of cellular protection. In addition, reactive oxygen species may have induced an antioxidant defense system, which may also be required for relief from other stresses.

Some studies have tried to address the question whether *R. solanacearum* bv2 strains such as UW551 and 1609 are "cold-adapted" by changes in their gene content (Gabriel et al. 2006, Guidot et al. 2009b). No genes have so far been found that could be related to cold adaptation/tolerance, nor to the relative ease with which the bacterium establishes in colder climates where brown rot was never a problem before.

Plant infestation and development of disease

When *R. solanacearum* colonizes a susceptible host plant, it may first attach to root surfaces and form microcolonies, preferentially at sites of "natural wounds" that occur during normal plant development. The root elongation zones and sites of lateral root emergence appear to be targets for initial attachment, probably due to the high density of root exudates and/or weakened epidermal barriers at these sites (Kelman & Sequira 1965). Roots may also become more easily accessible as a result of the physical wounds caused by agricultural practices (damage by equipment) or of nematodes present in the soil. It has been shown that swimming motility in *R. solanacearum* contributes significantly to these early stages of attachment to the host, probably because motile cells move towards and accumulate near the root segments of such plants. Both host and non-host plants can be colonized, although attraction is much less in the latter case (Mao & He 1998, Tans-Kersten et al. 2001). The movement towards plant roots is induced by chemotaxis, i.e the presence of diverse amino acids, organic acids and root exudates (Yao & Allen 2006). Thus, mutants defective in the CheA or CheW proteins, which are essential chemotaxis signal transduction agents, showed significantly reduced virulence as compared to the wild-type. This suggested that specific directed motility is required for full virulence rather than random motion (Yao and Allen 2006). The root-derived substrates that attract bacteria (including *R. solanacearum*) may vary between species or even within strains of a single species, suggesting that chemotactic responses can be specific for different hosts or ecological niches (Zhulin et al. 1995, Yao and Allen 2006). *R. solanacearum* cells proliferating at the sites of infection rapidly invade the intercellular spaces of the root cortex, followed by colonization of the intercellular spaces in the inner cortex and the vascular parenchyma. After 4-5 days, bacterial cells invade the xylem vessels, which is probably facilitated by the action of cellulolytic enzymes or by release of tyloses from parenchyma or xylem cells which contribute to vascular dysfunction (Grimoult et al. 1994, Nakaho et al. 2000). Once the bacteria have invaded the xylem vessels, they can travel rapidly to the upper parts of the host plant. These later stages in virulence require a highly specialized process of interacting genes and protein products of the pathogen as well as of the plant. This

eventually leads to massive amounts of bacterial cells inside the plant vascular tissue. The development of wilting symptoms is probably the result of production of large amounts of EPS that block water traffic. Denny et al (2005) suggested that bacteria that colonize plant tissue might fluctuate between the low and high cell density phenotypes, because recently invaded tissues will have low-density pathogen communities, while cell numbers are much higher in parts invaded earlier. Transition between saprophytic (low-density) to virulent/parasitic (high-density) lifestyles is controlled by the global virulence regulator PhcA.

Pathogenicity and virulence factors

During plant infestation, *R. solanacearum* produces so-called pathogenicity factors that are essential for disease. Next to this, many virulence factors are produced that enhance the pathogen's ability to colonize host tissue, allowing it to reach population sizes that often exceed 10^8 CFU/g plant tissue. Important factors that determine the successful invasion of a host include the abilities to circumvent recognition by the plant (HR), to attach to plant cells and to produce cell-wall-degrading enzymes (CWDEs). The best-studied pathogenicity factor is probably the T3SS, which is encoded by the *hrp* genes. The system constitutes a "needle" complex, or translocon, that delivers so-called "effector" proteins into the host cell. *R. solanacearum* bv3 strain GMI1000 is non-pathogenic on tobacco and, in fact, induces an HR when infiltrating tobacco leaves. In addition, proteins that are secreted by the conserved type II secretion system (T2SS) also have great impact on the development of wilting. Moreover, there may be "cross-talk" between the T3SS and T2SS, which is probably mediated by the HrpB and HrpG transcriptional regulators (Valls et al. 2006, Hikichi et al. 2007).

The type III secretion system (T3SS) and its exported products

Many plant and animal pathogens require a T3SS for virulence. To function in Gram-negative bacteria, the T3SS must cross three biological membranes, i.e the inner and outer bacterial membranes and the host plasma membrane. This occurs through the proteinaceous needle complex, which allows the translocation of effector proteins into host cells upon contact with host (plant) tissue (Aldon et al. 2000, Cunnac et al. 2004). In *R. solanacearum* bv3 strain GMI1000, the T3SS is encoded by a gene cluster located on a 23-Kb region on the megaplasmid (van Gijsegem et al. 1995). The system is composed of nine hrc (*hrp* conserved) genes that are essential for the core secretion apparatus. The *hrp* genes encode proteins that control transcription and function of effector proteins. In *R. solanacearum* bv2 strains, the region harbors an insertion of nine genes, of which six appear to be unique for bv2 (Gabriel et al. 2006, Figure 6) One of these additional genes encodes an IS*1021* (IS*Rso3*) transposase, of which multiple copies are present in the genome of *R. solanacearum*.

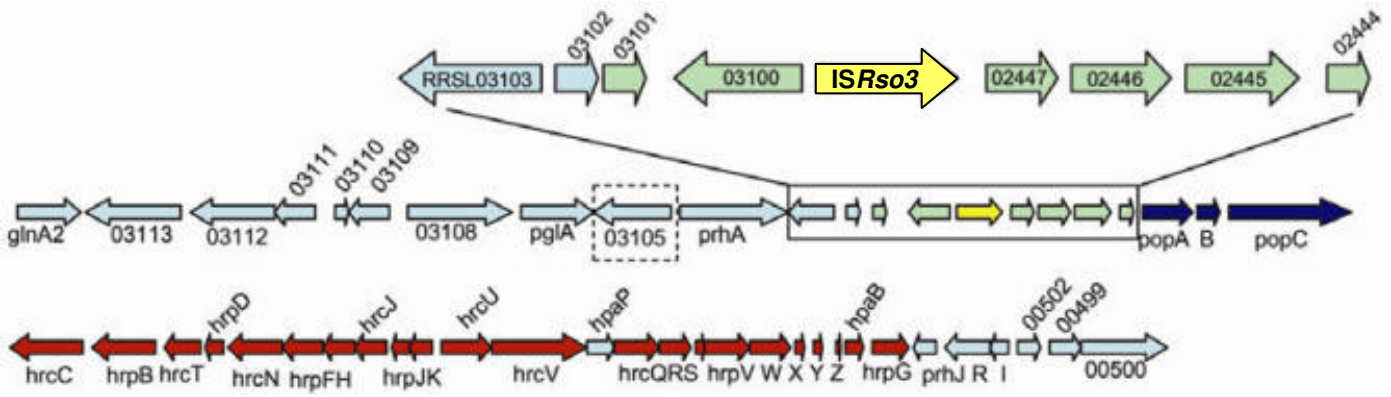

Figure 6. The *R. solanacearum* bv2 *hrp* gene cluster. Adapted from Gabriel et al (2006). ORFs are indicated using the UW551 nomenclature. Boxed regions indicate genes that are either not present, or organized differently, in bv3 strain GMI1000. Gene colors: red, hrp/hrc; dark blue, pop; yellow, transposase; green, not in GMI1000. The gene in the dashed-line box is found in the opposite orientation in GMI1000. An insertion of nine genes, including an IS*RSo3* transposase, was found in bv2 strain UW551 as compared to GMI1000.

The expression of T3SS is controlled by HrpG, a two-component response regulator, of which the transcription is induced upon contact with plant cells (Brito et al.1999, 2002, Aldon et al. 2000, Cunnac et al. 2004). It is negatively controlled by the PhcA confinement system (Schell 2000, Genin et al. 2005). HrpG strongly enhances the transcription of hrpB, which is an AraC-family transcriptional regulator that is required for the transcription of other *hrp* genes. HrpG appears to play a key role that extends beyond its functioning in T3SS. For instance, it has also been shown to control the expression of T3SS-independent pathways such as the production of CWDEs, EPS and the phytohormones ethylene and auxin (Valls et al. 2006).

HrpB-induced genes share a plant-inducible promoter (PIP) consensus motif (TTCG-N16-TTCG-N32/33-A-N3-T). By using micro-array analysis (Cunnac et al. 2004, Occialini et al. 2005), genome analysis (Mukaihara et al. 2009) and transposon mutagenesis (Mukaihara et al. 2004), over 80 candidate effector compound genes have been identified in strain GMI1000. Interestingly, the number of (predicted) effector genes in strain UW551 was lower than that for strain GMI1000, which might be related to differences in the host ranges of these strains (Gabriel et al. 2006).

Secreted effector proteins may act as "double agents" by promoting disease in susceptible hosts or eliciting a hypersensitive response (HR) in resistant plants (also called avirulence proteins). For instance, secretion of the well-studied protein AvrA of strains AW1 and GMI1000 (both race 1 and different biovars) triggered a rapid defense response in resistant plants, which resulted in rapid cell death (necrosis), thereby preventing further spread of the bacterium. Strain AW1, which was inactivated by a transposable element in *avrA*, did not elicit an HR response on tobacco leaves and was pathogenic on tobacco (Carney & Denny 1990). Variation of the *avrA* gene in *R. solanacearum* in the United States demonstrated that recognition of AvrA, and its allelic forms, determined the aggressiveness of strains on tobacco plants (Robertson et al. 2004). Strain UW551 showed an intermediate phenotype on tobacco leaves (no HR and mild chlorosis), which could be explained from the UW551 genome sequence. The analysis revealed the presence of an insertion sequence in the promoter region of *avrA*. Also, the characteristic PIP-box was missing, which might explain the differential behavior of these strains on tobacco (Gabriel et al. 2006).

In the GMI1000 genome, multiple genes are present that show homology to the avirulence genes of other plant-pathogenic bacteria (Salanoubat et al. 2002). Whether these putative *avr* genes are involved in the induction of plant defense responses is not clear. One would expect that the presence of multiple *avr* genes limits the host range and monogenic resistance would be observed more often than it is now. It is likely that the Avr proteins collectively act as pathogenicity factors on a large set of host plants (Kjemtrup et al. 2000). Besides Avr proteins, *R. solanacearum* might possess other general mechanisms that prevent plant defense responses to be triggered.

Chemotaxis and motility

As already indicated in the foregoing, another virulence factor that contributes to virulence is swimming motility mediated chemotaxis (Tans-Kersten et al. 2001, Yao & Allen 2006). Mutants that are inactivated in *fliC*, the flagellin structural gene, were shown to be reduced in virulence, but only when applied to tomato plants through a soil drench method. This suggested that flagellar action is most important at the early stages of infection. It was also shown by Yao and Allen (2006) that chemotaxis rather than random movement is required for full virulence.

Plant cell-wall-degrading enzymes (CWDE)

During plant infestation, CWDEs and many other proteins are secreted through the T2SS. *R. solanacearum* depends on this system, as T2SS-mutants are often severely impaired in colonization and multiplication *in planta* (Kang et al. 1994). Seven extracellular enzymes - secreted by T2SS - have been identified, i.e. a β-1,4-endoglucanase (Egl), an exoglucanase (CbhA), an endopoly-galacturonase (PehA or PglA), two exopoly-galacturonases (PehB and PehC), a pectin methylesterase (Pme) and Tek. Tek is a 28-kD protein which is the most abundant extracellular protein of many *R. solanacearum* strains. Schell (2000) actually suggested that Tek associates with EPSI. However, because Tek mutants have wild-type levels of EPSI and virulence, the function of this protein is still unclear (Denny et al. 1996). Moreover, it appears to vary between strains. For instance, it was shown in *R. solanacearum* strain K60 (bv1), that inactivation of PehA and PehB, but not PehC or Pme, reduced the colonization and wilt severity of tomato plants. A triple mutant, PehA-PehB-PehC, was, however, more virulent than the PehA-PehB double mutant (Tans-Kersten et al. 1998, Huang & Allen 2000, Gonzalez & Allen 2003). It was suggested that the absence of pectin breakdown in the triple mutant reduced plant defense, as a result partially restoring the reduced virulence of the double mutant. In *R. solanacearum* strain GMI1000, however, inactivation of one to four pectinolytic enzymes did not significantly affect its virulence on tomato. On the other hand, inactivation of EglI and CbhA both reduced virulence of strain GMI1000. Because a GMI1000 mutant lacking all six CWDEs was more virulent than a mutant defective in T2SS, it was suggested that other (as-yet unknown) secreted proteins also contribute to the ability of GMI1000 to colonize tomato plants (Liu et al. 2005). It is unclear to what extent CWDEs contribute to the virulence of *R. solanacearum* bv2 on its plant hosts.

Extracellular polysaccharide (EPS) production

The major virulence factor acting in susceptible host plants is probably EPSI, a long polymer which consists of repeat units of three N-acetylated monosaccharides (Orgambide et al. 1991). The EPSI biosynthetic pathway is encoded by the 16-Kb *eps* operon. It is estimated that 85% of the produced EPSI is released as a cell-free slime layer (McGarvey et al.1998). Mutants unable to

produce EPSI rarely wilt plants. They generally only colonize the roots and lower stems and do not spread well throughout the plant (Saile et al. 1997). Lipopolysaccharide, which is a major component of the outer leaflet of the outer membrane of Gram-negative bacteria, probably contributes to virulence as well. However, the exact mechanisms remain unclear (Newman et al. 2001, Denny 2006)

Phytohormones

The production by *R. solanacearum* of the phytohormones ethylene and auxin also appears to contribute to the virulence of this organism (Hirsch et al. 2002, Valls et al. 2006). Surprisingly, the transcription of the genes encoding these phytohormones was significantly reduced in a HrpG deficient mutant, which indicated that this regulator controls the expression of T3SS independent pathways (Valls et al. 2006). It was also shown by Valls et al. (2006) that ethylene produced by GMI1000 affects the transcription of various plant ethylene-response genes during infection. They concluded that *R. solanacearum* GMI1000 is able to modulate the expression of host genes and can therefore interfere with plant defense through production of ethylene.

Regulation of virulence

The PhcA confinement sensing system (i.e., response to high cell density) of *R. solanacearum* is a unique system that forms the core of the complex network that regulates virulence and pathogenicity (Schell 2000, Denny 2006). In the early stages of infection, plant signals are recognized by the sensor PrhA, which is located in the bacterial outer membrane. PhcA becomes activated in a quorum-sensing-dependent manner and controls the production of a range of virulence genes (Brumbley et al. 1993, Schell 2000). PhcA is a LysR-type transcriptional regulator that activates and represses many genes (Schell 2000). The levels of functional PhcA are controlled by a sensing system that is encoded by the *phcBSR* operon. PhcB is likely to be a small-molecule methyltransferase that synthesizes 3-OH palmitic acid methyl ester (3-OH-PAME) (Flavier et al. 1997). When bacteria are in a confined environment (such as the xylem vessels of a host plant), 3-OH-PAME, which is highly diffusible, accumulates in the extracellular environment. Threshhold levels of 3-OH-PAME are then sensed and transduced by the PhcS/PhcR two-component regulatory system via phosphotransfer leading to elevated levels of PhcA and hence the Phc regulon is activated (Schell 2000). However, addition of 3-OH-PAME to low-density cultures does not cause immediate expression of PhcA-activated genes. Possibly, an additional cell-density-related internal signal is required (Denny 2006).

The increase in PhcA affects the transcription of many genes that are involved in a variety of functions. Traits that are directly or indirectly upregulated by PhcA are the production of EPSI, EglI, Pme, competence for transformation by DNA and an acyl homoserine lactone (AHL) quorum sensing system (Schell 2000, Kang et al. 2002). Traits that are known to be negatively regulated by PhcA are the production of PehA, staphyloferrin B siderophore, type IV pili (necessary for twitching motility, autoaggregation and biofilm formation), flagellar motility, salt tolerance and activity of the HrpG transcriptional regulator (Schell 2000, Bhatt& Denny 2004, Genin et al. 2005).

Although PhcA has a central role in regulating virulence, other regulatory proteins and at least three other two-component systems also play roles (Schell 2000, Denny 2006). Sometimes, spontaneous loss of virulence occurs in culture as well as *in planta*. This PC phenomenon is characterized by the loss of pathogenicity and is associated with changes in morphology and motility. Poussier et al. (2003) showed that the change from wildtype to PC mutant is often the result of rearrangements and insertions in the *phcA* gene. Insertion sequences as well as inversions and (small) deletions were shown to inactivate the *phcA* gene. There is no evidence that *phcA* mutates spontaneously at a high rate. It is more likely that the *phcA* mutants (PC types) accumulate during stressful conditions such as prolonged stationary phase in culture or *in planta* (Denny et al. 1994). Cells at low density have little functional PhcA and, like PhcA mutants, exhibit a low-virulence phenotype.

It has been suggested that the PC state represents a survival state, optimized for survival in the open environment and invasion of plant tissues, although no evidence exists to prove this hypothesis. On the other hand, it was suggested that plant signals, in some cases, may induce a shift from the PC form back to the wildtype (Poussier et al. 2003, 2005). This suggests the possible involvement of PC types in survival and transition from saprophytic to pathogenic forms in natural settings.

Aim and outline of this thesis

The main aim of this thesis was to provide a better understanding of the ecology and adaptation of *R. solanacearum* bv2 in temperate climate habitats, in which it is not known to be historically endemic. The *R. solanacearum* bv2 strain(s) that have entered the Dutch ecosystem may thus be regarded as alien invading species. We hypothesized that, as the invading *R. solanacearum* bv2 has, since its introduction, persisted in the Dutch local waterways for over 15 years, it may have experienced conditions that were strongly selective for particular genotypic and/or phenotypic adaptations. The selective forces that could be regarded as being most selective were the seasonal low temperatures and the fluctuating but often low nutrient (carbon) availabilities. These forces may have incited changes in the genome that enhance the survivability of the organism.

Given the possibility that fitness-enhancing traits may have emerged and selected in the local *R. solanacearum* populations, we were interested in the diversity of the organism and its ability to survive prolonged periods of low temperature (winter) and absence of (carbonaceous) nutrients provided by true hosts. Overall, we thus hypothesized that, to accomplish its persistence in temperate climates, the bacterium may have been forced to adapt its genetic structure, its metabolism (at a transcriptional level) or both.

From the general hypotheses, the following research questions were derived:

- To what extent has *R. solanacearum* bv2 diversified in temperate climate conditions?
- Has *R. solanacearum* bv2 been able to adapt to low temperature?
  Do potato-derived and environmental R. solanacearum bv2 strains respond differently to cold stress?
- What are the key mechanisms of genome adaptation in *R. solanacearum* bv2?
  Does the activity of IS elements play a role?
  Do genomic modifications occur at specific loci?
- Is there a relationship between changed saprophytic fitness and pathogenic fitness (virulence)?

The main objectives of this study were derived from the above research questions. They can be formulated as follows:

- To assess the genetic diversity of Dutch *R. solanacearum* bv2 strains obtained from the open (water) environment,
- To assess the genomic differences between a presumably cold-adapted Dutch environmental and a tropical potato strain,
- To identify genes or genomic islands that have been acquired/deleted after the presumed introduction of *R. solanacearum* bv2 in the Dutch water ecosystem,
- To compare the ecological behavior of environmental versus potato strains under semi-natural conditions.
- To correlate the *R. solanacearum* bv2 genomic differences to differences in ecological behavior

In this thesis, I will provide data that provide important insight to the above research questions. A general introduction on the topic and view on the current status of our understanding of *R. solanacearum* bv2 ecology and genomics will be provided in **chapter I**. In **chapter II**, the occurrence of *R. solanacearum* strains in Dutch local waterways is presented, next to a thorough analysis of the genetic and phenotypic diversity of a set of 42 environmental strains. To gain insight in the virulence properties of the newly isolated strains, genomic regions involved in virulence were included in the analysis. **Chapter III** focuses on the genomic differences between a new environmental strain, KZR-5, and a selected tropical potato strain, 715. A suite of putative strain-specific sequences were found, identifying the deletion of a 17.6 Kb genomic island in the Dutch environmental strain. A glimpse of the putative mechanisms driving genomic diversity of *R. solanacearum* bv2 is also provided. In **chapter IV**, the (preliminary) results of comparative genomic hybridizations between selected *R. solanacearum* bv2 strains using micro-array technology are described. Using this technique, the deletion identified in the Dutch environmental strain KZR-5 was confirmed and putative additional genomic differences were detected. The analyses included three environmental strains and two potato strains (tropical, i.e. 715 and Dutch, i.e. 1609). In **chapter V**, the ecological relevance of the genes encoded by the putative genomic island PGI-1 is investigated. The focus was on the potential relevance of the deletion of two genes: (i) a cellobiohydrolase (*cbhA*) gene which potentially affects interaction/virulence with the host plant and (ii) a hypothetical protein containing a RelA/SpoT domain, possibly playing a role in the response to stress. Clear differences in behavior, i.e. survival at low temperature and competitiveness in tomato invasion, were found in the environmental strain KZR-5 as compared to the potato strains 1609 and 715. Finally, in **chapter VI**, I describe the response of (starved) *R. solanacearum* bv2 strains to oxidative stress following starvation. Across the board, the selected *R. solanacearum* bv2 strains were found to respond to starvation by enhancing their tolerance to oxidative stress, and there was a trend towards a higher stress tolerance in the environmental strain KZR-5 as compared to the potato strains 715 and 1609. However, any differences between strains KZR-5, 1609 and 715 await confirmation from additional work. The data shown in chapters II through VI are discussed in the **General discussion and concluding remarks (Chapter VII),** and a broader perspective on the significance of these data and prospects for further research are provided.

References

Aldon D, BritoB, Boucher C, Genin S (2000) A bacterial sensor of plant cell contact controls the transcriptional induction of *Ralstonia solanacearum* pathogenicity genes. EMBO J 19: 2304-2314

Alfano JR., Charkowski AO, Deng WL, Badel JL, Petnicki-Ocwieja T, van Dijk K, Collmer A (2000) The P*seudomonas syringae* Hrp pathogenicity island has a tripartite mosaic structure composed of a cluster of type III secretion genes bounded by exchangeable effector and conserved effector loci that contribute to parasitic fitness and pathogenicity in plants. Proc NatlAcad Sci USA 97: 4856-4851

Álvarez B, López MM, Biosca EG (2007) Influence of native microbiota on survival of *Ralstonia solanacearum* phylotype II in river water microcosms. Appl Environ Microbiol 73**:**7210-7217

Álvarez B, López MM, Biosca EG (2008a) Survival strategies and pathogenicity of *Ralstonia solanacearum* phylotype II subjected to prolonged starvation in environmental water microcosms. Microbiology154:3590-3598

Álvarez B, Vasse J, Le-Courtois V, Trigalet- Démery D, López MM, Trigalet A (2008b) Comparative behavior of *Ralstonia solanacearum* biovar 2 in diverse plant species. Phytopathol98**:**59-68

Anonymous (2000) Council directive 2000/29EC of 20 July 1998 on the control of *Ralstonia solanaceaurum* (Smith) Yabuuchi et al. Off J Eur Communities L235 pp 1-39

Argos P, Landy A, Abremski K et al. (1986) The integrase family of site-specific recombinases: regional similarities and global diversity. EMBO J 5: 433-440

Bertolla F, Frostegard A, Brito B et al. (1999) During infection of its host, the plant pathogen *Ralstonia solanacearum* naturally develops a state of competence and exchanges genetic material. Mol Plant Microbe Interact 12:467-472.

Bhatt G, Denny T P (2004) *Ralstonia solanacearum* iron scavenging by the siderophore staphyloferrin B is controlled by PhcA, the global virulence regulator. J Bacteriol 186: 7896-7904

Braeken K, Moris M, Daniels R, Vanderleyden J, Michiels J (2006) New horizons for(p)ppGpp in Bacterial and plant physiology. Trends Microbiol 14: 45-54.

Brito B, Aldon D, Barberis P, Boucher C, Genin S (2002) A signal transfer system through three compartments transduces the plant cell contact-dependent signal controlling *Ralstonia solanacearum hrp* genes. Mol Plant Microbe Interact. 15:109-119

Brito B, Marenda M, Barberis P, Boucher C and Genin S (1999) *prhJ* and *hrpG*, two new components of the plant signal-dependent regulatory cascade controlled by PrhA in *Ralstonia solanacearum*. Mol Microbiol 31: 237–251

Breukers A, Hagenaars T, van der Werf W and Oude Lansink A. (2005) Modelling of brown rot prevalence in the Dutch potato production chain over time: from state variable to individual- based models. Nonlinear Analysis: Real World Appl 6:797-815

Buddenhagen I, Sequeria L, Kelman A (1962) Designation of races of *Pseudomonas solanacearum.* Phytopathol 52:726.

Burrus V, Pavlovic G, Decaris B, Guedon G (2002) Conjugative transposons: the tip of the iceberg. Mol Microbiol 46:601–610

Brumbley SM, Carney BF, Denny TP (1993) Phenotype conversion in *Pseudomonas solanacearum* due to spontaneous inactivation of PhcA, a putative LysR transcriptional regulator. J Bacteriol 175:5477–5487

Calderón-Flores A, Du Pont G, Huerta-Saquero A, Merchant-Larios H, Servin-Gonzalez L, DuranS (2005) The stringent response is required for amino acid and nitrate utilization, nod factor regulation, nodulation, and nitrogen fixation in *Rhizobium etli*. J Bacteriol 187: 5075-5083

Carney BF, Denny TP (1990) A cloned avirulence gene from *Pseudomonassolanacearum* determines incompatibility on *Nicotiana tabacum* at the host species level. J Bacteriol 172:4836-4843.

Caruso P, Gorris MT, Cambra M, Palomo JL, Collar J, López MM (2002) Enrichment DASI-ELISA For sensitive detection of *Ralstonia solanacearum* in asymptomatic potato tubers using a specific Monoclonal antibody. Appl Environ Microbiol 68: 3634–3638

Caruso P, Palomo JL, Bertolini E, Álvarez B, López MM, Biosca EG (2005) Seasonal variation of *Ralstonia solanacearum* biovar 2 populations in a spanish river: recovery of stressed cells at low temperatures. App Environ Microbiol 71:140-148.

Cashel M, Gentry DR, Hernandez VJ, Vinella D (1996) The stringent response In: Neidhardt FC, Curtiss R (eds) *Escherichia coli* and *Salmonella:* Cellular and Molecular Biology. Washington D.C: ASM Press, pp 1458-1496

Castillo JA, Greenberg JT (2007) Evolutionary dynamics of *Ralstonia solanacearum.* App Environ Microbiol 73**:**1225-1238.

Cook D, Barlow E, Sequeira L (1989) Genetic diversity of *Pseudomonas solanacearum:* Detection of restriction fragment length polymorphisms that specify virulence and the hypersensitive response. Mol Plant Microbe Interact 2:113-121.

Cook D & Sequeira L (1994) Strain differentiation of *Pseudomonas solanacearum* by molecular genetic methods. In: Hayward AC, Hartman GL (eds). Bacterial wilt: the Disease and its Causative Agent, *Pseudomonas solanacearum*. Wallingford, UK: CAB International, pp 77-93

Cunnac S, Boucher C, Genin S. (2004) Characterization of the *cis*–acting regulatory element controlling HrpB–mediated activation of the Type III secretion system and effector genes in *Ralstonia solanacearum*. J. Bacteriol 186, 2309–2318.

Darling AE, Miklós I, Ragan MA (2008) Dynamics of genome rearrangement in bacterial Populations. PLoS Genet 4:1000128.

Das B, Bhadra RK (2008) Molecular characterization of *Vibrio cholerae* ΔrelA Δ spot double mutants. Archiv Microbiol 189: 227-238.

Daubin V, Perrière G (2003) G+C3 structuring along the genome: a common feature in prokaryotes. Mol Biol Evol 20:471-483

Denny TP (2005) A short history of the biochemical and genetic research on *Ralstonia solanacearum* pathogenesis. In: C. Allen, P. Prior, and A. C. Hayward (eds.), Bacterial wilt disease and the *Ralstonia solanacearum* species complex. APS Press, St. Paul, MN, pp 323-334

Denny TP (2006) Plant pathogenic *Ralstonia* species. In: Gnanamanickam SS (ed) Plant Associated Bacteria. Springer, Dordrecht, pp 573-644

Denny TP, Brumbley SM, Carney BFm Clough SJ, Schell MA (1994) Phenotypic conversion of *Pseudomonas solanacearum*; its molecular basis and potential function. In: Hayward AC and Hartman GL (eds) Bacterial wilt: The disease and its causative agent, *Pseudomonas solanacearum* Wallingford, UK, CAB international pp 137-143

Elphinstone JG (2005) The current bacterial wilt situation: A global overview In: Allen , Prior P,Hayward AC (eds) Bacterial wilt disease and the *Ralstonia solanacearum* species complex.American Phytopathological Society Press, St. Paul, pp 9-28

Elphinstone JG, Hennessy J, Wilson JK, Stead DE (1996) Sensitivity of different methods for the Detection of *Pseudomonas solanacearum* (Smith) smith in potato tuber extracts. EPPO Bull 26:674-678.

Elphinstone JG, Stanford HM, Stead DE (1998) Detection of *Ralstonia solancearum* in potato tubers,*Solanum dulcamara* and associated irrigation water. In: Prior PH, Allen C, Elphinstone J (eds) Bacterial wilt disease: molecular and ecological aspects. Springer-Verslag, Berlin, Germany, pp 133-139.

Fayet O, Ramond P, Polard P, Prere MF, Chandler M (1990) Functional similarities between Retroviruses and the IS3 family of bacterial insertion sequences Mol Microbiol 4:1771–1777

Fegan M, Prior P (2005) How complex is the *Ralstonia solanacearum* species complex? In: Allen C, Prior P, Hayward AC (eds) Bacterial wilt disease and the *Ralstonia solanacearum* species complex. American Phytopathological Society Press, St. Paul, pp 449-461

Fegan M, Taghavi M, Sly LI, Hayward AC (1998) Phylogeny, diversity and molecular diagnostics of *Ralstonia solanacearum* In: Prior PH, Allen C, Elphinstone J (eds) Bacterial wilt disease: molecular and ecological aspects. Springer, Berlin, pp 133–139

Flavier AB, Clough, SJ, Schell MA et al (1997) Identification of 3-hydroxypalmitic acid ethyl ester as a novel autoregulator controlling virulence in *Ralstonia solanacearum*. Mol Microbiol 26: 251-259

Gabriel DW, Allen C, Schell M et al. (2006) Identification of open reading frames unique to a select agent: *Ralstonia solanacearum* race 3 biovar 2. Mol Plant Microbe Interact 19:69-79.

Genin, S., Brito, B., Denny, TP, Boucher, C (2005) Control of the *Ralstonia solanacearum* Type III secretion system (Hrp) genes by the global virulence regulator PhcA. FEBS Letters 579, 2077–2081.

Gillings, MR, Fahy P (1994) Genomic fingerprinting: towards a unified view of the *Pseudomonas solanacearum* species complex,. In: Hayward AC and Hartman GL (eds), Bacterial wilt: the disease and its causative agent, *Pseudomonas solanacearum*. CAB International, Wallingford, United Kingdom, pp 95–112

Gogarten JP, Townsend JP (2005) Horizontal gene transfer, genome innovation and evolution. Nature rev Microbiol 3 (9): 679-87

Gonzalez, ET, Allen C (2003) Characterization of a *Ralstonia solanacearum* operon required forpolygalacturonate degradation and uptake of galacturonic acid. Mol Plant Microbe Interact 16:536-544.

Grey BE, Steck TR (2001) The viable but non-culturable state of *Ralstonia soanacearum* may be Involved in long-term survival and plant infection. App Environ Microbiol 67:3866-3872.

Griep RA, Van Twisk C,Van Beckhoven JRCM, Van der Wolf J, Schots A (1998) Development of specific recombinant monoclonal antibodies against the lipopolysaccharides of *Ralstonia solanacearum* race 3. Phytopathol 88: 795–803

Grompoult V, Gélie B, Lemattre P, Prior P, Schmidt J (1994) Comparative histology of resistant and susceptible tomato cultivars infected by *Pseudomonas solanacearum*. Phys Mol Plant Pathol 44: 105–123

Guidot A, Coupat B, Fall S, Prior P, Bertolla F (2009a) Horizontal gene transfer between *Ralstonia solanacearum* strains detected by comparative genomic hybridization on microarrays. Isme J 3:549-562.

Guidot A, Elbaz M, Carrere S, Siri MI, Pianzzola MJ, Prior P *et al* (2009b**)** Specific genes from the potato brown rot strains of *Ralstonia solanacearum* and their potential use for strain detection. Phytopathol 99: 1105-1112.

Guidot A, Prior P, Schoenfeld J, Carrere S, Genin S, Boucher C (2007) Genomic structure and phylogeny of the plant pathogen *Ralstonia solanacearum* inferred from gene distribution analysis. J Bacteriol 189:377-387.

Hacker J, Carniel E (2001) Ecological fitness, genomic islands and bacterial pathogenicity. EMBO Rep 2:376-381

Hacker J, Kaper JB (2000) Pathogenicity islands and the evolution of microbes. Annu Rev Microbiol 23:1089-1097

Hayward AC (1991) Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*Ann Rev Phytopathol 29:65-87.

Hengge-Aronis R (2002) Signal transduction and regulatory mechanisms involved in control of the {sigma}S (RpoS) subunit of RNA polymerase. Microbiol Mol Biol Rev 66:373-395

Hikichi Y, Takeshi Yoshimochi Shintaro Tsujimoto, Rena Shinohara, Kazuhiro Nakaho, Ayami Kanda Akinori Kiba, Kouhei Ohnishi (2007) Global regulation of pathogenicity mechanism of *Ralstonia solanacearum* Plant Biotech 24: 149–154

Hill CW (1999) Large genomic sequence repetitions in bacteria: lessons from rRNA operons and Rhs elements. Res Microbiol 150: 665-674

Hill CW, Feulner G, Brody MS, Zhao S, Sadosky AB, Sandt CH (1995) Correlation of Rhs elements with *Escherichia coli* population structure. Genetics 141:15–24

Hill KE, Fry JC, Weightman AJ (1994) Gene transfer in the aquatic environment: persistence and mobilization of the catabolic recombinant plasmid pD10 in the epilithon. Microbiology 140: 1555-1563

Hirsch J, Deslandes L, Feng DX, Balagué C, Marco Y (2002) Delayed symptom development in ein2-1, an Arabidopsis ethylene insensitive mutant, in response to bacterial wilt caused by *Ralstonia solanacearum*. Phytopathol 92:1142-1148

Hogg T, Mechold U, Malke H, Cashel M, Hilgenfeld R (2004) Conformational antagonism between opposing active sites in a bifunctional RelA/SpoT homolog modulates (p)ppGpp metabolism during the stringent response. Cell 117: 57-68

Huang Q, Allen C (1997) An exo-poly-alpha-D-galacturonosidase, PehB, is required for wildtype Virulence *of Ralstonia solanacearum*. J Bacteriol 179: 7369-78.

Janse JD (1998) Experiences with potato brown rot *Ralstonia solanacearum* biovar 2,race 3 in the Netherlands. In: Prior PH, Allen C, Elphinstone J (eds) Bacterial wilt disease: molecular and Ecological aspects. Springer-Verslag, Berlin, Germany, pp 146-152.

Jenkins DE, Schultz JE, Matin A (1988) Starvation-induced cross protection against heat or H_2O_2 challenge in *Escherichia coli.* J Bacteriol 170:3910-3914.

Jenkins DE, Chaisson SA, Matin A (1990) Starvation-induced cross protection against osmotic challenge in *Escherichia coli*. J Bacteriol 172:2779-278 1.

Kelman A, Sequeira L (1965) Root-to-root spread of *Pseudomonas solanacearum* Phytopathology 55:304-309

Kang Y, Liu H, Genin S, Schell MA, Denny TP (2002) *Ralstonia solanacearum* requires type 4 pili to adhere to multiple surfaces and for natural transformation and virulence. Mol Microbiol 6: 427–437

Kang Y, Huang, JZ, Mao GZ, He LY, Schell MA (1994) Dramatically reduced virulence of mutants of *Pseudomonas solanacearum* defective in export of extracellular proteins across the outer membrane. Mol. Plant-Microbe Interact. 7:370-377.

Kjemtrup S, Nimchuk Z, Dangl J (2000) Effector proteins of phytopathogenic bacteria: Bifunctional signals in virulence and host recognition (Review). Curr Opin Microbiol 3:73-78

Kültz D (2003) Evolution of the stress proteome: from monophyletic origin to ubiquitous function. J. Exp. Biol. 206: 3119–3124

Lambert CD (2002) Agricultural Bioterrorism Protection Act of2002: possession, use, and transfer of biological; agents and toxins;interim and final rule (7 CFR Part 331). Fed Regist 67:76908–76938.

Lange R, Hengge-Aronis R (1991) Growth phase-regulated expression of *bolA* and morphology of stationary-phase *Escherichia coli* cells are controlled by the novel sigma factor sigma S. J Bacteriol 173:4474-4481

Lemos JA, Lin VK, Nascimento MM, Abranches J, Burne RA: Three gene products govern (p)ppGpp production by Streptococcus mutans. *Mol Microbiol* 2007, 65: 1568-1581

Lin, RJ, Capage M, Hill CW (1984) A repetitive DNA sequence, rhs, responsible for duplications Within the *Escherichia coli* K-12 chromosome. J Mol Biol 177:1–18

Liu H, Zhang S, Schell MA, Denny TP (2005) Pyramiding unmarked deletions in *Ralstonia solanacearum* shows that secreted proteins in addition to plant cell-wall-degrading enzymes contribute to virulence. Mol Plant Microbe Interact 18:1296-1305.

Lobry JR, Sueoka N (2002) Asymmetric directional mutation pressures in bacteria. Genome Biol. 3:RESEARCH0058

Mahillon J, Chandler M (1998) Insertion sequences. Microbiol Mol Biol Rev 62:725-774

Mao GZ, He LY (1998) Relationship of wild type strain motility and interaction with host plants In *Ralstonia solanacearum.* In: Prior PH, Allen C, Elphinstone J (eds) Bacterial wilt disease: molecular and ecological aspects. Springer-Verslag, Berlin, Germany, pp. 184-191.

McGarvey JA, Bell CJ, Denny TP, Schell MA (1998) Analysis of extracellular polysaccharide I in Culture and *in planta* using immunological methods: new insights and implications. In P. Prior, C. Allen, and J. Elphinstone (eds.), Bacterial wilt disease and the *Ralstonia solanacearum* species complex. American Phytopathological Society Press, St. Paul, pp 157–163

Mercier A, Bertolla F, Passelègue-Robe E, Simonet P (2007) Natural transformation-based foreign DNA acquisition in a *Ralstonia solanacearum mutS* mutant. Research in microbiology 158: 537–544

Mittenhuber G (2001) Comparative genomics and evolution of genes encoding bacterial (p)ppGpp synthetases/hydrolases (the Rel, RelA and SpoT proteins). J Mol Microbiol and Biotechn 3: 585-600

Morita RY (1997) Starvation-survival lifestyle. In: Reddy CA, Chakrabarty MA, Demain AL, Tiedje JM (eds). Bacteria in oligotrophic environments New York, Chapman & Hall. pp 368–385

Mukaihara T, Tamura N, Murata Y, Iwabuchi M (2004) Genetic screening of Hrp type III-related pathogenicity genes controlled by the HrpB transcriptional activator in *Ralstonia solanacearum*.
Mol Microbiol. 54:863–875.

Mukaihara T, Tamura N (2009) Identification of novel *Ralstonia solanacearum* type III effector proteins through translocation analysis of hrpB-regulated gene products. Microbiology 155: 2235-2244

Nanamiya H, Kasai K, Nozawa A, Yun CS, Narisawa T, Murakami K *et al*. (2008) Identification and functional analysis of novel (p)ppGpp synthetase genes in *Bacillus subtilis*. Mol Microbiol 67:291-304.

Nakaho K, Hibino H, Miyagawa H (2000) Possible mechanisms limiting movement of *Ralstonia solanaceaurm* in resistant tomato tissues. Journal of phytopathology 148:181-190

Newman MA, Dow JM, Daniels MJ (2001) Bacterial lipopolysaccharides and plant–pathogen-interactions. European Journal of Plant Pathology 107, 95–102.

Occhialini A, Cunnac S, Reymond N, Genin S, Boucher C (2005) Genome-wide analysis of gene expression in *Ralstonia solanacearum* reveals that the hrpB gene acts as a regulatory switch controlling multiple virulence pathways. Mol Plant Microbe Interact 18:938–949

Oliver JD (2000) The viable but nonculturable state and cellular resuscitation In: Bell CR, Brylinsky M, Johnson-Green P (eds.) Microbial biosystems: new frontiers. Atlantic Canada Soc Microb Ecol, Halifax, Canada, pp 723-730

Oliver JD (2005) The viable-but-non-culturable state in bacteria. J.Microbiol 43:93–100

Oliver JD, Bockian R (1995) In vivo resuscitation, and virulence towards mice, of viable but nonculturable cells of *Vibrio vulnificus*. Appl Environ Microbiol 61:2620–2623

Orgambide G, Montrozier H, Servin P, Roussel J, Trigalet-Démery D, Trigalet A (1991) High heterogeneity of the exopolysaccharides of *Pseudomonas solanacearum* strain GMI 1000 and the complete structure of the major polysaccharide. J Biol Chem 266: 8312–8321

Palleroni NJ, Doudoroff M (1971) Phenotypic characterization and deoxyribonucleic acid homologies of *Pseudomonas solanacearum*. J Bact 107:690

Persson P (1998) Successful eradication of *Ralstonia solanacearum* from Sweden. EPPO Bull 28:113-119.

Poussier S, Prior P, Luisetti J, Hayward C, Fegan M (2000a) Partial sequencing of the hrpB and endoglucanase genes confirms and expands the known diversity within the *Ralstonia solanacearum* species complex. Syst Appl Microbiol 23:479–486.

Poussier S, Thoquet P, Trigalet– Démery D, Barthet S, Meyer D, et al. (2003) Host plant-dependent phenotypic reversion of *Ralstonia solanacearum* from nonpathogenic to pathogenic forms via alterations in the *phcA* gene. Mol Microbiol 49: 991–1003.

Poussier S, Trigalet-Démery D, Vandewalle P, Goffinet B, Luisetti J, Trigalet A (2000b) Genetic diversity of *Ralstonia solanacearum* as assessed by PCR-RFLP of the *hrp* gene region, AFLP and 16S rRNA sequence analysis, and identification of an African subdivision. Microbiology 146:1679-1692.

Poussier S, Trigalet-Démery D,, Barhtet P, Thoquet P, Arlat M , Trigalet A (2005) Phase reversion from phenotypic conversion mutants to wild type may be induced in *Ralstonia solanacearum* by a susceptible host plant. In: Bacterial wilt disease and the *Ralstonia solanacearum* species complex.American Phytopathol Soc Press, St. Paul, MN pp 351-357.

Poussier S, Vandewalle P, Luisetti J (1999) Genetic diversity of African and worldwide strains of *Ralstonia solanacearum* as determined by PCR-restriction fragment length polymorphism analysis of the *hrp* gene region. App Environ Microbiol 65:2184-2194.

Ralston E, Palleroni NJ, Doudoroff M (1973) *Pseudomonas pickettii*, a new species of clinical origin related to *Pseudomonas solanacearum*. Internat J of System Bacteriol 23:15–9

Rezsohazy R, Hallet B, Delcour J, Mahillon J (1993) The IS*4* family of insertion sequences: evidence for a conserved transposase motif. Mol Microbiol 9:1283-1295.

Roberts SJ, Eden-green SJ, Jones P, Ambler DJ (1990) *Pseudomonas syzygii* sp. nov., the cause of Sumatra disease of cloves. Syst Appl Microbiol 13:3443

Robertson AE, Wechter WP, Denny TP. Fortnum BA, Kluepfel DA (2004) Relationship between avirulence gene (*avrA*) diversity in *Ralstonia solanacearum* and bacterial wilt incidence. Mol Plant Microbe Interact 17:1376-1384.

Roszak DB, Colwell RR (1987) Survival strategies of bacteria in the natural environment. Microbiol Rev 51: 365–379

Rousseau P, Gueguen E, Duval-Valentin G, Chandler M (2004) The helix-turn-helix motif of Bacterial insertion sequence IS*911* transposase is required for DNA binding. Nucleic Acids Res 32:1335-1344.

Saddler GS (2005) Management of bacterial wilt disease In: Allen C, Prior P, Hayward AC (eds) Bacterial wilt disease and the *Ralstonia solanacearum* species complex. American Phytopathol Soc Press, St. Paul pp 121-132

Saile E, McGarvey JA, Schell MA, Denny TP (1997) Role of extracellular polysaccharide and endoglucanase in root invasion and colonization of tomato plants by *Ralstonia solanacearum*. Phytopathol 87:1264–1271

Salanoubat M, Genin S, Artiguenave F et al. (2002) Genome sequence of the plant pathogen *Ralstonia solanacearum*. Nature 415:497-502.

Schell MA (2000) Control of virulence and pathogenicity genes of *Ralstonia solanacearum* by an Elaborate sensory network. Annu Rev Phytopathol 38:263-292.

Spira B, Hu X, Ferenci T (2008) Strain variation in ppGpp concentration and RpoS levels in laboratory strains of *Escherichia coli* K-12. Microbiology 154: 2887-2895

Supriadi (2005) Present status of blood disease in Indonesia . In: Allen C, Prior P, Hayward AC (eds) Bacterial wilt disease and the *Ralstonia solanacearum* species complex. American Phytopathological Society Press, St. Paul, pp 395-404

Sun W, Roland KL, Branger CG, Kuang X, Curtiss R (2009) The Role of relA and spoT in *Yersinia Pestis* KIM5+ Pathogenicity. PLoS ONE 4: e6720

Swanson J, Yao J, Tans-Kersten J, Allen C (2005) Behavior of *Ralstonia solanacearum* race 3 biovar 2 during latent and active infection of geranium. Phytopathol 95:136-143

Taghavi M, Hayward C, Sly LI, Fegan M (1996) Analysis of the phylogenetic relationships of strains of *Burkholderia solanacearum, Pseudomonas syzygii*, and the blood disease bacterium of banana based on 16S rRNA gene sequences. Int J Syst Bacteriol 46:10–15

Tans-Kersten J, Huang H, Allen C (2001) *Ralstonia solanacearum* needs motility for invasive virulence on tomato. J Bacteriol 183:3597-3605

Tobes R, Pareja E (2006) Bacterial repetitive extragenic palindromic sequences are DNA targets for insertion sequence elements. BMC Genomics 7:62

Thomson N, Bentley S, Holden M et al. (2003) Fitting the niche by genomic adaptation. Nature Rev Microbiol 1:92-93

Valls M, Genin S, Boucher C (2006) Integrated regulation of the type III secretion system and other virulence determinants in *Ralstonia solanacearum*. PLoS Pathog 2:798-807

Van der Wolf JM, Bonants PJM, Smith JJ, Hagenaar M, Nijhuis E, van Beckhoven JRCM, Saddler GS, Trigalet A, Feuillade R (1998) Genetic diversity of *Ralstonia solancaerum* Race 3 in Western Europe determined by AFLP, RC-PFGE and Rep-PCR. In: Prior PH, Allen C, Elphinstone J (eds) Bacterial wilt disease: molecular and ecological aspects. Springer-Verslag, Berlin, Germany, pp 44-49.

Van Elsas JD, Bailey MJ (2002) The ecology of transfer of mobile genetic elements. FEMS Microbiol Ecol 42:187–197

Van Elsas JD, van Overbeek LS, Trigalet A (2005a) The viable but non culturable state in *Ralstonia solanacearum*: Is there a realistic threat to our strategic concepts? In: Allen C, Prior P, Hayward AC (eds) Bacterial wilt disease and the *Ralstonia solanacearum* species complex. American Phytopathological Society Press, St. Paul, MN pp 103-119

Van Elsas JD, van Overbeek LS, Bailey MJ, Schönfeld J, Smalla K (2005b) Fate of *Ralstonia solanacearum* biovar 2 as affected by conditions and soil treatments in temperate climate zones In: Allen C, Prior P, Hayward AC (eds) Bacterial wilt disease and the *Ralstonia solanacearum* species complex. American Phytopathological Society Press, St. Paul, MN pp 39-49

Van Elsas JD, Fry JC, Hirsch P, Molin S (2000a) Ecology of plasmid transfer and spread. In: Thomas CM (ed) The horizontal gene pool; bacterial plasmids and gene spread. Harwood Scientific Publishers, Amsterdam pp 175-206

Van Elsas JD, Kastelein P, de Vries PM, van Overbeek LS (2001) Effects of ecological factors on the survival and physiology of *Ralstonia solanacearum* bv2 in irrigation water. Canadian J Microbiol 47:842-854.

Van Elsas JD, Kastelein P, van Bekkum P, van der Wolf JM, de Vries PM, van Overbeek LS (2000b) Survival of *Ralstonia solanacearum* biovar 2, the causative agent of potato brown rot, in field and microcosm soils in temperate climates. Phytopathol 90:1358-1366.

Van Gijsegem F, Gough C, Zischek C, Niqueux E, Arlat M et al (1995) The *hrp* gene locus of *Pseudomonas solanacearum*, which controls the production of a type III secretion system, encodes eight proteins related to components of the bacterial flagellar biogenesis complex. Mol Microbiol 15:1095–1114

Van Overbeek LS, Eberl L, Givskov M et al. (1995) Survival of, and induced stress resistance in, carbon-starved *Pseudomonas fluorescence* cells residing in soil. Appl Environ Microbiol 61:4202-4208

Van Overbeek LS, Bergervoet JHH, Jacobs FHH, van Elsas JD (2004) The low-temperature-induced viable-but-non-culturable state affects the virulence of *Ralstonia solanacearum* biovar 2. Phytopathol 94:463-469

Villa JE, Tsuchiya K, Horita M, Opina N, Hyakumachi M (2005) Phylogenetic relationships of *Ralstonia solanacearum* species complex strains from Asia and other continents based on 16S rDNA, endoglucanase, and *hrpB* gene sequences. J Gen Plant Pathol 71:39–46

Wakimoto IU, Matsuo N, Angot A (1982) Multiplication of *Pseudomonas solancearum* in pure water, Ann Phytopath Soc Japan, 48:620-627

Wang YD, Zhao S, Hill CW (1998) Rhs elements comprise three subfamilies which diverged prior to acquisition by *Escherichia coli*. J Bacteriol 180: 4102-4110

Weber H, Polen T, Heuveling J, Wendisch VF, Hengge R (2005) Genome-wide analysis of the general stress response network in *Escherichia coli*: σ^S-dependent genes, promoters, and sigma factor selectivity. J Bacteriol 187:1591–1603

Weller SA, Elphinstone JG, Smith NC, Boonham N, Stead DE (2000) Detection of *Ralstonia solanacearum* strains with a quantitative, multiplex, real-time fluorogenic PCR (TaqMan) assay. Appl Environm Microbiol 66:2853–2858

Wendrich TM, Marahiel MA (1997) Cloning and characterization of a *relA/spoT* homologue from *Bacillus subtilis*. Mol Microbiol 26: 65-79

Wenneker M, Verdel MSW, Groeneveld RMW, Kempenaar C, van Beuningen AR, Janse JD (1999) *Ralstonia (Pseudomonas) solanacearum* race 3 (biovar 2) in surface water and natural weed hosts: First report on stinging nettle (*Urtica dioica*). Euro J Plant Pathol 105:307-315.

Whitesides MD, Oliver JD (1997) Resuscitation of *Vibrio vulnificus* from the viable-but-non-culturable state. Appl Environ Microbiol 64:3025-3028.

Williamson L, Nakoho K, Hudelson B, Allen C (2002) *Ralstonia solanacearum* race 3, biovar 2 strains isolated from geranium are pathogenic on potato. Plant Dis 86:987-991

Yabuuchi E, Kosako Y, Yano I, Hotta H, Nishiuchi Y (1995) Transfer of two *Burkholderia* and an *Alcafigenes* species to *Ralstonia* gen. nov.: proposal of *Ralstonia pickettii* (Ralston, Palleroni and Doudoroff 1973) comb. nov., *Ralstonia solanacearum* (Smith 1896) comb. nov. and *Ralstonia eutropha* (Davis 1969) comb. nov. Microbiol Immunol 39897-904

Yao J, Allen C (2006) Chemotaxis is required for virulence and competitive fitness in the bacterial wilt pathogen *Ralstonia solanacearum*. J Bacteriol 188:3697-3708.

Zhulin IB, Taylor BL (1995) Chemotaxis in plant-associated bacteria: the search for the ecological niche, In: Fendrik I, Del Gallo M, Vanderleyden L, de Zamaroczy M (eds.) *Azospririllum* VI and related microorganisms: genetics, physiology, and ecology. Springer-Verlag, Berlin, Germany, pp 451-459

Chapter II

Genetic and phenotypic diversity of *Ralstonia solanacearum* biovar 2 strains obtained from Dutch waterways

Patricia Stevens
Jan Dirk van Elsas

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Abstract

A novel set of *Ralstonia solanacearum* biovar 2 isolates was obtained, at several sampling occasions, from Dutch waterways, sediment and bittersweet plants and their genetic and phenotypic diversity was investigated. As reference strains, two previously-described strains obtained from diseased potato plants, denoted 1609 (Netherlands) and 715 (Bangladesh), were included in the analyses.

All novel isolates showed BOX and GTG5 PCR based genomic profiles similar to those of the reference strains. Also, PCR-restriction fragment length polymorphism (RFLP) analysis of the *phcA* and *hrp* genomic regions as well as sequence analysis of six selected genomic loci, revealed great homogeneity across the strains. In contrast, pulsed field gel electrophoresis (PFGE) of restricted genomic DNA revealed the distribution of all strains across four groups, denoted pulsotypes A through D (pulsotypes C and D had one representative each). Moreover, pulsotype B, consisting of five strains, could be separated from the other pulsotypes by a divergent genomic fingerprint when hybridized to a probe specific for insertion element IS*Rso3*.

Representatives of pulsotypes A, B and C were selected for growth and metabolic studies. They showed similar growth rates when grown aerobically in liquid media. Assessment of their metabolic capacity using BIOLOG GN-2 revealed a reduced utilization of compounds as compared to the reference strains, with some variation between strains.

Introduction

Ralstonia solanacearum biovar (bv) 2, the causal agent of wilting disease (brown rot) in potato and other plant species, is responsible for large economic losses in agriculture worldwide. The organism is thought to have its origin in tropical regions, in which it is often endemic. Its occurrence in temperate climate regions may relate to an initial introduction from infested plant material followed by spread from the infestation points. Hence, fostering our understanding of the behavior (survival, growth and diversification) of this organism in the open temperate climate environment is important, as it may eventually aid in the design of containment strategies for this organism.

A major issue in such epidemiological work is the correct identification of *R. solanacearum* isolates. Traditionally, the organism has been identified using plant infection tests (defining races), metabolism-based criteria (defining biovars) and molecular criteria. The taxonomy of *R. solanacearum* has recently undergone a major revision, and in the currently proposed scheme (mainly based on strains obtained from infested plants - (Fegan & Prior 2005) *R. solanacearum* bv2 (also denoted as race 3) belongs to the newly-proposed phylogroup II, sequevar 1. Given the ongoing discussions, we will in this paper use the traditional classification into biovars.

Over the last decades, *R. solanacearum* bv2 has increasingly been observed in infested areas in soils in temperate climate regions such as in The Netherlands, Belgium, France, Sweden, Spain and the UK (Janse 1996; Janse 1998; van Elsas et al. 2000; Caruso et al. 2005). 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 1998; Elphinstone et al. 1998). In particular bittersweet may have catalyzed the spread of the organism, given its capacity to serve as a colonizable host (Wenneker et al. 1999; van Overbeek and van Elsas 2006; Alvarez et al. 2008b).

Ecological work in microcosms has shown that *R. solanacearum* bv2 can persist, for varying periods of time, in different soil and water systems, as well as in the rhizosphere, plant residues and/or inside host plants (Granada GA & Sequira L 1983; van Elsas et al. 2000; Gorissen et al. 2004). The survival time (time to extinction) varied from weeks to months depending on the ecological conditions, offering possibilities for the organism to reach aquatic refuge niches from infested plants in soil, such as aquatic sediment or the aforementioned bittersweet.

Specifically, the survival in aquatic habitats was shown to strongly depend on temperature and to be negatively affected by light, salinity and the presence of other bacteria, lytic phages and protozoa (van Elsas et al. 2001; Alvarez et al. 2007). On the other hand, the presence of sediment in the water was shown to exert a positive effect on the survival of a population at low temperature (van Elsas et al. 2001). The bv2 strain used in such experiments, strain 1609, showed highest (>100 days) survival in sterile water at 20^{o}C (van Elsas et al. 2000), as well as in agricultural water at 12^{o}C (van Elsas et al. 2001). The organism was also shown to be an excellent scavenger of nutrients, even multiplying in sterilized demineralized water (Wakimoto et al. 1982).

In all work published to date, it was postulated that bv2 strains (Hayward 1991; Janse 1996; van der Wolf et al. 1998; Timms-Wilson et al. 2001) are genetically almost clonal. However, this conclusion was based on observations of strains that were mostly derived directly from infested

(potato) plants. There is a true paucity of knowledge on the level of diversity across bv2 strains obtained from the open environment, for the simple reason that such isolates have only been sparsely studied. Hence, the aim of this study was to determine to what extent *R. solanacearum* bv2, since its presumed introduction with infested plant material in the Netherlands in the 1990's, established and diversified in aquatic habitats. To achieve this aim, a set of isolates was obtained at different sampling times in two consecutive years. These were identified as true bv2 strains and subsequently analyzed molecularly and phenotypically. The molecular analyses consisted of GTG5 and BOX PCR based fingerprintings (Versalovic et al. 1994), hybridization with probes for insertion sequence IS*Rso3* (Jeong & Timmis 2000), analyses of the virulence regions *phcA* (Poussier et al. 2003) and *hrp* (Poussier et al. 1999; Poussier et al. 2000) and pulsed field gel electrophoresis of genomic DNA (Smith et al. 1995; van der Wolf et al. 1998). Also, oligolocus sequence typing (OLST) based on six genomic loci as well as analysis of a variable tandem repeat region were applied to selected strains. The phenotypic analyses included plant (tomato) pathogenicity tests, growth rate assessments and analyses of metabolic capacities.

Materials and methods

Bacterial strains - sampling, isolation and growth

Isolates were obtained at three different occasions (1st sampling in May 2004, 2nd in June 2004 and 3rd in October 2005) on four locations (denoted KZR, A, B and C) in the Northern part of the Netherlands, i.e. in Kommerzijlsterriet (KZR) and Reitdiep (locations A, B and C). In these regions, major outbreaks of potato brown rot had occurred in the 1990's. At each site, samples were taken from canal water, sediment and bittersweet plants. Per location, 2 x 50 ml canal water (sampled at 10-20 cm depth, approximately 2 meter away from the edge), one to several bittersweet plants growing at the canal side (roots in the water sediment), as well as 100-500g canal sediment were collected and analyzed for the presence of *R. solanacearum* bv2. Water temperatures ranged mostly from 13°C to 17 °C and were occasionally as low as 6°C; an overview of the samples is provided in Table 1.

Processing of the samples to obtain *R. solanacearum* bv2 were done as described elsewhere (Wenneker et al. 1999), with modifications. Briefly, for isolation from water, cells were pelleted by centrifugation (10,000 x *g* for 15 min) or collected on 0.2µm nitrocellulose filters. The cell pellets or concentrates were resuspended in 1 or 2 ml 0.01M phosphate buffer (pH 7.0) and dilution-plated on modified semi-selective SMSA medium(Elphinstone 1996). For isolation of *R. solanacearum* bv2 from bittersweet, stem and root material was surface-sterilized (10-30 s) with 70% ethanol and homogenized in 5-10 ml phosphate buffer using a sterilized mortar. For isolation from sediment, the sediment samples were left standing for 1 h, after which the upper water layer was discarded and the remaining sediment mixed with 50-100 ml phosphate buffer. Homogenized plant material or sediment was then incubated for 2 h with shaking at 28°C in 50 ml 0.01M phosphate buffer before dilution plating on modified SMSA medium. Plates were incubated for 4-5 days at 28°C. Screening for *R. solanacearum* bv2 was performed via colony PCR using the bv2 specific primer sets D2-B (Boudazin et al. 1999) and fliC (Schönfeld et al. 2003). This provided presumptive evidence for identity. Presumptive bv2 isolates were further purified on 0.1xTSBS (10% strength

Trypticase Soy Broth [Becton Dickinson and Company, Sparks, Md, USA], 0.1% sucrose; pH 7.2) or BGT (Bacto peptone 10 g, yeast extract 1 g, casamino acids 1 g, glucose 5 g; H_2O 1L; pH 7.2) agar (1.5%) plates and incubated for 2 days at 28°C, and rechecked using the same methods. The identity of the positive strains was confirmed by PCR using the *R. solanacearum* race 3-specific primer pair 630/631 (Fegan et al., 1998).

Table 1. Detection of *R. solanacearum* bv2 in bittersweet, water and sediment at five locations from a Dutch waterway

			number of positive samples/number of samples			total number of		
month/year	water temp.	location	bittersweet	water	sediment	samples	positive	isolates purified
May 2004	14°C	KZR	1/3	0/4	0/3	10	1	12
		NZ	0/1	0/4	0/1	6	0	
June 2004	17°C	A	3/3	1/1[1]	1/2	6	5	13
		B	0/2	1/1[1]	1/2	5	2	3
		C	0/2	1/1[1]	0/2	5	1	2
October 2005	13°C	A	1/1	1/2	0/2	5	2	11
		B	1/1	0/2	0/2	5	1	1
		C	0/1	0/2	0/2	5	0	
Februari 2005	6°C	A	nd	0/2	0/2	4	0	
		B	nd	0/2	0/2	4	0	
		C	nd	0/2	0/4	6	0	

nd not determined, as no bittersweet plants were found at that sampling event.
[1] The water samples analyzed in June 2004 were of larger volume than the others (4 9 50 ml), which was combined before enrichment and plating on SMSA culture plates.

The confirmed new isolates (42 in total), next to the reference strains used, are listed in Table 2. All reference strains, including those kindly received from T. Timms-Wilson, Oxford, UK, had already been identified as *R. solanacearum* bv2 (Timms-Wilson et al. 2001). Confirmation of the identity of the novel isolates was further obtained via sequence analysis of the 16S ribosomal RNA gene. For this, the representative isolates KZR-5, PA1, PA5, SA31 and WC76 were used, using primers B8-F/B27F and 1492-R (GATC biotech, Germany). See Table 3.

Table 2. *R. solanacearum* bv2 strains characterized in this study

strain	month/year of isolation	location/ country	bittersweet plant nr.	source[1]	virulence	pulsotype XbaI [2]	ISRso3 group [3]	TR0578 [4]	OLST [5]
1609	1995	Netherlands	-	potato	moderate	A	1	5,6	+
715	unknown	Bangladesh	-	potato	Vir	A	2	5,5	+
KZR-1	May 2004	KZR	1	s	Vir	A	1	5,7	+
KZR-2	May 2004	KZR	1	s	Vir	A	1	5,7	+
KZR-3	May 2004	KZR	1	s	Vir	A	1	5,7	nd
KZR-5	May 2004	KZR	1	s	Vir	C	1	5,7	+
KZR-6	May 2004	KZR	1	s	nd	A	1	nd	nd
KZR-7	May 2004	KZR	1	s	nd	A	1	nd	nd
KZR-8	May 2004	KZR	1	s	nd	A	1	nd	nd
KZR-9	May 2004	KZR	1	s	nd	A	1	nd	nd
KZR-10	May 2004	KZR	1	s	nd	A	1	nd	nd
KZR-12	May 2004	KZR	1	s	nd	A	1	nd	nd
KZR-13	May 2004	KZR	1	s	nd	A	1	nd	nd
KZR-14	May 2004	KZR	1	s	nd	A	1	nd	nd
PA1	June 2004	A	2	s	Vir	B	2	5,6	+
PA2	June 2004	A	2	s	nd	B	2	5,6	nd
PA4	June 2004	A	2	s	nd	B	2	5,6	nd
PA5	June 2004	A	2	s	nd	A	1	5,6	nd
PA8	June 2004	A	3	s	Vir	A	1	5,6	nd
RA9	June 2004	A	3	r	Vir	B	2	5,6	+
RA12	June 2004	A	3	r	Vir	A	1	5,6	nd
RA13	June 2004	A	3	r	nd	A	1	5,6	nd
RA16	June 2004	A	4	r	nd	A	1	5,6	nd
RA18	June 2004	A	4	r	nd	A	1	nd	nd
WA19	June 2004	A	-	water	Vir	B	2	nd	nd
WA20	June 2004	A	-	water	nd	A	2	5,6	+
SA31	June 2004	A	-	sediment	Vir	A	1	5,6	nd
WB48	June 2004	B	-	water	Vir	A	1	5,7	+
WB49	June 2004	B	-	water	nd	A	1	5,6	nd
SB63	June 2004	B	-	sediment	Vir	A	1	5,6	nd
WC76	June 2004	C	-	water	Vir	D	1	5,6	nd
WC78	June 2004	C	-	water	nd	A	1	5,6	+
RA05-9	Oktober 2005	A	5	r	nd	A	1	5,6	+
RA05-10	Oktober 2005	A	5	r	nd	A	1	5,6	+
RA05-11	Oktober 2005	A	5	r	nd	A	1	5,6	+
RA05-12	Oktober 2005	A	5	r	nd	A	1	nd	nd
RA05-13	Oktober 2005	A	5	r	nd	A	1	nd	nd
PA05-16	Oktober 2005	A	5	s	nd	A	1	nd	nd
PA05-17	Oktober 2005	A	5	s	nd	A	1	nd	nd
PA05-18	Oktober 2005	A	5	s	nd	A	1	nd	nd
PA05-21	Oktober 2005	A	5	s	nd	A	1	5,6	+
PA05-22	Oktober 2005	A	5	s	nd	A	1	nd	nd
WA05-6	Oktober 2005	A	-	water	nd	A	1	nd	nd
PB05-28	Oktober 2005	B	6	s	nd	A	1	5,6	+

[1] *R. solanacearum* cells were isolated from either stems (s) or roots (r) of *S. dulcamara*

[2] pulsed field gel electrophorisis pattern (pulsotype) after analysis of *XbaI* digested genomic DNA

[3] IS*Rso3* duplication was scored as a single polymorphic band. Group 2 : presence of a 8Kb hybridising band. Group 1: no 8Kb hybridising band present

[4] Tandem repeat region TR0578 as defined by Neil Parkinson, Central science laboratory, York

[5] Oligolocus sequencing, as shown in Table 4, was performed for selected strains (+) nd: not determined

For routine analyses, *R. solanacearum* bv2 strains were grown in 0.1xTSBS at 28°C. To determine the doubling times at either 16°C or 28°C of the selected strains 1609, 715, KZR-1, KZR-5, PA1 and PA5, cultures were grown (in duplicate) in 0.1xTSBS at the indicated temperatures and CFU numbers were

determined over time. For experiments at 28°C, the absorption values at 660 nm were measured at 40, 185, 260, 345 and 430 min and converted to CFU via a calibration curve. For experiments at 16°C, we used direct dilution plating for CFU enumerations at 0, 19, 43, 48 and 54 hours.

Virulence tests

Selected *R. solanacearum* bv2 strains (n=15) were tested for pathogenicity (Arlat & Boucher 1991) on at least eight 2-4 week old tomato plants (*Solanum lycopersicon cv Maribel*) in sterile peat microcosms. Strain introduction was performed by watering the substrate (50 g dry weight sterile peat soil) with 25 ml of tenfold diluted (in sterile demineralized water) bacterial suspension that had grown overnight in liquid 0.1xTSBS (final inoculum density approximately 10^8 CFU/ml). Prior to inoculation, plants were kept without added water for 2 days and roots were slightly damaged by gently moving the plant up and down in the soil. This procedure (cell density, root wounding) offered the correct window for virulence testing with the plant/peat combination that was available. Plants were incubated in the greenhouse under a 26°C (day, 14 h) / 21°C (night, 10 h) regime, and disease development in the plants was scored at regular time intervals using a disease matrix ranging from 0 (no wilting symptoms) to 4 (all leaves wilted) (Winstead & Kelman, 1952).

DNA extraction

For routine analyses, total genomic DNA was extracted from 4-ml liquid 0.1xTSBS cultures grown at 28°C, using the Ultraclean™ microbial DNA extraction kit according to the manufacturer's protocol (MoBIO Laboratories Inc. Carlsbad, UK). This consistently yielded 0.1-5µg/µl DNA of high quality, as measured via agarose gel electrophoresis and staining with ethidium bromide.

Plasmid extraction was done according to Birnboim and Doly for plasmids 5-100Kb in size (Birnboim & Doly 1979) or using the commercial Qiagen plasmid extraction kit for plasmids up to 50 Kb (Benelux B.V, Venlo, The Netherlands). Genomic DNA for use in Southern hybridisation experiments was extracted essentially as described elsewhere (Sambrook & Maniatis 1989). Genomic DNA of strain UW551 was kindly received from Caitilyn Allen (Wisconsin, Madison, USA).

Table 3. Primers and PCR conditions used in this study

Primer [1]	Sequence of primer (5'-3')	PCR conditions (°C) [2]	reference
D2-F	5' GTCCGGAAAGAAATCGCTTC 3'	60	Boudazin et al. 1999
B-R	5' GGCGGGACTTAACCCAACATC 3'	60	Boudazin et al. 1999
B8-F	5' AGAGTTTGATCMTGGCTCAG 3'	55	Lane et al. 1991
27-F	5' AGAGTTTGATCMTGGCTCAG 3'	55	Lane et al. 1991
1492-R	5' GGTTACCTTGTTACGACTT 3'	55	Lane et al. 1991
fliC-F	5' GAACGCCAACGGTGCGAACT 3'	60	Schönfeld et al. 2003
fliC-R	5' GGCGGCCTTCAGGGAGGTC 3'	60	Schönfeld et al. 2003
BOX A1R	5' CTACGGCAAGGCGACGCTGACG 3'	50	Versalovic et al. 1994
GTG5	5' GTGGTGGTGGTGGTG 3'	43	Versalovic et al. 1994
phcA-F	5' ATCAAGGTCGTGAGCTGGTA 3'	57	this study
phcA-F2	5' GCACGCCAAGGTTGTCGAGT 3'	57	this study
phcA-R	5' ATCAAGGTCGTGAGCTGGTA 3'	57	this study
phcA-R2	5' CAACGTACCGGCGAAGCTGA 3'	57	this study
GMIHrcV-F	5' ATCGGTATCGCCGCGCTAGT 3'	60	this study
GMIHrcV-R	5' TGCACCGTGGTGATGATCAG 3'	60	this study
pglA-F2	5' GCAGAACTCGCCCAACTTCC 3'	68 [3]	this study
hrcV-R2	5' CGCCTCCACCAAGTCCATTC 3'	68 [3]	this study
hrpB-F2	5' CGTGGTGTCGTGCCGCAATA 3'	68 [3]	this study
hrpB-R	5' TGCCGGAGTCGTCGTCATAC 3'	68 [3]	this study
cbhA-F	5'AGCTGCCTCACTACTAACTG 3'	52	this study
cbhA-R	5' CCGGCTGTAGTTCCTTGAAT 3'	52	this study
spoT-F	5' GAACTGCGTTGGAGGCCATC3'	60	this study
spoT-R	5' TATCCAAGAAGCAGGCTGAG 3'	60	this study
holC-F	5' CTACGGCGTGTTCGTCTTCA 3'	59	this study
holC-R	5' CATCAGCACCGACAGGATCT 3'	59	this study
mutS-F	5' GGCGACTTCTACGAGCTGTT 3'	59	this study
mutS-R	5' CGGTGTCCAGGCCGATGAAT 3'	59	this study
TR0578-F	5' CATACGCCGGCGTCAGCACGCT 3'	59	Parkinson pers. comm
TR0578-R	5' GTGGCCATCACGATCGCCTTGTC 3'	59	Parkinson pers. comm

[1] F, forward primer, R, reverse primer

[2] annealing temperatures. Standard PCR reactions were proceeded by a 5 min denaturation step at 96°C, followed by a final extension step for 5 min at 72°C.

[3] denaturation for 45s at 96°C, cycling of 20s at 96°C and a single annealing and extension step of 15 min at 68°C, followed by a final extension step for 13 min at 72°C.

Use of PCR to detect and analyze specific gene regions in *R. solanacearum*

16S rRNA gene (D2-B), fliC and phcA

PCR primers and reaction conditions used for amplification of the respective genomic regions are listed in Table 3. For PCR amplification, we used the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, USA). Standard PCR reaction mixtures contained 1x PCR buffer (1.5 mM MgCl, 10 mM Tris and 50 mM KCl, Roche Applied Science, Basel, Switserland), 2.5 mM $MgCl_2$, 10% DMSO, 200 μM each deoxynucleotide, 0.2 μM of each primer and 20 U/ml Taq DNA polymerase (Roche Applied Science, Basel, Switserland). When desired, PCR products were digested with 10 U of the appropriate enzyme according to the manufacturer's instructions (Fermentas Life sciences, EU) and analyzed on 1.5% agarose (Roche diagnostics, Mannheim, Germany) (Sambrook & Maniatis 1989)

BOX and GTG5

For BOX and GTG5 genomic fingerprintings, we used a twofold concentrated PCR buffer, 500 μM of each deoxynucleotide, 2 mg/ml bovine serum albumin (BSA), 0.4 μM primer and 80 U/ml Taq DNA polymerase (Roche Applied Science, Basel, Switzerland). Amplicons were analyzed by electrophoresis on 1.5% agarose gels.

Development of a PCR-based hrp fingerprinting system

For amplification of the *hrp* gene region (partial) we used a forward primer localized in the *pglA* gene (PglA-F2) and a reverse primer in the *hrcV* gene (HrcV-R2), which were both based on the sequences of these genes in strain 1609. To obtain these sequences, we determined the *hrcV* sequence for bv2 by cloning a strain 1609 derived 300 bp PCR product obtained using PCR primers GMIHrcV-F and GMIHrcV-R, based on the GMI1000 genome sequence (Salanoubat et al. 2002). Sequencing of this insert and comparison with *hrcV* sequences in the NCBI database indeed revealed a partial *hrcV* gene, on the basis of which a second, presumably bv2 specific, reverse primer was designed. The sequence of the bv2 *pglA* gene was kindly received from Christian Boucher (INRA, Toulouse, France).

For amplification of the genomic region between the *pglA* and *hrcV* primer target sites (28.2 Kb on the basis of information from bv2 strain UW551), we used the TaKaRa La Taq polymerase kit (code RR020AG, Takara BIO INC., Shiga, Japan), which is suitable for amplification of large DNA regions including high G+C content DNA. For amplification, the 2xGC buffer I supplied in the kit was used, and the supplied dNTP mixture (final concentration 400 μM of each nucleotide) following the manufacturer's instructions. The cycling program was as follows: denaturing at 96°C for 3 min; 30 cycles (96°C for 20 sec, 68°C for 15 min) and a final extension step of 72°C for 15 min. For comparison with GMI1000 we used two additional primers, hrpB-F2 (based on the *hrpB* sequence of strain 1609) and hrpB-R (based on the *hrpB* sequence of strain GMI1000 (Table 3).

Oligolocus sequence typing (OLST)

For fifteen selected strains (see Table 2), we applied OLST. PCR conditions were as described above (Section **Use of PCR to detect and analyze specific gene regions in *R. solanacearum***). We analyzed the partial sequences of the following six genes: *phcA* (global virulence regulator), *mutS* (DNA mismatch repair protein), *hol*C (DNA polymerase III halo enzyme subunit), *cbhA* (cellobiohydrolase), *relA*/*spoT* (stress response related hypothetical protein) and *fliC* (flagellar protein) by PCR amplification and sequencing. We also analyzed tandem repeat region TR0578 (N. Parkinson, unpublished data) which is present in hypothetical protein RRSL_04153 (strain UW551 - (Gabriel et al. 2006)) for a selected 27 strains (Table 2). Sets of primers were designed based on sequence information derived from either strain UW551 or 1609, to amplify internal fragments for all genes except *phcA*. For the latter, we designed a primer 349 bp upstream of the start codon (phcA-F2, Table 3). For all sequencing reactions, extension from the forward primer was used, with the exception of *cbhA*, for which we used the reverse primer (Table 4).

DNA sequencing was performed in Applied Biosystems 3130 or 3730XL sequencers. Sequences were aligned using the program MEGA4 (Tamura et al. 2007) and trimmed to obtain sequences of identical length. The length of the sequences used for comparison of strains is shown in Table 4.

Table 4 Selected loci, primers and sequence lengths used for OLST analysis

locus	nomenclature strain UW551	n strains	sequence primer	sequence length (bp)
phcA	RRSL_02699	15	F2	383
mutS	RRSL_01926	15	F	328
holC	RRSL_03599	15	F	308
cbhA	RRSL_02065	15	R	484
relA/spoT	RRSL_02057	15	F	502
fliC	RRSL_02321	15	F	277

[1] One of the selected strains (strain KZR-5) did not yield a PCR product

Cloning procedures

For cloning of the PCR products, we used the pGEM-T easy vector system (A1360) according to the manufacturer's protocol (Promega Corporation, Madison USA). For transformation we used competent *E. coli* MM294 cells (Sylphium Life Sciences, Groningen, Netherlands)

IS*Rso3* detection via Southern hybridization

For preparation of an IS*Rso3* DNA probe we used plasmid ePST001 that was obtained by cloning of the PCR product obtained with PCR primers PhcA-F2 and R2 on genomic DNA of a spontaneous *phcA* mutant of strain 1609. This plasmid (containing IS*Rso3* and part of the *phcA* gene) was restricted with the restriction enzymes *AvaI* and *RsaI*. The resulting 610-bp fragment - corresponding to position 296-905 of the IS*Rso3* sequence - was excised from agarose gel and purified using the Qiaex II gel extraction kit (Qiagen Benelux B.V, Venlo, Netherlands). The purified 610-bp fragment was used for labeling by the digoxigenin (DIG) DNA labelling kit (Roche Applied Science, Penzberg, Germany).

For Southern hybridization analysis, 5-10 µg of *PstI*-digested genomic DNA of all strains listed in Table 2 was transferred to a Hybond-N nitrocellulose membrane (Amersham Biosciences Benelux, Roosendaal, The Netherlands). Hybridization (at 48°C) was done using the DIG DNA detection kit (Roche) according to the manufacturer's protocol.

Pulsed field gel electrophoresis (PFGE)

0.5 OD_{660} units of a 0.1x TSBS overnight culture (OD 0.5-1) was collected and mixed with 150 µl $TE_{25}S$ (0.3 M sucrose, 25 mM Tris, 25 mM EDTA) and 200 µl 1% pulsed-field-certified agarose (Bio-Rad, Hercules, CA). The mixture was then added to a plug mold while still molten. After solidification, the block was placed in 500 µl $TE_{25}S$ + 2 mg/ml lysozyme (Merck AG, Darmstadt, Germany) and incubated for 2h at 37°C. The agarose plugs were then incubated in NDS solution (0.5 M EDTA, 10 mM Tris, 1% lauroyl sarcosine) containing 1 mg/ml proteinase K (Merck AG, Darmstadt, Germany) for 48h at 50 °C. The plugs were washed twice in $T_{20}E_{50}$ (20 mM Tris, 50 mM EDTA) and twice in $T_{10}E_{10}$ (10 mM tris, 10 mM EDTA) with 1 mM PMSF. Washing steps were performed for 1 h on ice. Finally, the plugs were washed once and stored in $T_{20}E_{50}$ at 4 °C.

Restriction of genomic DNA in agarose plugs with *XbaI* (New England Biolabs Inc. Beverly, MA) was done according to the manufacturer's instructions. First, the plugs were washed for 1h in 500 µl 0.5 x $T_{10}E_{10}$ at room temperature. After pre-incubation of 1 h in restriction buffer, the buffer was refreshed by restriction buffer containing 60U of *XbaI*. Plugs were incubated overnight at 37°C and the reaction was stopped by adding 1/10 volume of 0.5 M EDTA, pH 8.

Analysis of the samples was performed using a CHIEF-DR II pulsed field gel electrophoresis system. For separation of *XbaI*- digested genomic DNA, we used 1% agarose in 0.5 X TBE at 14°C. Electrophoresis was conducted for 22 h with a switch time of 1-80 s, angle of 120° and voltage of 6V/cm. A phage lambda size marker (New England Biolabs) was used to estimate band sizes. For separation of the two (intact) replicons we used - for each strain - 1/3 agarose plug containing unrestricted genomic DNA. Samples were separated in 0.8% pulsed-field-certified agarose (Bio-Rad, Hercules, CA) in 1 x TAE at 14°C. Electrophoresis was conducted for 48h with a switch time of 500s, 3 V/cm and an angle of 105^0. A high molecular weight *Hansenula wingei* Mb size marker (Bio-Rad, Hercules, CA) was used to estimate fragment sizes.

BIOLOG assay

To test selected strains for their ability to utilize different carbon sources, we used BIOLOG GN2 plates **(**Biolog Hayward, CA) in duplicate or triplicate experiments. Cells were grown overnight in 0.1xTSBS (OD_{660} 0.5-1,.0), washed twice in 0.85% NaCl and diluted to OD_{660} 0.10 in 0.85% NaCl. One hundred µl of washed cells was transferred to each well of the 96-well GN2 plates and the plates were incubated at 28°C. Each well was scored daily (up to 5 days) for colour formation (visually). Wells that showed weak colour formation were scored as +/-. Because not all strains scored positive for the wells containing D-cellobiose, D, L lactic acid and malonic acid (which are known to be utilized by bv2 strains; OEPP/EPPO, 2004), we tested our strains for growth on these substrates in liquid M63 medium (Amresco, USA) which contained 2.0 g/L ammonium sulfate, 13.6 g/L potassium phosphate, 0.5 mg ferrous sulphate x $7H_20$, 1mM $MgSO_4$ supplemented with 0.5% of the respective carbon source. Incubation was at 28°C, with shaking, and growth was monitored every 2 days by plating on 0.1x TSBS agar plates for up to 10 days.

Statistical analyses

The banding patterns obtained in the PFGE analysis (*XbaI*-fragmented DNA) and the IS*Rso3* hybridization fingerprints were used to generate a matrix indicating the presence or absence (scored as 0 or 1, respectively) of the bands detected using these tools. A total of 40 bands were scored (20 for PFGE and 20 for IS*RSo3* detection). A combined dendogram was constructed using UPGMA and Euclidean distance. For this analysis, we used the software package Statistica 8.0.Growth rates (doubling times) of strains were compared across strains using Student's t-test applied on the replicates. These were judged to be significant at $P<0.05$.

Nucleotide sequence accession numbers

The obtained sequences were deposited in the Genbank data library as follows: 16S rRNA gene sequences of selected strains: accession numbers GQ266171 to GQ266175 and GQ266265 to 266269, the 1609 *hrcV* partial sequence: accession number GQ266272, OLST-generated sequences: accession numbers GQ266176 to GQ266264 and GQ266273 to GQ266299 and the sequence of

plasmid ePST001 containing IS*Rso3* inserted in *phcA* region under accession numbers GQ266270 and GQ266271.

Results

Isolation and identification of *R. solanacearum* bv2 strains from water, sediment and bittersweet

For detection of *R. solanacearum* bv2 in Dutch local waterways, we analyzed 30 samples obtained at four sampling events from water, sediment and bittersweet plants. Five different locations were involved (Table 1). Overall, 12 out of 61 samples were positive for *R. solanacearum* bv2 (20%). Specifically, *R. solanacearum* isolates were not found in February 2005, when the water temperature was relatively low, i.e 6^{o}C (six samples from water and eight from sediment, Table 1). When the water temperature was higher (17^{o}C), putative *R. solanacearum* bv2 colonies were obtained from 50% of the samples (eight out of 16 samples analyzed, Table 1). These colonies, when sufficiently separated from other colonies, showed typical fluid irregular morphologies on SMSA. PCR screening with the bv2 specific primer sets D2-B and fliC (Boudazin et al. 1999; Schönfeld et al. 2003) identified all such irregularly-shaped colonies as presumptive *R. solanacearum* bv2 strains.

Following streaking to purity, a total of 42 new presumptive bv2 strains, encompassing all isolates from 2004 and 2005, were obtained from canal water, sediment and bittersweet stems and roots (Table 2). In the 1st sampling, twelve *R. solanacearum* bv2 strains were obtained exclusively from bittersweet (Table 2). In a 2nd sampling we obtained six canal water strains (locations A, B and C), two sediment strains (locations B and C) and ten strains from bittersweet. In the 3rd sampling, another set of strains was obtained from canal water as well as bittersweet at locations A and B (Table 2). To confirm that the 42 novel strains belonged to *R. solanacearum* bv2, we successfully applied PCR 630/631, which has been shown to be specific for bv2 strains (race 3 – potato-infective) (Fegan et al., 1998, Table 3).

In addition, the almost complete 16S rRNA gene sequences were determined (using primers 27-F and 1492-R) for a random selection of novel strains, i.e. KZR-5, PA1, PA5, SA31 and WC 76. Analyses of the sequences showed that they were all internally consistent and 100% identical to those of the reference bv2 strains 1609 and 715. Also, an expected similarity to bv3 strain GMI1000 was noted, with just 7 in 1,387 nucleotides different. The new strains were convincingly identified and distinguished from other biovars by a bv2 specific triplet (TTC) that is typically present at positions 458-460 (*Escherichia coli* numbering system; (Boudazin et al. 1999).

To identify the strains as plant pathogens that are typically able to cause wilting disease in susceptible plants, virulence tests on tomato were performed. All selected strains tested (15 out of 42, Table 2**)** caused wilting of the tomato plants. Specifically, 75-100% wilting was found within 21 days after inoculation. This level of virulence was similar to that exhibited by reference strain 715 in the same test.

Genomic fingerprintings

BOX and GTG5 fingerprintings were used to compare the genomic fingerprints of the new environmental strains with those of two reference potato strains, i.e. 1609 and 715, as well as with

that of bv3 strain GMI1000. For both systems, the patterns of all 42 strains were similar to each other as well as to those of reference strains 1609 (van Elsas et al. 2000) and 715 (Timms-Wilson et al. 2001). Bv3 strain GMI1000 (Salanoubat et al. 2002) produced divergent patterns (data not shown). In both the BOX and GTG5 patterns, a few (1–3) bands were shown to vary in intensity, but these different band intensities were not consistent across separate PCR reactions even on the same template DNA. This method-related variation, often seen in genomic fingerprinting, was not further taken into account (Svec et al. 2005).

Analysis of the *phcA* and *hrp* gene regions

To test whether the *phcA* and *hrp* gene regions of the novel environmental isolates showed polymorphisms as compared to the reference strains, we analyzed the entire strain set by PCR/RFLP analysis of the *phcA* and *hrp* genomic regions (Table 2) and compared these with the reference strains.

phcA region - For *phcA*, all novel strains produced amplicons of the expected 2.1 Kb, which was similar to the products obtained with reference bv2 strains 1609 and 715. In contrast, bv3 strain GMI1000 showed, as expected, a product of approximately 1.9 Kb. Indeed, alignment of the GMI1000 sequence with bv2 sequence data (strain 1609; kindly received from Christian Boucher, INRA, Toulouse, France) showed the presence of an additional 183 nucleotides in bv2 in the region upstream of the *phcA* gene (included in the amplicons). Digestion of all bv2 strain amplicons with either *PstI* or *EcoRV* resulted in bands that corresponded with the expected sizes of 282, 878 and 999 bp (*PstI*) and 136, 877 and 1,149 bp (*EcoRV)*, totalling about 2.1 kB. In this respect, no difference was detected between any of the novel strains and reference strains 1609 and 715.

hrp gene region - To compare the *hrp* regions of the 42 novel bv2 strains with those of reference strains 1609 and 715, we investigated the region between the *pglA* and *hrcV* genes, of approximately 28 Kb, by PCR/RFLP. Amplification (pglA-F2 / hrcV-R2 primers; Table 3) of genomic DNA of all new strains consistently yielded amplicons of sizes similar to those of reference strains 1609 and 715. To detect differences that might eventually have occurred due to small deletions/insertions or inversions, the amplicons were restricted with *BsrBI*, *HinfI* or *RsaI* in separate and analyzed by gel electrophoresis. Overall, 20-25 bands were produced per strain, depending on the restriction enzyme used. Again, no differences were detected between the patterns generated with any of the strains. The patterns of all new strains also closely resembled those of strains 1609 and 715. As a way of example, Fig. 1 shows the restriction patterns produced with strain KZR-5 in comparison to that of strain 1609.

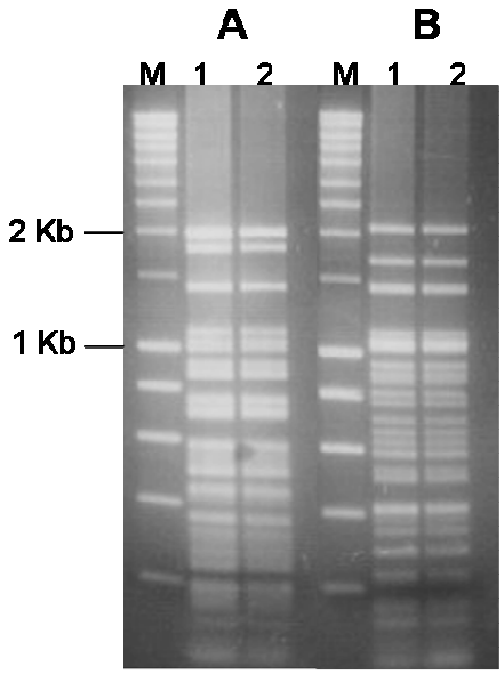

Figure 1. Agarose gel showing fingerprints of the *hrp* region (primerset pglA-F2 and hrcV-R2) after restriction with A) *HinfI* and B) *RsaI*. Lane M is Kb+ molecular size marker, lane 1 is strain 1609; lane 2 is strain KZR-5.

Analysis of genomic make-up by direct PFGE and screening for plasmids

To estimate genome sizes, we selected three novel isolates, i.e. strains KZR-5, PA2 and PA5, next to the reference strains 1609 and 715, for analysis by PFGE of unrestricted genomic DNA. Given that the genomic make-up of *R. solanacearum* bv3 is known, reference bv3 strain GMI1000 was also used (Fig. 2a).

The analysis showed that the genomic make-up of the novel isolates was internally consistent and similar to that of the reference strains 1609 and 715. Specifically and akin to strain GMI1000, all bv2 strain genomes showed the presence of two large replicons, estimated to be approximately 2 and 3.5 Mb in size, for a total genome size of about 5.5 Mb. This is in the range of the sizes of the two replicons of strain GMI1000, i.e. respectively 2.1 and 3.7Mb, for a total of 5.8 Mb (Fig. 2A). Using the plasmid extraction method of Birnboim and Doly (Birnboim & Doly 1979), we found no evidence for the presence of smaller plasmids (about 5 to 100 Kb range) in our novel isolates.This corroborated the lack of small plasmids in reference strains 1609 and 715 (data not shown).

Genomic variation between strains observed using PFGE of restricted genomic DNA

To detect putative differences between the genomes of the novel bv2 strains and compare these to the reference strains, *XbaI*-digested genomic DNA was analyzed via PFGE together with that of strains 1609 and 715. Replicate PFGE analyses revealed consistent patterns for each strain (not shown). A few bands that were polymorphic between several strains were apparent (Fig. 2B). Overall, four different banding patterns, hereafter denoted as pulsotypes A through D, were found. Specifically, the patterns of most novel strains (35 out of 42) were identical to those of reference strains 1609 and 715, giving rise to a large group, denoted as pulsotype A (see Table 2). Pulsotype B differed from pulsotype A by two bands, of approximately 85 and 110 Kb in size

(arrows 1 and 2, Fig. 2B). These were apparently correlated, as they were either both present or absent. This pulsotype encompassed five strains that had all been obtained from location A (Fig. 2B, lanes 7-9 and 11).

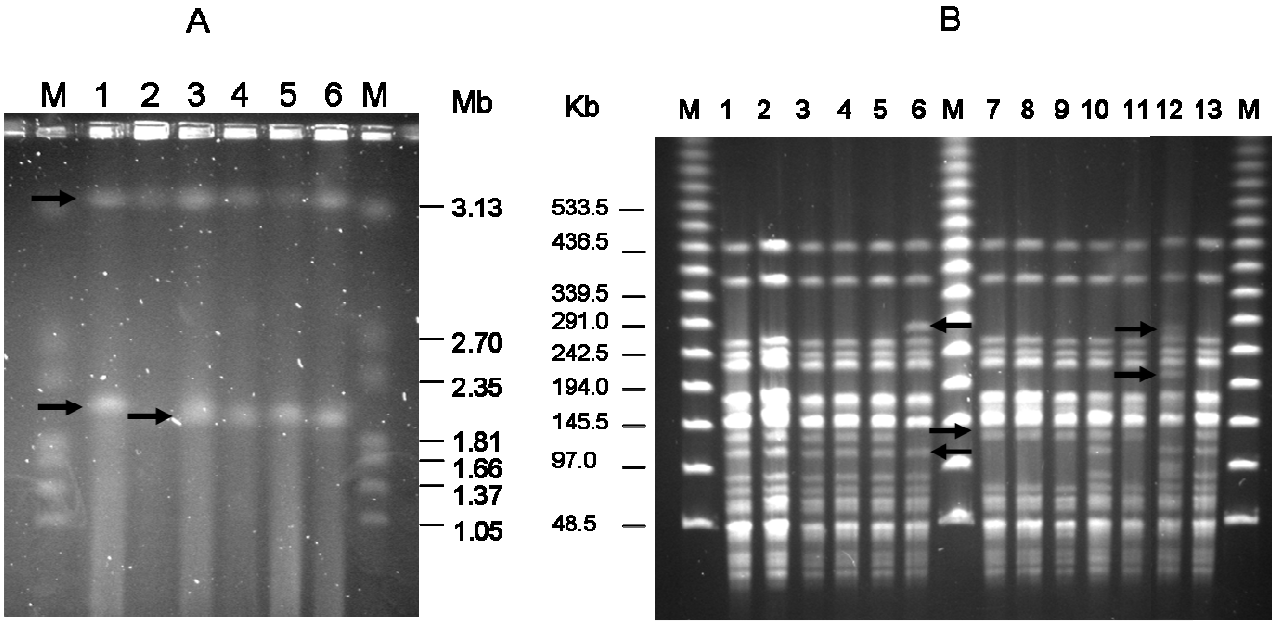

Figure 2 (A) Agarose gel of uncut genomic DNA of *R. solancearum* strains, showing the two circular replicons. Lane M is *H. wingei* chromosomal marker, lane 1: GMI1000 (bv3), lane 2: 1609, lane 3: 715 lane 4: KZR-5, lane 5: PA2, lane 6: PA5. Run conditions were: 0.8% chromosomal grade agarose (1 x TAE), switchtime of 500s, 3V/cm, 14^{o}C for 48 hours. **(B)** Agarose gel showing pulsed field gel electrophoresis profiles of *XbaI* digested genomic DNA of *R. solanacearum* strains. Lane M is *lambda* marker, lane 1: 715, lane 2: 1609, lane 3: KZR-1, lane 4: KZR-2, lane 5: KZR-3, lane 6: KZR-5, lane 7: PA1, lane 8: PA2, lane 9: PA4, lane 10: PA5, lane 11: WA19, lane 12: WC76, lane 13: WC78. Arrows: polymorphic bands. Run conditions were: 1% pulsed field certified agarose (0.5 x TBE), switchtime 1-80s, 6V/cm, 14^{o}C for 22 hours.

Two other pulsotypes, denoted C and D, were detected in single strains obtained from locations KZR and C. Specifically, pulsotype C consisted of one strain (out of twelve) from location KZR that differed from pulsotype A in that it showed an additional band of approximately 280 Kb, while a 110 Kb band was lacking (Fig. 2B, lane 4). Pulsotype D referred to one strain from location C (of two) that showed another divergent PFGE pattern (Fig. 2B, lane12).

Screening for IS*Rso3*

On the assumption that insertion element movement rather than mutation may have been a driver for the short-term genetic diversification in *R. solanacearum* bv2 in the open environment, we assessed the distribution of the IS*Rso3* element – an element typically found in *R. solanacearum* genomes - in the genomes of the 42 new and two reference strains. In all patterns generated by Southern hybridization with the IS*Rso3* probe, >20 bands were visualized, which ranged in size from 0.5 to 8 Kb. Overall, the patterns were quite similar across most strains, defining IS*Rso3* group 1. However, a conspicuous single band, of approximately 8 Kb (Fig. 3), was detected in six novel strains, all from location A (Fig. 3, lanes 5 and 6). This band was also found in reference

strain 715 but not in 1609 (data not shown). The group of strains that harbored this extra IS*Rso3* copy was denoted IS*Rso3* group 2.

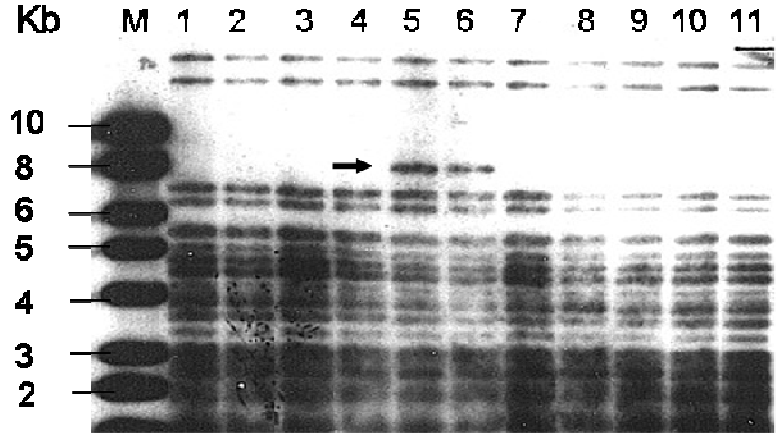

Figure 3. Southern blot analysis of *R. solanacearum* genomic DNA after restriction with *PstI* and hybridisation with an IS*Rso3-AvaI/RsaI* (610 bp) fragment as DNA probe. Lane M is Kb+ molecular size marker. Strains, lane 1: 1609, lane 2: KZR-2 lane 3: KZR-5, lane 4: KZR-1, lane 5: PA1, lane 6: PA2, lane 7: RA18, lane 8: SA31, lane 9: SB63, lane 10: WB48, lane 11: WC76.

R. solanacearum bv2 populations occur as different 'genomotypes' in aquatic habitats in The Netherlands

To depict the genomic diversity found across the novel environmental bv2 strains, we decided to use the banding patterns obtained in the PFGE analysis of *XbaI*-digested DNA and the IS*Rso3* analysis to generate a combined dendogram (Fig. 4). The analysis showed that the 42 new bv2 strains – along with the reference strains 1609 and 715 - fall into five groups, here denoted as genomotypes A1, A2, B2, C1 and D1 (Fig. 4).

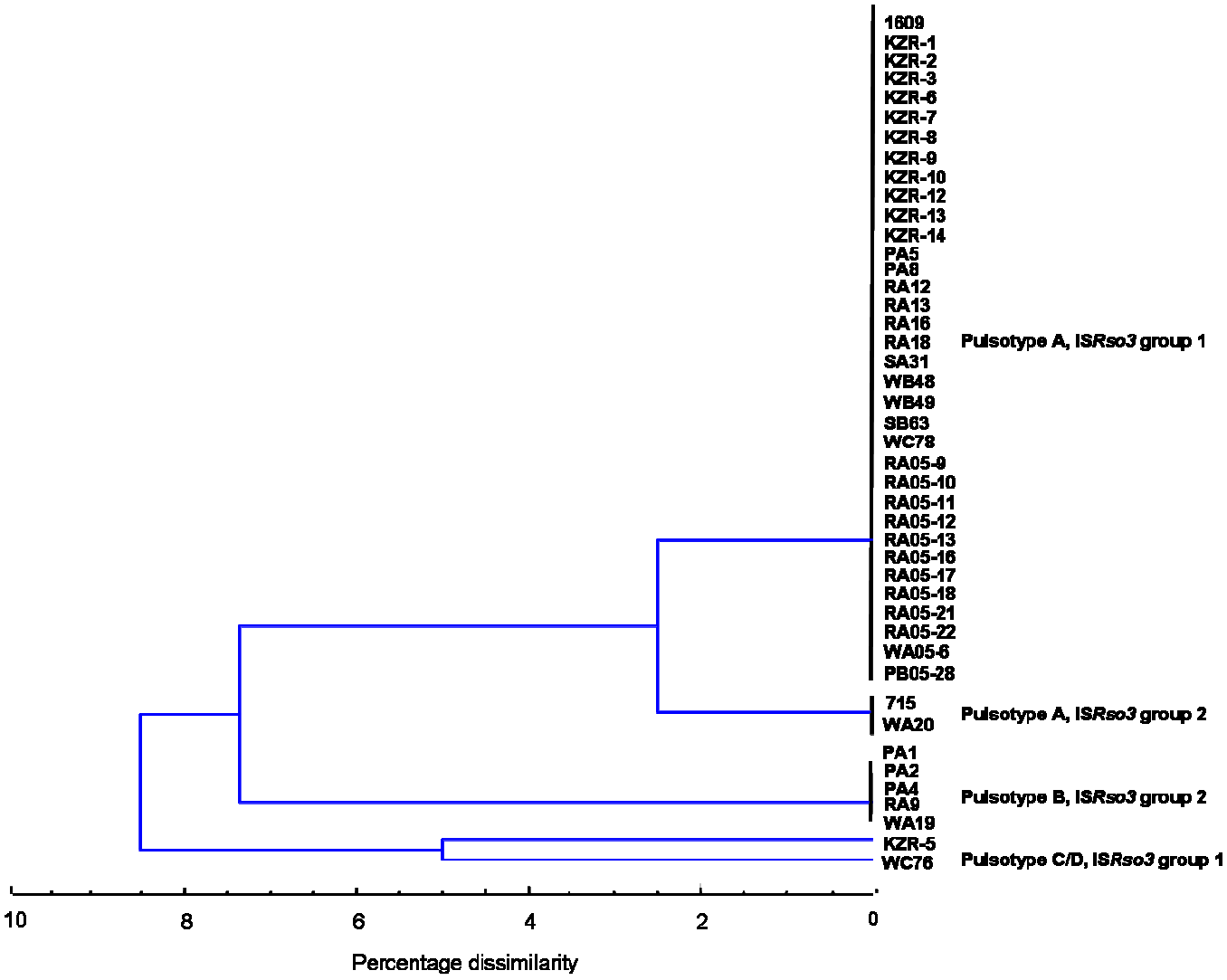

Figure 4. Dendogram obtained by cluster analysis with UPGA and Euclidean distance, using the PFGE and IS*Rso3* patterns of all *R. solanacearum* strains used in this study. Bands were scored as either present or absent. Genomotype defined by pulsotype (A-D) and IS*Rso3* group (1 or 2)

The majority of the strains, i.e. 37 (including strains 1609 and 715) fell in genomotype A1. Two strains, i.e. reference strain 715 and strain WA20, revealed the presence of an extra copy of IS*RSo3*, defining genomotype A2. The five pulsotype B strains also revealed the extra IS*RSo3* copy, defining genomotype B2. Two strains, i.e. pulsotype C strain KZR-5 and pulsotype D strain WC76 (both of IS*Rso3* group 1), formed separate genomotypes, i.e. C1 and D1, respectively.

Oligolocus sequence typing (OLST) and analysis of tadem repeat region

To assess whether strains belonging to different genomotypes could still be shown to be diverse at the nucleotide sequence level, we sequenced seven genomic loci of fifteen selected strains and cross-compared these to the sequences of reference strains 1609 and 715 (Table 2 and 4). However, for none of the genomic regions *phcA*, *mutS*, *hol*C, *cbhA*, *relA/spoT and fliC* single nucleotide polymorphisms (SNPs) were found between the strains analyzed. The sequences were also identical to those of reference strains 1609 and 715, thus revealing clonality across all strains.

In contrast, three allelic forms of a tandem repeat region in hypothetical gene RRSL_04153 were detected across the novel bv2 strains. For the majority of strains (21), the region contained five stretches of CCCAAG and six of TCCGAG/C (thus denoted tandem repeat TR5,6). For five other strains, i.e. KZR-1, KZR-2, KZR-3, KZR-4 and WB48, we found TR5,7. Reference strain 715 was classed as TR5,5. This analysis thus showed a further sub-grouping of the strains of pulsotype A. Specifically, one set (from location KZR) plus one from site B, was of the TR5,7 type, while all other strains were TR5,5 or TR5,6. The analysis places, for instance, strain KZR-5 closer to the main cluster (genomotype A1) which comprised the majority of strains (Fig. 4).

Growth rates of selected *R. solanacearum* strains at 28°C and 16°C

To assess whether any correlation existed between genetic type and growth rate ander aerobic conditions in liquid, we compared the growth rates of selected strains of genomotypes A1, B2 and C1, i.e. KZR-1 and PA5 (A), PA1 (B) and KZR-5 (C) with those of reference strains 1609 and 715 at high (28°C) and low (16°C) temperatures in 0.1 x TSBS. The low temperature was selected as the lowest temperature at which the organism shows reasonable growth in liquid.

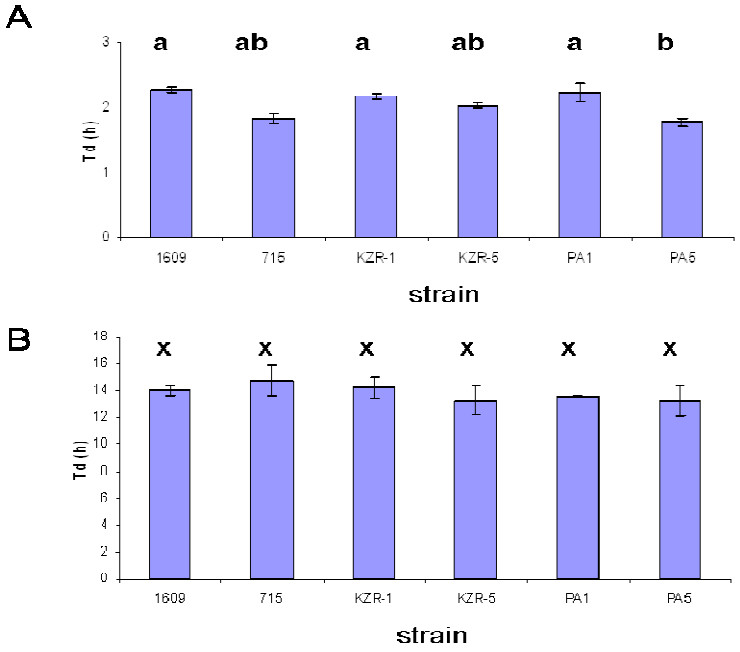

Figure 5. Doubling times in 0,1xTSBS of selected *R. solanacearum* strains in hours (h) at A) 28°C and B) 16°C. Statistical classes (a, ab, b and x) are indicated. P-values were < 0.05.

At 16°C, the doubling times (T_d) of the selected environmental strains were similar to each other. They also resembled those of both reference strains. Average doubling time thus was 13.9 h; range 13.2–14.8 h (Fig. 5B). At 28°C, the T_d of all strains varied from 1.8 to 2.3 h. Conspicuously, strain PA5 (similar to reference strain 715) was the fastest grower (T_d for both strains was 1.8h). Strains KZR-1 and PA1 (together with 1609), were the slowest (T_d between 2.2 and 2.3h). See Fig. 5A. The growth rates of strains PA1 and 715 on the one hand, and of strains KZR-1 and PA1 (and

1609) on the other hand, were indeed similar (t-test). Values were, when significant, at the threshold of significance (P-values between 0.01 and 0.05)

Determination of metabolic capacities

We applied BIOLOG GN-2 analysis to selected strains of all genomotypes (based on PFGE and IS*Rso3* distribution), to monitor the development of metabolic activity (response to substrate availability) over time. Thus, novel environmental strains KZR-1, KZR-5, PA1, PA5 and WC76, next to reference strains 1609 and 715, were analyzed. In addition, tests for the utilization of lactose, cellobiose and maltose were performed in separate. First, reference strains 1609 and 715 were shown to utilize the same 36 (of 95) carbon sources (Table 5). In contrast, all new strains consistently showed a metabolic response to a subset of only 29 of these 36 carbon sources. Thus, seven of the 36 substrates did not induce a metabolic response in the new strains. For three substrates, we confirmed the observation by direct utilization tests. The substrates consisted of the following organic acids, amino acids and other compounds: L-histidine, bromosuccinic acid, D,L-lactic acid, L-pyroglutamic acid, propionic acid, succinic acid, and γ-aminobutyric acid,.

Table 5. Carbon sources utilized by seven selected *R. solanacearum* strains using BIOLOG GN-2 plates

	strain						
C source [a]	**1609**	**715**	**KZR-1**	**KZR-5**	**PA1**	**PA5**	**WC76**
cellobiose [b]	+	+	+	+	+	+	+
Cis-Aconitic Acid	+	+	+	+	+	+	+
Citric Acid	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+
D-Galacturonic Acid	+	+	+	+	+	+	+
D-Gluconic acid	+	+	+	+	+	+	+
D-Glucuronic Acid	+	+	+	+	+	+	+
D-Saccharic Acid	+	+	+	+	+	+	+
Glucuronamide	+	+	+	+	+	+	+
lactic acid [b]	+	+	+	+	+	+	+
L-Alaninamine	+	+	+	+	+	+	+
L-Alanine	+	+	+	+	+	+	+
L-Asparagine	+	+	+	+	+	+	+
L-Aspartic Acid	+	+	+	+	+	+	+
L-Glutamic Acid	+	+	+	+	+	+	+
L-Serine	+	+	+	+	+	+	+
L-Threonine	+	+	+	+	+	+	+
malonic acid [b]	+	+	+	+	+	+	+
Pyruvic Acid Methyl Ester	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+
α-D-Glucose	+	+	+	+	+	+	+
α-Ketoglutaric Acid	+	+	+	+	+	+	+
β-Hydroxybutyric Acid	+	+	+	+	+	+	+
L-Proline	+	+	+	+	+	+	+/-
D-Alanine	+	+	+/-	+	+	+	+/-
m-Inositol	+	+	+/-	+/-	+/-	+/-	+/-
Quinic Acid	+	+	+/-	+/-	+/-	+/-	+/-
Tween 40	+	+	+/-	+/-	+/-	+/-	+/-
Tween 80	+	+	+/-	+/-	+/-	+/-	+/-
L-Histidine	+	+	-	-	-	-	-
D,L-Lactic Acid	+	+	-	-	-	-	-
Bromosuccinic Acid	+	+	-	-	-	-	-
L-Pyroglutamic Acid	+	+	-	-	-	-	-
Succinic Acid	+	+	-	-	-	-	-
Propionic Acid	+/-	+/-	-	-	-	-	-
γ-Aminobutyric Acid	+/-	+/-	-	-	-	-	-
total	36	36	29	29	29	29	29

[a] Utilization of carbon source was determined visually and scored as (+) if a clear color formation was seen at least twice out of 3 replicate experiments or (±) when the color formation was weak

[b] Utilization of these carbon sources was determined in liquid M63+ 0.5% cellobiose, lactose or maltose

Discussion

In this study, we assessed the genetic and phenotypic diversity of a set of new *R. solanacearum* bv2 strains obtained from Dutch local waterways as well as from bittersweet plants more than ten years after a major outbreak of bacterial wilt in potato (Janse 1998). We hypothesized that, following the infestation of fields from diseased potato the organism may have spread to local waterways and survived. Ecological theory dictates that under such conditions, genomic

adaptations might have occurred in the local populations, giving rise to fitter forms. Major ecological conditions that may have selected fitter forms include water temperatures ranging from about 16 to as low as 5-10°C (ditch bottoms) in large part of the year coming up to >20°C in summer. We thus successfully isolated a set of 42 presumed *R. solanacearum* bv2 strains and subjected these to molecular and phenotype analyses. Several molecular tools were first employed to look at the genetic make-up of these strains, with a focus on (1) regions involved in pathogenicity and (2) the overall genome. The identity of the presumed bv2 strains was then confirmed by bv2 (race 3) specific PCR, and corroborated by 16S rRNA gene sequencing and virulence testing on tomato. The successful isolation, in different years, of the organism from different local waterways in the Netherlands indicates the capacity of *R. solanacearum* bv2 to survive for long periods of time under local conditions, including the low temperature regime as sketched above.

The BOX and GTG5 PCR fingerprintings as well as the analysis of the *phcA* and *hrp* regions revealed genomic homogeneity across the 42 novel environmental strains, and the fingerprints were also akin to those of the reference strains 1609 and 715. With respect to the *hrp* gene region, low diversity across strains had previously also been observed in another set of 47 (plant-derived) *R. solanacearum* bv2 strains (Poussier et al. 1999; Poussier et al. 2000). This in spite of the fact that the *hrp* gene cluster can also be a target for genome reorganization or modification (Gabriel et al. 2006). Specifically, in bv2 strain UW551 - and potentially in other bv2 strains – the *hrp* region contains nine novel ORFs that were inserted between the *popA*/*B*/*C* and *hrcC* gene regions, as compared to that of bv3 strain GMI1000 (Gabriel et al. 2006). Next to transcriptional regulators RRSL_03103 and 03100 and hypothetical proteins RRSL_03102, 03101, 02446, 02447, 02445 and 02444, this included insertion sequence IS*Rso3*, which points to a role of this genetic element in genome flexibility. We compared the *RsaI*- and *BamH1*-generated fingerprinting patterns of the *pglA*-to-*hrpB* region of strain UW551 with those of bv2 strains 1609 and KZR-5 and bv3 strain GMI1000, and indeed found consistency among the bv2 strains and clearly different amplicon sizes between the bv2 and bv3 strains (not shown).

In spite of the fact that the *phcA* region can be genetically flexible (Poussier et al., 2003), we did not detect variation across our new bv2 strains. In addition, OLST of the six loci (2,282 scanned nucleotides, Table 4) showed no SNPs across the novel strains and consistency with the two reference strains. In contrast, the variable tandem repeat region TR0578 of hypothetical protein RRSL_04153 showed allelic versions TR5,5, TR5,6 and TR5,7 across the novel strains as well as reference strains. Variation in tandem repeat sequences is usually the result of slipped strand replication. This is in contrast to point mutations, which are dependent on proofreading activity. Possibly, proofreading and DNA repair is tightly controlled in *R. solanacearum* bv2, while slipped strand replication is under less tight control or, alternatively, specifically controlled.

We thus concluded, on the basis of the still limited analyses, that the new environmental bv2 strains had an almost clonal appearance of the analyzed regions and were similar to reference strains. Several previous studies showed virtual clonality across *R. solanacearum* bv2 strains (van der Wolf et al. 1998; Poussier et al. 1999; Poussier et al. 2000; Timms-Wilson et al. 2001; Castillo & Greenberg 2007), but these strains have mostly been isolated from infested plant material. For instance, in a recent multilocus sequence typing study (Castillo & Greenberg 2007), plant-derived bv2 strains showed low heterogeneity when compared to other *R. solanacearum* types.

However, both PFGE and IS*Rso3* hybridization fingerprinting revealed clear genomic differences among the novel environmental strains in our strain set. This even extended to strains obtained from single bittersweet plants. The differences were conspicuous in that a number of specific bands were either absent or present, thus indicating major genomic reshufflings, e.g. due to transpositions, deletions or insertions. Considering the co-existence of types, of 12 strains obtained at location A, five belonged to pulsotype B whereas the other seven were of pulsotype A (clustering with reference strains 1609 and 715). The five pulsotype B strains also revealed an extra copy of IS*Rso3*, thus forming genomotype B2 (Fig.3). Transposition of IS*Rso3* alone, however, does not fully explain the observed pulsotype B, as IS*Rso3* type 2 was also detected in pulsotype A strains 715 and WA20 (data not shown). Hence, combining the *XbaI*-PFGE and IS*Rso3* hybridization fingerprinting was a valid approach, as the events leading the changed PFGE patterns and this IS*Rso3* transposition are likely independent. Different pulsotypes have been described previously among bv2 strains (Smith et al. 1995; van der Wolf et al. 1998). Amplified fragment length polymorphism (van der Wolf et al. 1998; Poussier et al. 2000) has also revealed a glimpse of minor genomic heterogeneity among the (mainly) plant-associated bv2 strains. From a comparison with the former data, our pulsotype A was probably similar to the previously-determined dominant pulsotype (Smith et al. 1995), whereas B, C and D were different and novel.

Although, the presumed IS element driven genomic changes may be at the basis of the diversification of *R. solanacearum* bv2 in Dutch water systems, we currently ignore the true extent of diversity across extant bv2 strains. This is due to the rather low sample size analyzed. It has been cogitated that South American habitats, which allow good survival of bv2 strains, may incite more variation than that found in other continents (Poussier et al. 2000b). Clearly, the generally observed homogeneity of bv2 strains may be due to a generalized dissemination of pulsotype A from plant sources. It thus also relates to the bias in the analyses towards plant-derived strains. This is in contrast with the situation in other bacterial plant pathogens. For instance, in *Erwinia carotovora* subsp. c*arotovora*, a pathogen of potato, considerable genetic heterogeneity was observed between strains from a single field in a single season and from a single potato plant (Yap et al. 2004).

At the level of phenotype, our limited analysis did not allow a clear conclusion in respect of ecological fitness or adaptation. Thus, we did not observe a conspicuous difference in the virulence of the novel isolates towards tomato. Also, the growth rates at 16^{o}C in 0.1xTSBS were similar across genomotypes or when compared to the reference strains. Similarly, growth at 28^{o}C was not significantly different. The putative differences in growth rates might simply have been too small to detect with the method used, or growth conditions under which differences might become apparent were not used. The strategies used by the organism to survive in environmental waters might include reductions in cell size, entry into the viable but non culturable (VBNC) state and/or the formation of filamentous cells and cell aggregation (Alvarez et al. 2008a), however these aspects were not part of this study. In contrast, the analysis of the selected strains with BIOLOG GN2 plates did show some phenotypic variation. A striking apparent loss of the capacity to give a metabolic response to particular substrates was found in all new environmental strains as opposed to the two reference strains. All new strains were able to utilize the same "core" set of carbon sources as the reference strains, but seven other compounds were not used. This phenotypic difference with the reference strains, which both originated from (wilted) potato, might hint at different selective forces under which the environmental strains have survived.

Whereas, in the BIOLOG assays, the two reference strains may show the full complement of functions needed in relation to their ecological success in potato, such functions may be less essential for strains that have survived for prolonged periods in water, sediment or bittersweet. For instance, γ-aminobutyric acid, L-histidine, lactic acid and succinic acid, all carbon sources known to be present in a crop plant like tomato, were utilized by the reference strains and in none of the novel environmental strains. The differential phenotypes of the new strains might be the result of differences in gene expression.

The heterogeneous *R. solanacearum* bv2 populations encountered by us on two occasions in the same habitat – including bittersweet - might reflect mechanisms that ensure optimal adaptive capabilities. Bittersweet plants under temperate conditions usually do not show symptoms of disease in the presence of *R. solanacearum*. Thus, a commensalistic relationship might have evolved in which *R. solanacearum* may behave as an endophyte (Hardoim et al. 2008), whereas it may also have to persist in water, allowing colonization of a next plant host. The novel *R. solanacearum* pulsotypes B, C and D could represent classes that are potentially better equipped for plant-associated and open survival in temperate climates.

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References

Álvarez B, López MM, Biosca EG (2007) Influence of native microbiota on survival of *Ralstonia solanacearum* Phylotype II in river water microcosms. Appl Environ Microbiol 73:7210–7217

Álvarez B, López MM, Biosca EG (2008a) Survival strategies and pathogenicity of *Ralstonia solanacearum* phylotype II subjected to prolonged starvation in environmental water microcosms. Microbiology 154:3590–3598

Álvarez B, Vasse J, Le-Courtois V, Trigalet-Démery D, López MM, Trigalet (2008b) Comparative behavior of *Ralstonia solanacearum* biovar 2 in diverse plant species. Phytopathology 98:59–68

Arlat M, Boucher C (1991) Identification of a Dsp DNA region controlling Aggressiveness of *Pseudomonas solanacearum*. Mol Plant Microbe Interact 4:211–213

Birnboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 7:1513–1523

Boudazin G, Le Roux AC, Josi K, Labarre P, Jouan B (1999) Design of division specific primers of *Ralstonia solanacearum* and application to the identification of European isolates. Eur J Plant Pathol 105:373–380.

Caruso P, Palomo JL, Bertolini E, Álvarez B, López MM, Biosca EG (2005) Seasonal variation of *Ralstonia solanacearum* biovar 2 populations in a Spanish river: recovery of stressed cells at low temperatures. Appl Environ Microbiol 71:140–148

Castillo JA, Greenberg JT (2007) Evolutionary dynamics of *Ralstonia solanacearum*. Appl Environ Microbiol 73:1225–1238

Elphinstone JG, Hennessy J, Wilson JK, Stead DE (1996) Sensitivity of different methods for the detection of *Pseudomonas solanacearum* (Smith) smith in potato tuber extracts. Bull OEPP 26:674–678 .

Elphinstone JG, Stanford HM, Stead DE (1998) Detection of *Ralstonia solancearum* in potato tubers, *Solanum dulcamara* and associated irrigation water. In: Prior PH, Allen C, Elphinstone J (eds) Bacterial wilt disease: molecular and ecological aspects. Springer, Berlin, pp 133–139

Fegan M, Prior P (2005) How complex is the *Ralstonia solanacearum* species complex? In: Allen C, Prior P, Hayward AC (eds) Bacterial wilt disease and the *Ralstonia solanacearum* species complex. American Phytopathological Society Press, St. Paul, pp 449–461

Fegan M, Taghavi M, Sly LI, Hayward AC (1998) Phylogeny, diversity and molecular diagnostics of *Ralstonia solanacearum*. In: Prior PH, Allen C, Elphinstone J (eds) Bacterial wilt disease: molecular and ecological aspects. Springer, Berlin, pp 133–139

Gabriel DW, Allen C, Schell M et al (2006) Identification of open reading frames unique to a select agent: *Ralstonia solanacearum* race 3 biovar 2. Mol Plant Microbe Interact 19:69–79

Gorissen A, van Overbeek LS, van Elsas JD (2004) Pig slurry reduces the survival of *Ralstonia solanacearum* biovar 2 in soil. Can J Microbiol 50:587–593

Granada GA, Sequira L (1983) Survival of *Pseudomonas solanacearum* in soil, rhizosphere and plant roots. Can J Microbiol 29:433–440

Hardoim PR, van Overbeek LS, van Elsas JD (2008) Properties of bacterial endophytes and their proposed role in plant growth. Trends Microbiol 16:463–471

Hayward AC (1991) Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*.Annu Rev Phytopathol 29:65–87

Janse JD (1996) Potato brown rot in western Europe-history, present occurence and some remarks on possible origin, epidimology and control strategies. Bull OEPP 26:679–695

Janse JD (1998) Experiences with potato brown rot *Ralstonia solanacearum* biovar 2, race 3 in the Netherlands. In: Prior PH, Allen C, Elphinstone J (eds) Bacterial wilt disease: molecular and ecological aspects. Springer, Berlin, pp 146–152

Jeong EL, Timmis JN (2000) Novel insertion sequence elements associated with genetic heterogeneity and phenotype conversion *in Ralstonia solanacearum*. J Bacteriol 182:4673–4676

Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. Wiley, London, pp 115–175.

OEPP/EPPO (2004) EPPO Standards PM 7/21, diagnostic protocols for regulated pests.Bull OEPP 20:255–262.

Poussier S, Vandewalle P, Luisetti J (1999) Genetic diversity of African and worldwide strains of *Ralstonia solanacearum* as determined by PCR-restriction fragment length polymorphism analysis of the *hrp* gene region. Appl Environ Microbiol 65:2184–2194

Poussier S, Trigalet-Démery D, Vandewalle P, Goffinet B, Luisetti J, Trigalet A (2000) Genetic diversity of *Ralstonia solanacearum* as assessed by PCR-RFLP of the *hrp* gene region, AFLP and 16S rRNA sequence analysis, and identification of an African subdivision. Microbiology 146:1679–1692

Poussier S, Thoquet P, Trigalet-Démery D, Barthet S, Meyer D, Arlat M, Trigalet A (2003) Host plant-dependent phenotypic reversion of *Ralstonia solanacearum* from non-pathogenic to pathogenic forms via alterations in the phcA gene. Mol Microbiol 49:991–1003

Salanoubat M, Genin S, Artiguenave F et al (2002) Genome sequence of the plant pathogen *Ralstonia solanacearum*. Nature 415:497–502

Sambrook J, Maniatis T (1989) Molecular cloning -a laboratory manual. Cold Spring Habour Laboratory Press, New York.

Schönfeld J, Heuer H, van Elsas JD, Smalla K (2003) Specific and sensitive detection of *Ralstonia solanacearum* in soil on the basis of PCR amplification of *fliC* fragments. Appl Environ Microbiol 69:7248–7256

Smith JJ, Offord LC, Holderness M, Saddler GS (1995) Genetic diversity of *Burkholderia solanacearum* (synonym *Pseudomonas solanacearum*) race 3 in Kenya. Appl Environ Microbiol 61:4263–4268

Svec P, Vancanneyt M, Seman M, Snauwaert C, Lefebvre K, Sedlacek I, Swings J (2005) Evaluation of (GTG)(5)-PCR for identification of *Enterococcus spp*. FEMS Microbiol Lett 247:59–63

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

Timms-Wilson TM, Bryant K, Bailey MJ (2001) Strain characterization and 16S–23S probe development for differentiating geographically dispersed isolates of the phytopathogen *Ralstonia solanacearum*. Environ Microbiol 3:785–797

Van der Wolf JM, Bonants PJM, Smith JJ, Hagenaar M, Nijhuis E, van Beckhoven JRCM, Saddler GS, Trigalet A, Feuillade R (1998) Genetic diversity of *Ralstonia solancaerum* Race 3 in Western Europe determined by AFLP, RC-PFGE and Rep-PCR. In: Prior PH, Allen C, Elphinstone J (eds) Bacterial wilt disease: molecular and ecological aspects. Springer-Verslag, Berlin, Germany, pp 44-49.

Saddler GS, Trigalet A, Feuillade R (1998) Genetic diversity of *Ralstonia solancaerum* Race 3 in Western Europe determined by AFLP, RC-PFGE and Rep-PCR. In: Prior PH, Allen C, Elphinstone J (eds) Bacterial wilt disease: molecular and ecological aspects. Springer, Berlin, pp 44–49.

Van Elsas JD, Kastelein P, van Bekkum P, van der Wolf JM, de Vries PM, van Overbeek LS (2000) Survival of *Ralstonia solanacearum* biovar 2, the causative agent of potato brown rot, in field and microcosm soils in temperate climates. Phytopathology 90:1358–1366

Van Elsas JD, Kastelein P, de Vries PM, van Overbeek LS (2001) Effects of ecological factors on the survival and physiology of *Ralstonia solanacearum* biovar 2 in irrigation water. Can J Microbiol 47:842–854

Versalovic J, Schneider M, de Bruijn FJ, Lupski JR (1994) Genomic fingerprinting of bacteria using repetitive sequence based PCR (rep-PCR). Methods Mol Cell Biol 5:25–40

Wakimoto IU, Matsuo N, Angot A (1982) Multiplication of *Pseudomonas solanacearum* in pure water. Ann Phytopathol Soc Jpn 48:620–627

Wenneker M, Verdel MSW, Groeneveld RMW, Kempenaar C, van Beuningen AR, Janse JD (1999) *Ralstonia (Pseudomonas) solanacearum* race 3 (biovar 2) in surface water and natural weed hosts: first report on stinging nettle (*Urtica dioica*). Eur J Plant Pathol 105:307–315

Winstead NN, Kelman A (1952) Inoculation techniques for evaluating resistance to *Pseudomonas solanacearum*. Phytopathol 42:628–634.

Yap MN, Barak JD, Charkowski AO (2004) Genomic diversity of *Erwinia carotovora subsp carotovora* and its correlation with virulence. Appl Environ Microbiol 70:3013–3023

Chapter III

A putative genomic island, PGI-1, in *Ralstonia solanacearum* biovar 2 revealed by subtractive hybridization

Patricia Stevens
Jan Dirk van Elsas

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Abstract

Ralstonia solanacearum biovar 2, a key bacterial pathogen of potato, has recently established in temperate climate waters. On the basis of isolates obtained from diseased (potato) plants, its genome has been assumed to be virtually clonal, but information on environmental isolates has been lacking. Based on differences in pulsed-field gel electrophoresis patterns, we compared the genomes of two biovar 2 strains with different life histories. Thus, genomic DNA of the novel environmental strain KZR-5 (The Netherlands) was compared to that of reference potato strain 715 (Bangladesh) by suppressive subtractive hybridization. Various strain-specific sequences were found, all being homologous to those found in the genome of reference potato strain 1609. Approximately 20% of these were related to genes involved in recombinational processes. We found a deletion of a 17.6-Kb region, denoted as a putative genomic island PGI-1, in environmental strain KZR-5. The deleted region was, at both extremes, flanked by a composite of two insertion sequence (IS) elements, identified as *ISRso2* and *ISRso3*. The PGI-1 region contained open reading frames that putatively encoded 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. The ecological behavior of strain KZR-5 was compared to that of reference strain 715. Strain KZR-5 showed enhanced tolerance to 4°C as compared to the reference strain, but was not affected in its virulence on tomato.

Introduction

The species *Ralstonia solanacearum* encompasses a wide range of organisms that interact with plants, often causing plant disease. In fact, *R. solanacearum* has been suggested to form a true species complex (Fegan & Prior 2005), containing several types that consistently differ in biochemical properties(defining biovars), genome content and plant host range (defining races). The traditional robust classification of the organism into biovars has recently been challenged (Fegan & Prior 2005), resulting in a classification into four so-called phylotypes, denoted I through IV. Adhering to the classical taxonomy, we here will use the biovar (bv) designation.

R. solanacearum bv2 is a quite homogeneous group of phytopathogens (van der Wolf et al. 1998; Castillo & Greenberg 2007; Stevens & Van Elsas 2010) that cause bacterial wilt in potato as well as other crops (Janse 1998; Janse et al. 2004; Gabriel et al. 2006). It probably originated and evolved in South America, being frequently found in varying regions in Peru (Janse 1996), the cradle of potato (Ciampi & Sequeira 1980; Swanepoel 1990). In the late eighties to early nineties, the bacterium may have spread, from infested potato material, into temperate climate regions such as in the Netherlands. More than 20 years after its presumed introduction, the bacterium can still be found in Dutch local waterways, that is, in surface water, ditch sediment as well as in *Solanum dulcamara* (bittersweet). Microcosm experiments have shown that *R. solanacearum* bv2 can survive for relatively long periods in bulk and rhizosphere soils (Granada & Sequira 1983; van Elsas et al. 2000), in agricultural drainage or run-off water and in canal sediment (van Elsas et al. 2001). Furthermore, the bittersweet plants growing at the sides of waterways may serve as refuges for the organism. In these environments, the organism may show physiological responses leading to reductions in cell size, entry of (part of) the population into the viable-but-non-culturable (VBNC) state and the formation of filamentous cells and cell aggregation (Grey & Steck 2001; Alvarez et al. 2008a).

During winter, *R. solanacearum* is often difficult to detect (as CFUs) in Dutch waterways, with only few or no typical *R. solanacearum* colonies appearing on semi-selective (SMSA) agar plates (Elphinstone et al. 1996). This suggests a decline in local population sizes, the emergence of VBNC cells (which are undetectable via plating), or both. When the water temperature rises again in spring and summer, bacterial cells may be released into the surface water from ditch sediment and bittersweet plants. Re-growth of the population (or resuscitation from a VBNC state) may then lead to elevated cell densities. As many crop production fields in The Netherlands are localized close to infested waterways, the bv2 cells present in these waters pose a continuous threat to potato production (Janse 1998; Elphinstone et al. 1998).

To understand the short-term evolution of the environmental bv2 populations in local waterways, genomic comparisons of recent environmental isolates with potato-derived strains are necessary. In support, the draft genomes of two plant-derived bv2 strains, i.e. potato strain 1609 (IPO1609; NW_002196568) and geranium strain UW551 (NZ_AAKL00000000) have very recently become available. Moreover, genomic information of the banana-derived bv1 strain Molk2 (YP_002254716) is also available and so is the genome sequence of bv3 strain GMI1000 (Salanoubat et al. 2002).

Guidot et al. (Guidot et al. 2007) investigated the *R. solanacearum* species complex by comparing the genome contents of 17 strains (representing all four groups of the species complex)

using microarray technology. Fifty three % of the genes present in bv3 strain GMI1000 (used as the reference strain) were also present in the strains of all groups, thus defining the core gene content of the species. Very recently, it was found that variable genes are often located in mobile genetic elements, which could be characterized by lower G+C contents, or in regions of alternative codon usage, ACURs (Guidot et al. 2009b). The variable genes may have been acquired through horizontal gene transfer (HGT), while the localization of these genes is likely to be influenced by recombination events (Bertolla et al. 1999; Terol et al. 2006; Guidot et al. 2009a). Thus far, little variation at the nucleotide level has been found among the two plant-derived bv2 (i.e. 1609 and UW551) genomes (Gabriel et al. 2006), which might corroborate the presumed recent spread of bv2 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 bv2, in particular with respect to environmental strains. In many bacteria, genome diversification is mediated by HGT events as well as the activity of insertion sequence (IS) elements and transposons. Moreover, *R. solanacearum* is naturally transformable and its genome (both bv2 and bv3) contains a plethora of IS elements (Salanoubat et al. 2002). Together with recombinational hot spot (Rhs) elements (Wang et al. 1998), the IS elements have the potential to induce major chromosomal rearrangements, such as deletions, inversions, duplications and transpositions.

In a previous study (Stevens & Van Elsas 2010), we described a suite of 42 novel environmental *R. solanacearum* bv2 strains isolated from Dutch canal waters, sediment and bittersweet. Using genomic fingerprinting techniques, we compared these environmental strains with the potato-derived reference bv2 strains 715 (Bangladesh) and 1609 (Netherlands), and revealed a distribution among four major groups based on genetic techniques. Several new environmental strains, in particular strain KZR-5, stood out on the basis of the divergent pulsed-field gel electrophoresis (PFGE) patterns of the genomic DNA.

To better understand the putative genetic changes incurred in environmental strain KZR-5, we here decided to analyze its genome via suppressive subtractive hybridization (SSH) using tropical potato strain 715 as the comparator. We identified a set of strain-specific sequences that together revealed the excision of a genomic region in strain KZR-5. To delineate the ecological characteristics of KZR-5 in comparison to the reference strain, we assessed its virulence on tomato and its population dynamics in water at two ecologically-relevant temperatures.

Materials and methods

Bacterial strains

The *R. solanacearum* strains used in this study are listed in Table 1. Bacterial strains were stored in 20% glycerol at –80°C. Prior to each experiment, cultures from this stock were grown in 0.1 x TSBS (10% strength Trypticase Soy Broth [Becton Dickinson and Company, MD, USA], 0.1% sucrose; pH 7.2) at 27°C with shaking at 180 rpm.

Genomic DNA isolation

Genomic DNA of strains KZR-5, 715, 1609, PA1, PA5, SA31 and SB63 was isolated using the

Ultraclean ™ microbial DNA extraction kit according to the manufacturer's protocol (MoBIO Laboratories Inc. Carlsbad, UK). This yielded 50-100 ng/µl DNA of high quality, as measured via agarose gel electrophoresis followed by staining with ethidium bromide.

Table 1. *R. solanacearum* strains used in this study

strain	isolation source	year	pulsotype [1]	reference
KZR-5	Dutch waterway, bittersweet	2004	C	Stevens and van Elsas (2010)
715	Bangladesh, potato	?	A	Timms-Wilson et al. (2001)
1609	The Netherlands, potato	1995	A	van Elsas et al. (2000)
PA1	Dutch waterway, bittersweet	2004	B	Stevens and van Elsas (2010)
PA5	Dutch waterway , bitterweet	2004	A	Stevens and van Elsas (2010)
SA31	Dutch waterway, sediment	2004	A	Stevens and van Elsas (2010)
SB63	Dutch waterway, sediment	2004	A	Stevens and van Elsas (2010)

[1] Pulsotype was defined using pulsed field gel electrophoresis of *XbaI* digested genomic DNA (Stevens and van Elsas 2010)

Suppressive substractive hybridization (SSH)

Genes unique to either *R. solanacearum* strain KZR-5 or 715 were identified by SSH using strain 715 as the tester and strain KZR-5 as the driver, as well as the reverse, i.e. strain KZR-5 as the tester and strain 715 as the driver. PCR SSH was performed using the CLONTECH PCR-Select bacterial genome subtraction kit (BD Biosciences, Palo Alto, CA) according to the manufacturer's instructions with a few modifications. We performed the recommended control steps (i.e adapter ligation) including a control subtraction using *E. coli* genomic DNA that was enriched with ϕX174/*HaeIII* digest (supplied in the kit). To check the efficiency of adapter ligation, we used primers PglA-F and R (Table 3) to amplify a 875 bp fragment of the polygalacturonase gene of *R. solanacearum* bv2 (RSIPO_03945), not containing *RsaI* sites. PCR was carried out in 25 µl mixtures containing 200 µM of each nucleotide, 2.5 mM, $MgCl_2$, 2% DMSO, 0.4 µM primer, 1 µl of template (prepared as instructed in the manual) and 200 U/ml of Taq polymerase (Roche Applied Science, Basel, Switzerland). The amplification conditions were 72°C for 2 min, 94°C for 30s followed by 34 cycles of 94°C for 10s, 58°C for 30s and 68°C for 1min with a final extension at 72°C for 5 min.

For the primary PCR using the adapter-specific primer SSH primer1 (Table 3), PCR was carried out as described above, except that we used a "hot start". To obtain the hot start, the Taq polymerase (Roche) was pre-treated by mixing it 1:1 with a hot start Taq antibody (Takara BIO Inc. Shiga, Japan) and incubation for 10 min at room temperature. PCR was carried out as described above, except that the cycling conditions were: 72°C - 2 min, followed by 34 cycles of 94°C - 30 s, 62°C - 30 s and 72°C - 90 s (no final extension).

For the nested PCR, primers SSH nest1 and SSH nest2 (Table 3) were used. PCR was performed as described above (including a hot start), except that the template was 1 µl of a 1:40 dilution of the PCR products obtained from the primary PCR. The amplification conditions were 15 cycles of 94°C - 30 s, 68°C - 30 s and 72°C - 90 s (no final extension).

Construction of strain-specific clone libraries

Products obtained from the nested PCR described above were cloned using the pGEM-T easy vector system according to the manufacturer's protocol (Promega Corporation, Madison USA).

For transformation, we used competent *E. coli* DH5α cells (Invitrogen, Groningen, The Netherlands). At first, 115 white colonies of strain KZR-5 (driver strain 715, tester strain KZR-5) and 50 white colonies of strain 715 (driver strain KZR-5, tester strain 715) were PCR-analyzed using primers SP6 and T7 (Table 3); all showed to contain inserts ranging in size between 0.1 to 1.2 Kb. All PCR products were then digested with *HaeIII* and the digests analyzed on agarose gel to allow grouping of the inserts, thus avoiding the sequencing of duplicates. Based on the PCR/*HaeIII* clustering of the inserts (thereby excluding the clones with inserts that appeared to be similar in size and restriction pattern), 67 strain KZR-5 and 28 strain 715 specific inserts were selected for sequence analysis.

DNA sequencing

For sequencing, the inserts were amplified using PCR primers SP6 and T7 (Table 3). DNA sequencing using the T7 primer was performed in Applied Biosystems 3130 or 3730XL sequencers. Inserts larger than 1 Kb were sequenced by GATC-Biotech (Konstanz, Germany).

PCR amplification

PCR primers and annealing temperatures used for different purposes in this study are listed in Table 3. For PCR amplification, we used the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, USA). Standard PCR reaction mixtures contained 1x PCR buffer (1.5 mM $MgCl_2$, 10 mM Tris and 50 mM KCl, Roche Applied Science, Basel, Switzerland), 2.5 mM $MgCl_2$, 10% DMSO, 200 μM each deoxynucleotide, 0.2 μM of each primer and 20 U/ml Taq DNA polymerase (Roche).
For amplification of large genomic regions (3-27 Kb), we used the TaKaRa La Taq polymerase kit (Takara BIO INC., Shiga, Japan), which is suitable for amplification of large DNA regions including high G+C content DNA. For amplification, we used the 2xGC buffer I supplied in the kit and the supplied dNTP mixture (final concentration 400 μM of each nucleotide) following the manufacturer's instructions. The cycling program was as follows: denaturing at 96˚C for 45s; 30 cycles (96˚C for 20 sec, 68˚C for 15 min) and a final extension step at 72˚C for 15 min.

Southern hybridization

For Southern blotting, 5-10 μg of *PstI* or *BamH1*-digested genomic DNA was transferred to a Hybond-N nitrocellulose membrane (Amersham Biosciences Benelux, Roosendaal, Netherlands). For preparation of the DNA probes, we excised PCR products from agarose gel and purified these using the Qiaex II gel extraction kit (Qiagen Benelux B.V, Venlo, Netherlands). For each DNA probe, up to 1 μg cleaned PCR product was labelled using the DIG DNA labelling kit (Roche Applied Science, Penzberg, Germany). Hybridization (at 48°C), washing and detection were done using the DIG DNA detection kit according to the manufacturer's recommendations (Roche Applied Science, Penzberg, Germany).

Survival in water at 4°C and 20°C

The survival of strains KZR-5 and 715 in sterile water microcosms kept at 20°C and 4°C was assessed as described elsewhere (van Overbeek et al. 2004). Microcosms were inoculated at densities of approximately 5.0 E^{+6} CFU/ml

Virulence tests

Strains KZR-5 and 715 were tested for virulence on eight 4-5 week old tomato plants (*Solanum lycopersicon cv Maribel*) using inoculum densities of 10^5 CFU/ml and 10^8 CFU/ml (to detect differences in virulence). Tomato plants are often used as a model system to test virulence of *R. solanacearum* because of high susceptibility and fast growth. Strain introduction was performed by watering the plant substrate (50 g dry weight sterile peat soil) with 25 ml of diluted (e.g.10,000x or 10x, in sterile demineralized water) bacterial suspension that had grown overnight in liquid 0.1 x TSBS. Prior to inoculation, plants were kept without added water for 2 days and roots were slightly damaged by gently moving the plant up and down in the soil. This procedure offered the correct window for virulence testing with the plant/peat combination that was available. Plants were incubated in the greenhouse at 26°C (day, 14 h) / 21°C (night, 10 h), and disease development in the plants was scored at regular intervals over time using a disease matrix ranging from 0, no wilting symptoms, to 4, all leaves wilted (Winstead & Kelman 1952)

Nucleotide sequence accession numbers

The obtained SSH sequences were deposited in the Genbank GSS data library under number GS557176 to GS557233, the sequence of clone KZR-5 covering the deletion under number GQ899141 and the sequence of the IS blocks of strains 715, 1609 and UW551 under number GU586290 to GU586294.

Results

Approach and selection of strains

In a previous study, 42 novel *R. solanacearum* bv2 isolates were obtained which, together with two reference strains, clustered into four groups denoted as genomotypes (Stevens & Van Elsas 2010). We used a subset of these strains in the current study (Table 1). To allow the analysis of any genomic changes incurred in strains from the open environment, we selected the novel environmental *R. solanacearum* bv2 strain KZR-5 for comparison of its genome to that of the tropical potato strain 715 using two-way SSH. Strain KZR-5 was selected as (i) it was a fresh environmental isolate representative of current water populations and (ii) it was genetically clearly divergent from the reference potato strains 715 and 1609 on the basis of PFGE (whereas they appeared genetically identical with respect to five genes at the nucleotide level as evidenced by oligolocus sequence typing).

Analysis of putative strain-specific sequences

After removal of sequences of poor quality as well as duplicates, a total of 58 sequences (40 presumably specific for KZR-5 and 18 for 715) remained for further analysis. Sequences were analyzed using the Megablast tool available at http://blast.ncbi.nlm.nih.gov/Blast.cgi, which is optimized for highly similar sequences. All sequences obtained had closest matches with sequences of *R. solancearum* bv2, showing best hits with particular regions of the strain 1609 draft genome (Table 2). One strain KZR-5 specific sequence (clone 2, Table 2) was also present in the strain 715 specific clone library (clone 42, Table 2).

Table 2. Analysis of *R. solanacearum* bv2 sequences obtained by SSH of strains KZR-5 and 715

strain	clone	size	%GC	E-value	% identity	location[1]	localisation gene	comment/ description locus [2]
KZR-5	1	435	25.2	0.0	99%	3562	non-coding	179 bp homology to 3' of RSIPO_04521 (repA)
KZR-5	2	199	50.5	2,00E-93	98%	9648	non-coding	806 bp 5' RSIPO_03105 (type III effector protein), 506 3' RSIPO_03106 (Rhs related protein)
KZR-5	3	451	53.5	8,00E-77	96%	204935	non-coding	356 bp 5' 'RSIPO_03280 (short-chain dehydrogenase/reductase Sdr protein)
								214 bp 3' RSIPO_03281 (transcriptional regulator protein)
KZR-5	5	158	60.7	6,00E-62	98%	629758	non-coding	210 bp 5' RSIPO_00581(HP)[3], 340 3' RSIPO_00582 (atp-dependent rna helicase protein)
KZR-5	6	127	49.1	2,00E-55	99%	768732	non-coding	283 bp 5' RSIPO_00719 (HP), 181 bp 3' RSIPO_00720 (HP)
KZR-5	7	300	51.8	3,00E-133	95%	1219358	non-coding	845 bp 5' RSIPO_04991 (transposase protein) 265 bp, 3' RSIPO_01131 (resolvase protein)
KZR-5	8	246	49.2	8,00E-123	99%	1343620	non-coding	305 bp 5' RSIPO_04138, 4806 bp 3' RSIPO_04140
KZR-5	9	301	55.2	2,00E-134	97%	1861380	non-coding	259 bp 5' RSIPO_04920 (ankyrin-repeat protein), 1981 bp 3' RSIPO_04515 (HP)
KZR-5	10	405	50.4	3,00E-174	94%	2846904	rRNA-IPO_02633	rRNA
KZR-5	11	329	56.9	1,00E-141	97%	57808	RSIPO_00042/00043	hypothetical proteins
KZR-5	12	311	57.3	2,00E-129	98%	109112	RSIPO_00088	hypothetical protein
KZR-5	13	807	66.6	0.0	99%	231128	RSIPO_00199/00200	n-acetyl-gamma-glutamyl-phosphate reductase (arganine biosynthesis, HP
KZR-5	14	252	59.9	6,00E-74	94%	406260	RSIPO_00363	polyphenol oxidase with tyrosine hydroxylase activity protein
KZR-5	15	458	64.6	0.0	96%	485927	RSIPO_00449	na/pi cotransporter II-related; protein
KZR-5	16	320	55.4	6,00E-120	94%	792075	RSIPO_00742	Rhs-like protein
KZR-5	17	224	59.8	4,00E-105	98%	959516	RSIPO_00893	hypothetical protein
KZR-5	18	201	55.8	2,00E-98	99%	1181831	RSIPO_01096	hypothetical protein
KZR-5	19	280	55	5,00E-140	99%	1826012	RSIPO_01668	dna glycosylase protein
KZR-5	20	498	55.6	0.0	94%	1931367	RSIPO_01774/01775	hypothetical protein, signal peptidase I
KZR-5	21	299	60.5	1,00E-112	96%	1932709	RSIPO_01776	ggtp-binding protein LepA
KZR-5	22	360	59.5	9,00E-94	95%	1938599	RSIPO_01781	rna polymerase sigma-epsilon factor
KZR-5	23	382	56.8	0.0	99%	2093883	RSIPO_01920/01921	zn-dependent alcohol dehydrogenase, HP
KZR-5	24	419	49.4	0.0	96%	2294401	RSIPO_02102	hypothetical protein
KZR-5	25	598	61.4	0.0	97%	2321310	RSIPO_02126	dna gyrase (subunit a)(type II topoisomerase) protein
KZR-5	26	165	51	1 e-72	97%	2642532	RSIPO_02440	twitching motility protein
KZR-5	27	341	61.7	3,00E-133	97%	2655192	RSIPO_02453/02455	HP, aldehyde dehydrogenase oxidoreductase protein
KZR-5	28	231	57.2	6 e-109	98%	2818819	RSIPO_02606	porin gram negative type
KZR-5	29	399	57.4	2,00E-175	99%	2974086	RSIPO_02766	preprotein translocase SecY (membrane subunit)
KZR-5	30	283	60.4	9,00E-138	98%	3172522	RSIPO_02930	Rhs related protein
KZR-5	31	540	47.4	0.0	99%	10190	RSIPO_03106	Rhs related protein
KZR-5	32	245	60.4	2,00E-98	96%	44903	RSIPO_03132	helicase 6 related protein
KZR-5	33	250	59.2	5,00E-80	94%	569851	RSIPO_03564	Papd-like protein
KZR-5	34	339	59.9	2,00E-134	96%	776950	RSIPO_03723/03724	general secretion pathway GspG-related protein, HP
KZR-5	35	364	58.2	7e -160	97%	933881	RSIPO_03831	phospholipase D/transphosphatidylase protein
KZR-5	36	445	55.9	0.0	98%	1059080	RSIPO_03940/03941	dioxygenase protein, hypothetical protein
KZR-5	37	121	54.5	5,00E-52	97%	1108407	RSIPO_03983	hypothetical protein
KZR-5	38	306	53.9	2,00E-149	98%	1299888	RSIPO_04110	transporter protein
KZR-5	39	223	51.6	5,00E-109	99%	1509251	RSIPO_04250/04251	hemagglutinin-related protein
KZR-5	40	250	57.6	8,00E-78	94%	274019	RSIPO_04911	hypothetical protein
KZR-5	41	419	48.9	0.0	99%	1823187	RSIPO_04916	hemolysin-type calcium-binding protein-Rtx
715	42	215	51.7	1,00E-90	95%	9441	non-coding	808 bp 5' RSIPO_03105 (type III effector protein), 506 bp 3' RSIPO_03106 (Rhs-related protein)
715	43	632	52.7	0.0	94%	395517	non-coding	209 bp 5' RSIPO_03417 (hemagglutinin-related protein)
715	44	303	53.5	3,00E-123	95%	921595	non-coding	23 bp 5' RSIPO_00863 (inorganic pyrophosphatase protein)
								276 bp 3' RSIPO_00864 (protein of unknown function duf482)
715	45	447	53.3	0.0	97%	3088314	non-coding	197 bp 5' RSIPO_02866 (general secretory pathway protein f)
								2721bp 3' RSIPO_02867 (transcriptional regulator protein)
715	46	577	52.9	0.0	99%	2843767	rRNA-IPO_02626/02627	rRNA
715	47	220	58.6	9,00E-92	94%	299320	RSIPO_00260	methylmalonyl-coa mutase protein
715	48	143	60.9	1,00E-63	97%	1533044	RSIPO_01404	outer membrane chaperone, Skp-related protein
715	49	139	66.2	1,00E-48	94%	2947883	RSIPO_02738	fimbrial Type-4 assembly protein
715	50	718	53.9	0.0	99%	3321126	RSIPO_03045	Rhs-related protein
715	51	130	63.1	4,00E-48	94%	1132348	RSIPO_04887	hypothetical protein
715	52	260	55	6,00E-104	93%	240086	RSIPO_03302	hypothetical protein
715	53	299	55.2	1,00E-141	97%	238021	RSIPO_04909	hypothetical RelA/SpoT domain protein
715	54	442	51.1	0.0	97%	238458	RSIPO_04909	hypothetical RelA/SpoT domain protein
715	55	219	42.9	1,00E-100	97%	238461	RSIPO_04909	hypothetical RelA/SpoT domain protein
715	56	291	50.9	4,00E-146	99%	238966	RSIPO_04909	hypothetical RelA/SpoT domain protein
715	57	439	49.4	0.0	97%	2293969	RSIPO_04939	helix-turn-helix domain transcription regulator protein
715	58	625	41.6	0.0	99%	943358	RSIPO_04975	transposase protein
715	59	216	50.4	5,00E-104	99%	1015410	RSIPO_04978/00955	hypothetical protein, fatty acid desaturase protein

[1] Genome location of the first nucleotide of the cloned sequence is given with respect to the 1609 genome nomenclature (NW_002196568).

[2] Description of the functional genes as annotated for strain 1609. For the non-coding sequences, the distances to neighboring genes at the 3′ and 5′ sides are given.

[3] HP hypothetical protein

The box indicates the five clones (52-56) that initially indicated a deleted region (See Fig. 1)

These sequences were homologous to non-coding regions in the 1609 genome, with nine nucleotides difference. The average G+C content of all strain-specific sequences was 54% (ranging from 25.2% to 66.2%, see Table 2), which is considerably lower than the 64% average of the *R. solanacearum* bv2 genome (Gabriel et al. 2006). For 38 sequences (30 specific for KZR-5 and 8 for 715), we did not find homologues (using Megablast) in strain GMI1000, which indicates that these sequences are possibly bv-specific or, alternatively, divergent between these strains.

Of the putative strain-specific sequences, 48% (28/58) were homologous to sequences of a variety of functional genes, 18% (11/58) to genes encoding hypothetical proteins and another 14% to putative non-coding regions or as-yet-unassigned genes (8/58). In addition, almost 20% (11/59) were homologous, or closely related, to genes known to be potentially involved in genome flexibility and recombination processes (Table 2; clones 2, 5, 7, 16, 30, 31, 32 of strain KZR-5 and clones 42, 47, 50 and 58 of strain 715). These thus potentially identified Rhs-related proteins, transposases or related sequences. Four strain 715 specific sequences (clones 53-56) were found to be similar to a single gene (RSIPO_04909), which putatively encodes a protein with a conserved RelA/SpoT domain. A fifth cloned sequence (clone 52) was localized 1 Kb upstream of this presumed *relA*/*spoT* gene (Figure 1A). As nearly 40% of all sequenced clones of strain 715 (11/28, including duplicates) localized to the region identified by the putative *relA*/*spoT* gene, we decided to place a focus on these sequences in our further analyses.

Identification and characterization of a putative genomic island in *R. solanacearum* bv2 which is absent from strain KZR-5

Using comparisons with the strain 1609 draft genome, the 11 strain 715 specific sequences were found to localize to one single genomic region of approximately 2.4 Kb in size. This region was predicted to encode two hypothetical proteins, one of which possessed a RelA/SpoT domain (Figure 1A). To test whether the region was indeed unique for strain 715 (as well as other bv2 strains) and absent from strain KZR-5, we used PCR primers spoT-F and spoT-R to amplify the region from different genomes (Figure 1A, Table 3). Using genomic DNA of strains 715, 1609, KZR-5 and four other bv2 strains (PA1, PA5, SA31 and SB63 (Stevens & Van Elsas 2010)), we obtained products of the expected size (1.6 Kb) for all strains except KZR-5. Southern hybridization analysis using the *relA/spoT* PCR fragment of strain 1609 as the DNA probe confirmed the presence of the locus in strains 715, 1609, PA1, PA5, SA31 and SB63 and its absence in strain KZR-5 (Fig 1B).

To investigate the exact size of the deleted region in strain KZR-5, we designed eight primer sets (ps: 1-8) to amplify regions present in regions 1 to 20 Kb upstream as well as downstream of the *relA/spoT* gene (Table 3, Figure 2A). The total region under investigation had stretches of unassigned nucleotides in the draft genome sequence of strain 1609, which indicated the presence of repeat sequences characteristic for IS elements (Figure 2A).

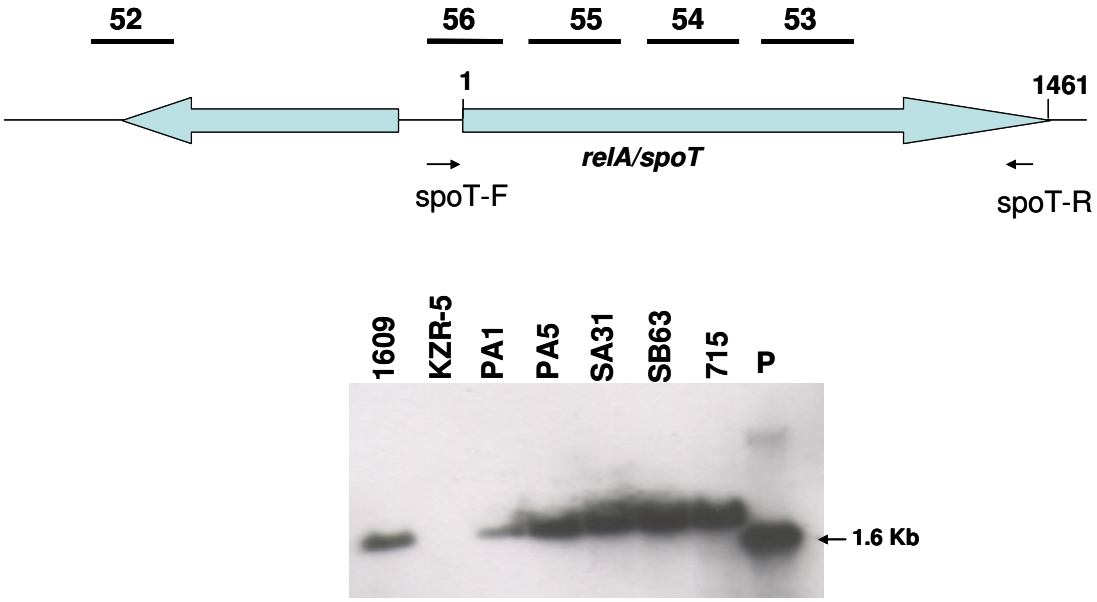

Figure 1. Detection of a deletion incurred in *R. solanacearum* bv2 strain KZR-5. **(A)** Localization of five sequences of strain 715 in the strain 1609 genome. The numbers 52-56 correspond to sequences of clones shown in Table 2. The spoT-F and spoT-R: indicate primers used for PCR amplification of the *relA/spoT* 1.6 Kb fragment. Positions 1 and 1461 of *relA/spoT* (RSIPO_04909) correspond to positions 238741 and 23728 in the genome of strain 1609. **(B)** Southern blot analysis of genomic DNA of different *R. solanacearum* strains after restriction with *PstI* using a 1.6 Kb *relA/spoT* fragment as DNA probe. The *R. solanacearum* strains used for hybridization are indicated in the figure. Lane P is unlabeled DNA probe.

We used primer combinations ps9-F/ps11-R and ps6-F/ps14-R to amplify these regions, which both gave products of approximately 3 Kb in size on the basis of the genomic DNA of

strains 715 and 1609. Sequence analyses of these amplicons showed that, for both strains, they consisted of IS*14231* (*ISRso2*) and IS*1021* (*ISRso3)* elements, with predicted transcription in opposite directions (Figure 2B).

Table 3. PCR primers used in this study

Primer	Sequence of primer (5'-3')	T annealing (°C)[1]	reference
cbhA-F	5'AGCTGCCTCACTACTAACTG 3'	52	Stevens & van Elsas 2010
cbhA-R	5' CCGGCTGTAGTTCCTTGAAT 3'	52	Stevens & van Elsas 2010
spoT-F	5' GAACTGCGTTGGAGGCCATC3'	60	Stevens & van Elsas 2010
spoT-R	5' TATCCAAGAAGCAGGCTGAG 3'	60	Stevens & van Elsas 2010
PglA-F2	5' GCAGAACTCGCCCAACTTCC 3'	58	this study
PglA-R	5 CTTCAGCGGCACGAAGGCAT 3'	58	this study
SSH primer 1	5' CTAATACGACTCACTATAGGGC 3'	62	BD bioscience
SSH nest1	5' TCGAGCGGCCGCCCGGGCAGGT 3	68	BD bioscience
SSH nest 2	5'AGCGTGGTCGCGGCCGAGGT 3'	68	BD bioscience
SP6	5' ATTTAGGTGACACTATAGGG 3'	55	this study
T7	5' TAATACGACTCACTATAGGG 3'	55	this study
ps1-F	5' TCACCGACCGCGCTACGAAT 3'	59	this study
ps1-R	5' TCGGTAGCGGCGGAAGTCAT 3'	59	this study
ps2-F	5' ACGTCGTCGGCAAGAGCTAC 3'	59	this study
ps2-R	5' GGTGTGGAAGTCGCCAATGT 3'	59	this study
ps3-F	5' GCCACGTTCCTGTCTTGGAT 3'	59	this study
ps3-R	5' ACTGCGAACGAGCCTGTTAG 3'	59	this study
ps4-F	5' CGGTGTGGTGATTGCACAGA 3'	59	this study
ps4-R	5' ACAAGGCCAGAACGCAGAGT 3'	59	this study
ps5-F	5' GCAAGGTCTGGCTAAGACTG 3'	59	this study
ps5-R	5' CGACGACATGATCGACTACG 3'	59	this study
ps6-F	5' AGACCGTTGTCGCAAGTTAC 3'	59	this study
ps6-R	5' GCGCTCAAGGATTGACTGAA 3'	59	this study
ps7-F	5' CGGCAGTCGCATGATTATCT 3'	59	this study
ps7-R	5' AATGGTGCCGTCTGTTGAAG 3'	59	this study
ps8-F	5' CTCACGCGATGGATACAGGA3'	59	this study
ps8-R	5' GAGCTGGTGAACGTGTATGG 3'	59	this study
ps9-F	5' TGCAGAAGTCGCAAGCTCAT 3'	60	this study
ps9-R	5' TTGTACCGGCTCTAGTGGAA 3'	60	this study
ps10-F	5' GGTCATCGCAAGGTTCGTTA 3'	58	this study
ps10-R	5' CGAGTCATGCCATCTTGGTT 3'	58	this study
ps11-F	5' CTTGCTGCCTCCTTGAATGA 3'	58	this study
ps11-R	5' GACGCTGCTCGTGTAATGAT 3'	58	this study
ps12-F	5' TCGAAGCGGCTCTGACTTAT 3'	55	this study
ps12-R	5' ATGACAGCCGGTGGTATGAA 3'	55	this study
ps13-F	5' ATGCCGTGCCGCTTAAGATA 3'	55	this study
ps13-R	5' ATCCACCTTGGATGCGATTC 3'	55	this study
ps14-F	5' CAACATCACAGCGGATGCTA 3'	55	this study
ps14-R	5' TCGCGATGTACGACACGATA 3'	55	this study
ps15-F	5' ACGCCTACCGACAGATAACG 3'	55	this study
ps15-R	5' GACGGTGGTGCGATTGAAGT 3'	55	this study
IS2/3-seq	5' ACGCTGCACGATCATTGACC 3'	seq. primer	this study

F, forward primer, R, reverse primer

[1] Annealing temperatures. Standard PCR reactions were preceded by a 5 min denaturation step at 96°C, followed by a final extension step for 5 min at 72°C.

Because we were interested in how these *ISRso2* and *ISRso3* elements are linked to each other in these IS "blocks", we designed an additional primer (IS2/3-seq, Table 3) for further sequencing. For both IS blocks, we found a 107-bp sequence connecting the two IS elements, with no apparent homology to other known sequences, except for a 21-bp stretch with 100% homology to a Holiday structure resolvase of *Bifidobacterium longum* (bp 56-76 of the 107 bp element (Figure 2B). Hence, although functional evidence is still lacking, the full region determined by the blocks likely contained several functions of relevance for transposition / recombination processes.

All PCR amplifications, except those with primer sets ps5 and ps6 (Table 3), performed on genomic DNA of strains 715, 1609 and KZR-5, yielded amplicons of the expected sizes (between 150 and 600 bp). Primer sets ps5 and ps6 also yielded such products for strains 715 and 1609, but not for strain KZR-5 (Table 3, Figure 2A), thus indicating the presence of a deletion. To more precisely determine the size of the deletion, we designed seven additional primer sets (Table 3; ps9 through ps15) for comparison of this region between the strains. Primer sets ps9, ps10 and ps13 through ps15 yielded products of the expected sizes for all tested strains, indicating that these regions were not part of the deletion. In contrast, primer sets ps11 and ps12 gave products of the expected sizes for strains 715 and 1609, whereas amplification on KZR-5 genomic DNA yielded no products (Figure 2A).

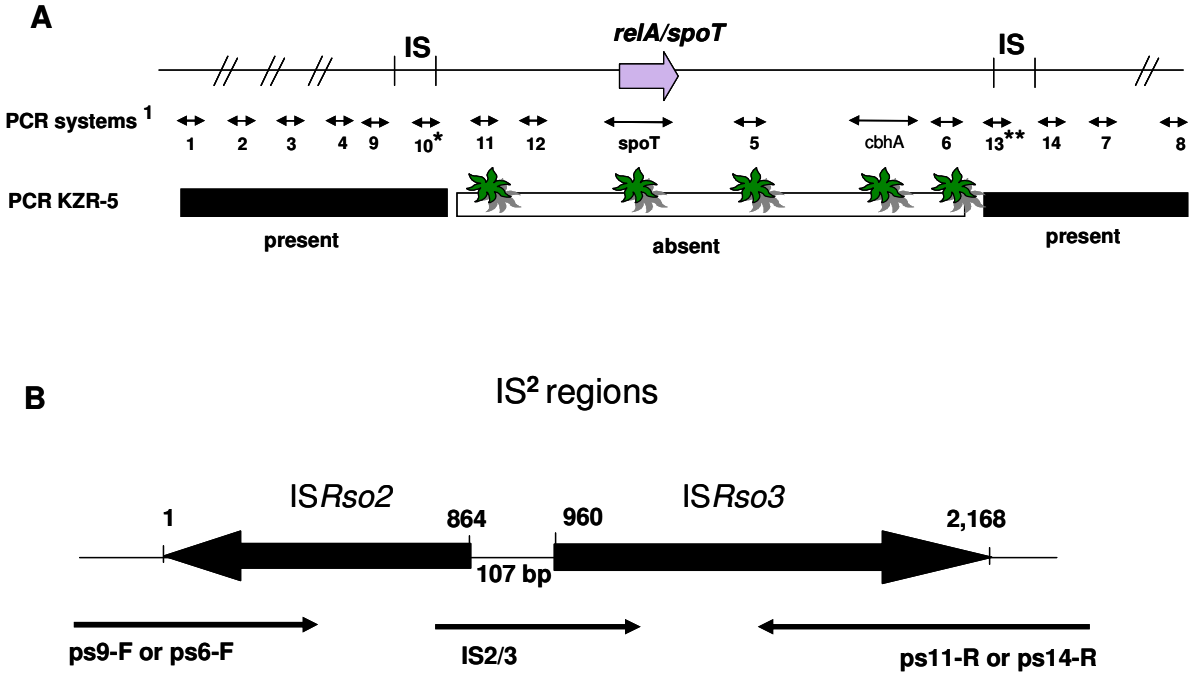

Figure 2. Primer systems used to characterize the identified region (A) and the composition of the IS blocks (B). **(A)** Upper line: inferred localization of IS elements and the *relA/spoT* region and PCR systems based on genomic information of strain 1609. IS: unassigned nucleotides in the 1609 genome which represent insertion sequences. [1] : primer systems, corresponding to primers shown in Table 3 (ps1 through ps15; ps15 is not indicated). PCR systems consisted of two PCR primers (e.g 1: ps1-F/ps1-R, 2: ps2-F/ps2-R, etc.) *: ps10 amplifies IS*Rso3* **: ps13 amplifies IS*Rso2*. Second line: Presence and absence of sequences in strain KZR-5 on the basis of PCR and hybridization : The absence in strain KZR-5 was confirmed by Southern blot analysis using the corresponding PCR products of strain 1609 as DIG labeled DNA probes. **(B)** Insertion sequence (IS) regions determined by sequence analysis of PCR products of strains 715 and 1609 that were obtained with primer combinations ps9-F/ps11-R or ps6-F/ps14-R. The position of the sequencing primers (ps9-F, ps6-F, ps11-R, ps14-R and IS2/3) is indicated. [2]: IS regions correspond to the two IS regions, which are similar, shown in A (IS).

To allow a cross-comparison of the identified genomic region between strains 715, 1609, UW551 and KZR-5, we performed PCR with a range of different primer combinations across most of the deleted region (see Figure 2). Using the combinations ps4-F with ps14-R/ps7-R or ps9-F with ps11-R/ps14-R/ps7-R, amplification products of the expected sizes (ranging from 1 to over 10 Kb) were obtained for strains 715, 1609 and UW551, as visualized on agarose gel (data not shown). However, these bands were lacking from the PCR reactions performed on strain KZR-5 (data not shown). PCR amplification across the IS blocks (using primers ps9-F/ps11-R and ps6-F/ps14-R) produced amplificons of approximately 3 Kb that were similar across strains 715, 1609 and UW551. This suggests that, for these strains, the PGI-1 region localizes within a similar genomic context.

To amplify the region around the putative deletion from strain KZR-5 genomic DNA, we used primers ps4-F/ps14-R and ps4-F/ps7-R, for which the amplificons were expected to range between 2 and 4 Kb. However, no such products were obtained. We also performed a PCR using primer pair ps11-F/ps14-R on strain KZR-5 genomic DNA, and, unexpectedly, obtained a clear 3.6 Kb product. To elucidate its sequence, the PCR product was cloned and partially sequenced using primers ps11-F, ps13-R, IS2/3 and ps14-R. Thus, the region to which primer ps11-F had annealed was identified as follows: 861 bp upstream of the ps4-F target site (Figure 2A), the stretch tgtgcctacg**ttgaatga** was present, which showed strong homology at the 3'-end with primer ps11-F (cttgctgcctc**ttgaatga**) and thus a ps11-F "landing" site was identified.

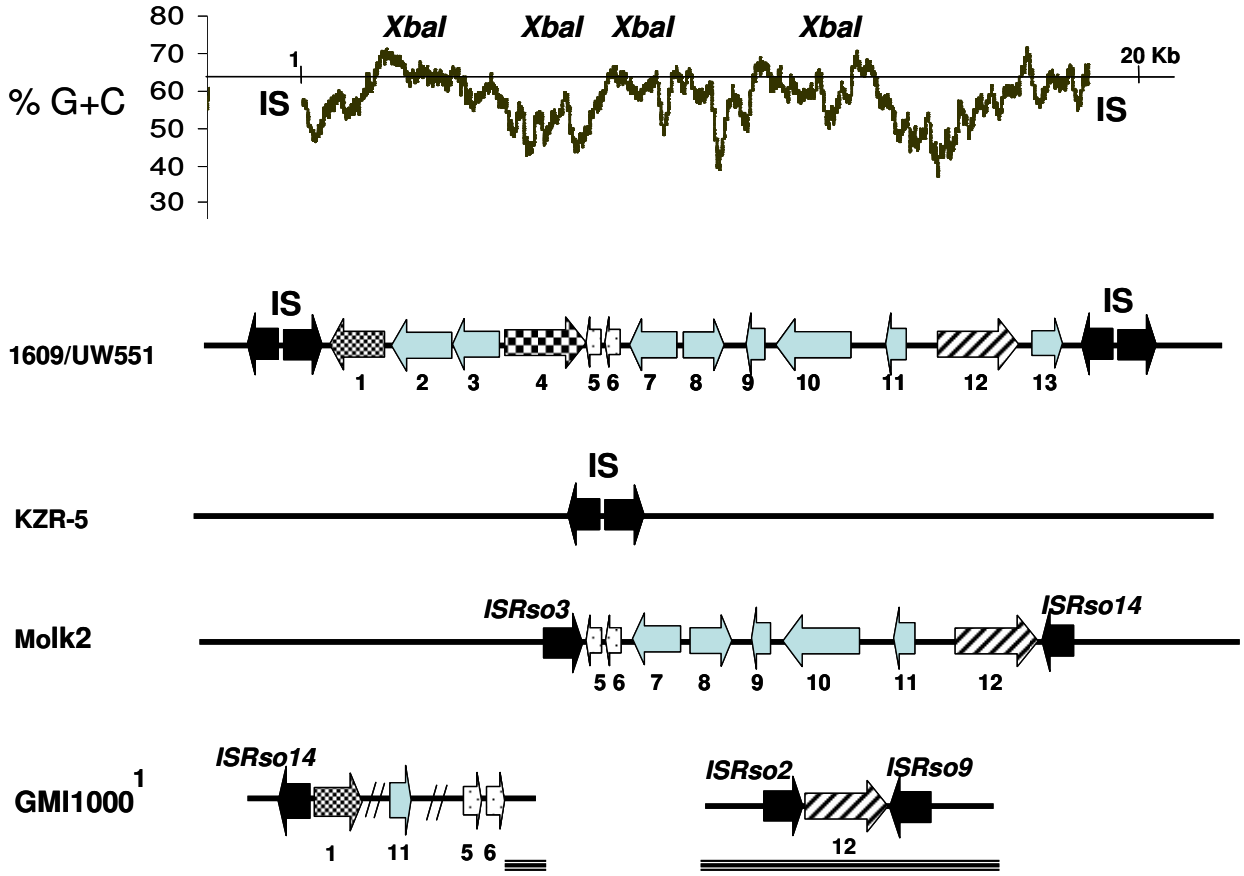

Figure 3. The PGI-1 region in strains 1609 and UW551, the deletion in strain KZR-5 and the related regions in strains Molk2 and GMI1000. ——— The average G+C content of PGI-1 is shown over 200 bp intervals (upper graph). The location of four *XbaI* sites (positions 3,614, 5,330, 8,156 and 13,293) is also indicated. Positions 1 and 20 Kb correspond to position 243,626 (1) and 225,013 (20 Kb) of strain 1609. The location of the IS elements and the PGI-1 region (in between the IS blocks) in strains 1609 and UW551 are indicated (second graph). The organization of this region is, based on PCR and Southern hybridization results, the same in strain 715. Numbers below arrows indicate ORF numbers as in Table 4. ORF 1 through 3 encode hypothetical proteins, ORF 4 encodes a RelA/SpoT domain protein, ORF 5 a site specific integrase/recombinase, ORF 6 a bacteriophage related protein, ORF 7 a drug metabolite transporter (DMT) protein, ORF 8 a transcriptional regulator protein, ORF 9 through 11 hypothetical proteins, ORF 12 a cellobiohydrolase and ORF 13 a hypothetical protein. The organization of the relevant regions in strains KZR-5, Molk2 and GMI1000 are also shown. [1] ORF 1, 11, 5 and 6 localize on the chromosome, ORF 12 localizes on the megaplasmid.
≡ Alternative codon usage region (ACUR)

Alignment of the resulting sequence with that of the strain 1609 genomic region showed that a region of 19.8 Kb, spanning the complete region in between the IS elements (17.6 Kb) plus one *ISRso2*/*ISRso3* block of 2.2 Kb (Figure 3), was present in strain 1609 and, by inference, 715, but had been deleted from strain KZR-5. The analysis also showed that the region harbors four *XbaI* restriction sites, which explains the observed PFGE pattern (*XbaI* digested genomic DNA) seen for strain KZR-5 as compared to that of strains 715 and 1609 (Stevens & Van Elsas 2010)

Characterization of the region

Based on information from the strain 1609 draft genome, the identified region has an average G+C content of 55%, which is considerably lower than the 64% average of the complete genome (Figure

3). Comparison of the sequences of the PGI-1 region (17.6 Kb size in between the IS elements) in the genomes of strains 1609 and UW551 (position 25,398 to 43,007) showed complete identity between the two regions, although the number of genes that had been annotated was somewhat different (Table 4). According to the strain 1609 annotation, the region contained genes encoding (1) a protein with a RelA/SpoT domain, which is a putative (p)ppGpp synthetase (ORF4, RSIPO_04909), (2) a transporter protein of drugs or metabolites (ORF7, RSIPO_04908), (3) a transcriptional regulator (ORF8, RSIPO_03301), (4) a cellobiohydrolase, which is involved in the degradation of cellulose (ORF 12, RSIPO_03298) and six hypothetical proteins (Table 4).
In addition, according to the strain UW551 annotation (Gabriel et al. 2006) there was a (bacteriophage-related) site-specific integrase/recombinase (ssi/r) (ORF5, RRSL_02058), a bacteriophage-related hypothetical protein (ORF6, RRSL_02059) and one additional hypothetical protein (ORF13, RRSL_02066). See Figure 3 and Table 4.

Table 4 Open reading frames (ORFs) present on PGI-1 in strains 1609, UW551, Molk2 and GMI1000

			nomenclature			
ORF	size (AA)[1]	gene/function [2]	IPO_1609	UW551	Molk2	GMI1000 [3]
1	324	hypothetical protein	RSIPO_03304	RRSL_02055	absent	RSc 0830
2	431	hypothetical protein	RSIPO_03303	RRSL_02056	absent	absent
3	302	hypothetical protein	RSIPO_03302	NA	absent	absent
4	486	RelA/SpoT domain protein	RSIPO_04909	RRSL_02057	absent	absent
5	63	site specific integrase/recombinase	NA	RRSL_02058	RSMK02625	RSc 0890
6	49	bacteriophage related protein	NA	RRSL_02059	RSMK02626	Rsc 0891
7	316	drug metabolite transporter (DMT) protein	RSIPO_04908	RRSL_02060	RSMK02627	absent
8	295	transcriptional regulator protein	RSIPO_03301	RRSL_02061	RSMK02628	absent
9	58	hypothetical protein	RSIPO_03300	NA	RSMK06220	absent
10	519	hypothetical protein	RSIPO_03299	RRSL_02063	RSMK02629	absent
11	133	hypothetical protein	RSIPO_04890	RRSL_02064	RSMK02632	RSc0834/0835
12	535	cellobiohydrolase	RSIPO_03298	RRSL_02065	RSMK02634	RSp 0583
13	137	hypothetical protein	NA	RRSL_02066	absent	absent

NA not annotated
[1] Size of the ORF based on annotation according to strain 1609 or UW551
[2] Gene function based on annotation for strains 1609, Molk2 or UW551
[3] ORFs identified in strain GMI1000 after Blast-P of annotated ORFs for strains 1609 and UW551

To further investigate genome flexibility, we assessed about 3 Kb of flanking sequence in the 1609 draft genome at both sides of the identified region. In the region flanking the left IS elements, we found a hypothetical protein and a hemagglutinin-related protein. At the right flank, we found genes homologous to those encoding TrbI (involved in bacterial conjugation), a VGR-related protein (VGR: In *E. coli* a protein with valine/glycine repeats and associated with Rhs elements (Wang et al. 1998), both potentially involved in genome flexibility, and two hypothetical proteins (data not shown).

To identify possible homologues of the *cbhA* and *relA*/*spoT* genes in the genome of strain KZR-5 we used BlastN and BlastP (available at NCBI) on the genome information of bv2 strains 1609 and UW551. However, we did not find close homologues of the *cbhA* or *relA*/*spoT* genes such as found on PGI-1 in the bv2 genomes (based on sequence homology and annotation). In addition, Southern hybridization with a *cbhA*-specific DNA probe showed single bands with genomic DNA of strains 715 and 1609 and no hybridization signal in strain KZR-5, similar to what was seen for

the putative gene with RelA/SpoT domain (Figure 1B). The *relA/spoT* gene of bv2 showed highest homology (using BlastP) to a similar gene from *Rhizobium etli* (46% identity) or *Exiguobacterium sp.* (40% identity), followed by a hypothetical phage-derived protein from *E. coli* (36% identity) and a putative *relA/spoT* gene from *Symbiobacterium thermophilium* (37% identity).

Comparison of the PGI-1 region across the sequenced *R. solanacearum* strains 1609, Molk2 and GMI1000 showed that several genes found in the region are indeed genetically flexible as they are flanked by IS elements and/or ACURs (Figure 3). For instance, the PGI-1 region in strain Molk2 was partially identical to that of strain 1609, as ORFs 5 through 12 appeared to be conserved. However, the Molk2 region completely lacked ORFs 1 to 4 as well as ORF13, which also were not present elsewhere in the genome (Table 4, Figure 3). Much like in strain 1609, the genes present were flanked by IS elements, but the nature of the IS elements differed. In contrast, the genes present in the PGI-1 regions of strains 1609, UW551 and Molk2 do not occur in a PGI-1-like island in the bv3 GMI1000 genome. In the latter strain, some of the genes are dispersed over the chromosome as well as the megaplasmid, while other genes are completely absent. For instance, the *cbhA* gene is encoded by a region on the megaplasmid, denoted Rsp0583. ORF 1 (hypothetical protein) and ORFs 5 (ssi/r) and ORF 6 (bacteriophage-related protein) localize 62 Kb apart from each other on the chromosome, with ORFs 5 and 6 co-localizing, like in PGI-1.

To determine whether islands like PGI-1 exist in other bacterial genomes, which might indicate a recent HGT event, we used the "string" database (http://string.embl.de), which aligns multiple ORFs against the 2,483,276 proteins of 630 organisms. Using this approach, we did not find any genomic regions with a similar gene order in other bacterial strains. However, in the genomes of *Polaromonas sp.* JS666 and *P. fluorescens* pfO1, we found that the transporter protein (RSIPO_04908) and the transcriptional regulator protein (RSIPO_03301) localize adjacent to each other, similar to the situation in PGI-1.

Phenotype and ecological behavior of environmental strain KZR-5 in comparison to the tropical potato-derived strain 715

To assess whether the loss of the genes for the putative RelA/SpoT domain protein and the cellobiohydrolase (CbhA), both uniquely present on PGI-1, conferred a discernable phenotype in strain KZR-5, we performed standard cellulose degradation and growth tests on strains KZR-5 and 715. Surprisingly, no differences in phenotypic behavior between strains KZR-5 and 715 were found. Then, to understand whether the novel environmental *R. solanacearum* strain KZR-5, in comparison to the potato-derived reference strain 715, had altered fitness in water under temperate climate conditions, we performed assessments of population dynamics at two temperatures in microcosms. Thus, the survival of strain KZR-5 was compared to that of strain 715 upon incubation at 4°C and 20°C (control). Strain KZR-5 persisted in a fashion similar to strain 715 in water at 20°C, with CFU numbers remaining roughly stable, between log 6.6 and log 7.1 from the onset of the experiment till day 130 (data not shown). At 4°C, the two strains behaved quite differently, with strain KZR-5 clearly being the best survivor over the experimental period (Figure 4A). At day 84, the CFU numbers of strain KZR-5 remained detectable at average levels between log 2.6 and 3.4 ml^{-1}, whereas those of strain 715 were at or below the limit of detection (Figure 4A). These counts were significantly different between strains KZR-5 and 715. (Fig. 4A; t test; $P< 0.05$). Thus, enhanced tolerance to cold stress was noted in strain KZR-5 as opposed to the comparator strain 715.

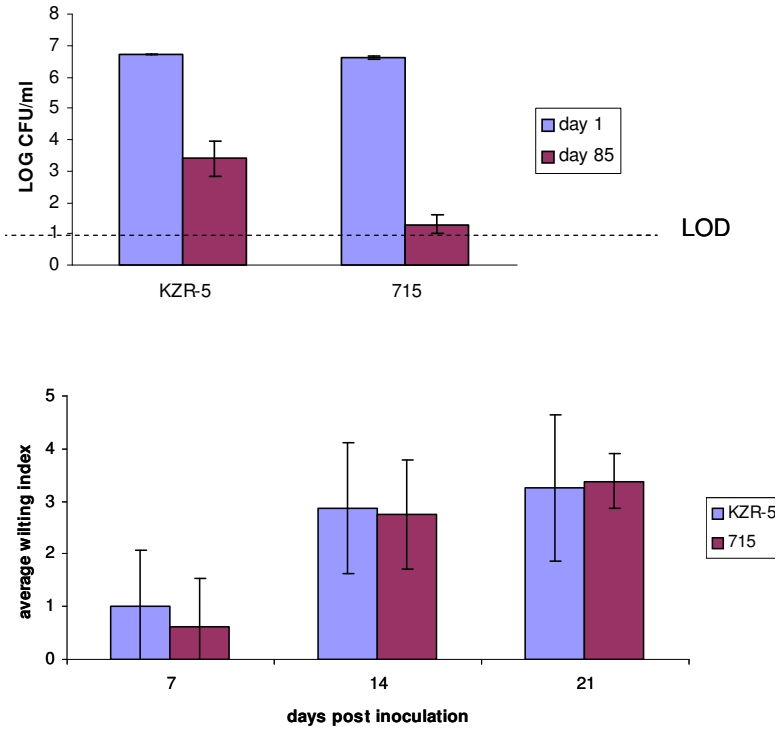

Figure 4 Survival of strains KZR-5 and 715 at 4°C (A) and virulence on tomato plants (B). **(A)** Culturability (the average of three replicates) of strains KZR-5 and 715 upon exposure to 4°C at t=1 (bar 1) and t= 84 days (bar 2). , -.-.-.-.- limit of detection (10 CFU/ml). Statistical classes (a and b) are indicated. P-values were < 0.05 **(B)** Average wilting of 4-5 week old tomato plants at 7, 14 and 21 days post inoculation. Plants were inoculated 25 ml of cell suspension containing 10^{+5} CFU/ml, incubated in the greenhouse at 26°C (day, 14 h) / 21°C (night, 10 h), and disease development in the plants was scored at regular intervals over time using a disease matrix ranging from 0, no wilting symptoms, to 4, all leaves wilted. The average wilting index was determined as wilting symptoms of all plants/number of plants.

Given the fact that a presumed *cbhA* gene was found to be present on PGI-1, we hypothesized that strain KZR-5 might have suffered a reduction in virulence on susceptible host plants as compared to the reference strain as well as, possibly, other environmental strains. Thus, virulence assays were performed on tomato using inoculum densities of 10^8 CFU/ml. The tests revealed complete wilting of the replicate test plants within 14 days (data not shown). Surprisingly, in none of the cases were significant differences in virulence found between strains KZR-5 and 715 (data not shown). As subtle differences might escape detection using high inoculum densities, we also performed a virulence assay using the same strains with inoculum densities of approximately 10^5 CFU/ml. At day 7, the wilting index of plants infested by strain KZR-5 was slightly higher (1.0) than that for strain 715 (0.6). At days 14 and 21, the wilting index was similar for both strains (2.8 vs 2.9 at day 14 and 3.3 vs 3.4 at day 21; P>0.05), suggesting they were, at least under these conditions, equally virulent on tomato.

Discussion

From among a larger set of novel environmental *R. solanacearum* bv2 strains, strain KZR-5 was specifically selected for a comparison of its genomic make-up to that of reference potato strain 715 using suppressive subtractive hybridization (SSH). This comparative analysis was undertaken in order to allow insight in the putative genomic changes incurred in strains present in the open environment for up to two decades, versus a tropical potato strain. The basis of the selection was a divergent PFGE pattern of *XbaI* digested genomic DNA that was previously revealed in strain KZR-5, which already provided a glimpse of genome diversity incurred by a genomic rearrangement (Stevens & van Elsas 2010).

Several studies have shown that SSH can be successful as long as the genomes that are subjected to the procedure are grossly homologous (Akopyants et al. 1998; Zhang et al. 2000; Janke et al. 2001; Parsons et al. 2003). Both *R. solanacearum* strains used here belong to bv2, which is known as a highly homogeneous group of organisms. Oligolocus sequence typing of over 2,000 nucleotides showed
that the strains were 100% homologous in the six regions analyzed (Stevens and van Elsas 2010). The SSH approach used in this study was effective in identifying genes or genomic regions that differ between the two selected *R. solanacearum* bv2 strains. All sequences found actually had homologues in the genome of strain 1609. Hence, we did not find sequences that had been newly acquired by strain KZR-5, pointing to an absence of major HGT events as drivers of short-term evolution in the water population exemplified by strain KZR-5.

A major finding was the presence, in the SSH library, of a large number of sequences that are known to be involved in genome flexibility (7/40 of KZR-5 and 4/19 of 715, see Table 2). This clearly points to a major role of genomic rearrangements in shaping the *R. solanacearum* bv2 genome under the local conditions. Moreover, the fact that we found a partial rRNA gene sequence in each library that localized to a single *rrn* operon (clones 10 and 46, Table 2) might indicate that such regions, together with the Rhs elements, mediate chromosomal rearrangements like those shown by Hill (Hill 1999). However, we have not further addressed this hypothesis.

The clearest evidence found in this study for the involvement of DNA rearrangements in genome diversification between the strains analyzed was the finding of a deletion of a putative genomic island, PGI-1, in strain KZR-5. In this case, strain 715 was the tester and strain KZR-5 driver in the SSH analysis. We then used multiple PCR systems to, firstly, define the size of the deletion in strain KZR-5 and, secondly, amplify and sequence the flanking regions. We cannot explain why amplification with primer sets ps4-F/ps14-R or ps9-F/ps14-R was unsuccessful in strain KZR-5, while the ps11-F/ps14-R set amplified a specific product. One possible explanation could be that other copies of *ISRso2* and *ISRso3* sequences (multiple copies are present in the *R. solanacearum* bv2 genome), hamper PCR amplification by acting as a primer sink due to "random" or "aspecific" annealing of the primers.

The PGI-1 region has key features of a genomic island that is potentially mobile because i) it has a lowered average G+C content compared to the average G+C content of the strain 1609 genome (55% vs 64%), (ii) it showed the presence of a site-specific integrase/recombinase (ssi/r) and a phage-related protein, (iii) there was a deletion of the region exactly at the IS blocks present at the island extremes, (iv) it revealed the presence of genes immediately at the right flank of the

right *ISRso3* element that encode TrbI (protein involved in conjugation) and a VGR-related protein, which (in *E. coli)* associates with Rhs and (v) it revealed the presence of genes such as *cbhA* and *relA*/*spoT* that are potentially involved in an ecologically relevant phenotype. Although we could assign potential functions to six ORFs, the function of eight other putative ORFs that were identified on the genomic island remained largely unknown. These ORFs encoded hypothetical proteins that lack known conserved domains. Hence, we cannot make any firm inferences about the function of these proteins and the possible effect of their deletion.

Comparison of the PGI-1 region with similar ones in bv1 strain Molk2, and in bv3 strain GMI1000 provided support for the notion that a genetically flexible region was found that was (1) consistently present in bv2 strains 1609 and UW551 and, by inference, 715, and (2) partially and differentially present across the other biovar strains, whereas it was absent from strain KZR-5. This finding supports the hypothesis that the region is a genomic island, as the PGI-1 region was flanked by IS elements in strains 715, 1609 and UW551. Also, in the bv3 strain strain GMI1000 some genes of the island, i.e. *cbhA* and the ssr/i and phage-related genes, were found to lie inside, or close to, ACURs which have probably been acquired through HGT (Salanoubat et al. 2002).

As a cellobiohydrolase gene was found to be present in the reference potato-derived *R. solanacearum* bv2 strains 1609 and 715 (as well as in bv1 strain Molk2 and bv3 strain GMI1000), but absent from non-phytopathogenic *Ralstonia* species (Liu et al. 2005), it might play a role in the interaction of *R. solanacearum* with host plants. Moreover, bv2 strain UW551 and bv3 strain GMI1000 produce, next to the cellobiohydrolase, at least five other enzymes, i.e a β 1,4-endoglucanase (Egl), an endopolygalacturonase (PglA), two exopolygalacturonases (PehB and PehC) and a pectin methyl esterase (Pme). Collectively, these enzymes probably assist the bacterium in the degradation of plant cell materials (Gabriel et al. 2006). It was shown that a GMI1000 mutant lacking the *cbhA* gene was reduced in virulence, corroborating the role of CbhA in the strain's ability to wilt plants (Liu et al. 2005). The finding of the loss of cellobiohydrolase in strain KZR-5 and the concurrent lack of an effect on plant invasion was puzzling. One explanation might be that for bv2 strains the *cbhA* gene is less important in plant invasion than for bv3 strains. Alternatively, a functional homologue of CbhA might be present in strain KZR-5, although we did not find other *cbhA*-like genes in the draft genome sequences of strains 1609 and UW551, nor additional bands with Southern blot analysis using a *cbhA* DNA probe. Therefore, it is unlikely that gene duplication had occurred in strain KZR-5, which would have maintained the functionality of the gene in spite of its deletion with PGI-1. As endo-and exoglucanases have a glycosyl hydrolase family 6 (GH6) domain (http://www.cazy.org/), we inspected the annotated 1609 and UW551 genomes to see whether other glycosyl hydrolases (with a presumed cellobiohydrolase activity) exist in the bv2 genome. We found four such genes (RSIPO_01357, 03533, 04005 and 03946), but these belong to other functional groups (GH15, GH18 or the AlgLyase superfamily) than CbhA (and Egl) and have different roles in carbohydrate metabolism. However, other functional homologues might still exist in *R. solanacearum* bv2, as the function of many genes in the bv2 genome is unknown.

The presence of a gene encoding a protein with a RelA/SpoT domain in the deleted PGI-1 region was striking. RelA/SpoT proteins are conserved across the bacteria, as they are thought to function in responses to starvation or other stress, as a result of their ppGpp(p) synthetase/hydrolase activity (ppGpp is a so-called alarmone). In *E. coli*, the *relA* and *spoT* genes become activated as part of the stringent (stress) response upon amino acid (*relA*) and carbon

(*spoT*) starvation. However, the function of RelA/SpoT in the stress response might differ between different bacterial species {Das & Bahdra, 2008, Chatterji & Ojha, 2001}. In *R. solanacaerum*, homologues of the *E. coli* RelA and spoT proteins exist (RSIPO_01119 and RSIPO_01943 respectively), but they were never studied in detail. In many gram-positive bacteria, only a single bifunctional RelA/SpoT homologue is responsible for balancing (p)ppGpp levels in the cell (Mittenhuber 2001). However, in *Bacillus subtilis* and *Streptococcus mutans* other functional ppGpp synthetases are also described (Lemos et al. 2007), (Nanamiya et al. 2008). These proteins have a RelA/SpoT domain, but lack the other conserved motifs found in traditional RelA and SpoT proteins and they appear to represent a different class of (p)ppGpp sythetases called SAS (small alarmone synthetase) proteins (Nanamiya et al. 2008). A comparison of the amino acid sequence of the *R. solanacearum* RelA/SpoT domain protein with the SAS proteins of *B.subtilis* and *Streptococcus mutans* showed they are indeed similar in composition and size (data not shown), thus indicating a putative similar function. The SAS proteins do not appear to be essential in the classical stress response but function in the synthesis of alarmone under other conditions (Nanamiya et al. 2008). Why a RelA/SpoT domain protein, which might be associated with presumed ecological fitness (survival) under stress, was deleted from the genome of strain KZR-5 and whether this led to enhanced fitness, is still unclear from the current work and thus remains speculative. The enhanced survival of KZR-5 in water at low temperature, though, provides food for the contention that there may be an advantage for the possession of the deletion under such stress conditions. Our results may indicate that life in an aquatic environment in a temperate climate (characterized by fluctuating but largely low temperature and nutrient conditions, persistence in bulk water, sediment and/or bittersweet) has incited a different survival modus, possibly also altering the function of other genes involved.

IS elements clearly played a major role in the deletion event, through an interaction between the two *ISRso2*/*ISRso3* blocks that flank the island. It seems likely that a recombination/cross-over occurred between the two 2.2 Kb large elements, which are 100% homologous to one another, thereby deleting a DNA loop of 17.6 Kb in between the *ISRso2*/*ISRso3* elements. IS element mediated genome diversification could play an important role in the structural flexibility of *R. solanacearum* bv2, like in other bacterial species such as *Burkholderia mallei* (Nierman et al. 2004)), *Yersinia* species (Darling et al. 2008)) and *Pseudomonas aeruginosa* (Battle et al. 2009). Unfortunately, although tens of *ISRso3* elements have recently been found using hybridisation (Stevens & Van Elsas 2010), we do not know the exact number and variability of diverse IS elements in the bv2 genome. We also ignore whether more IS blocks, such as found in PGI-1, exist in the genome, as these regions are often "missed" using shotgun genome sequencing as used for the bv2 strain 1609 draft genome.

One of our aims was to assess whether environmental strain KZR-5 was different in ecological behavior as compared to the tropical potato strain 715. First, upon inoculation of tomato plants at two inoculum densities, no changed ability of strain KZR-5 to cause wilting disease was observed, in spite of the deleted *cbhA* gene. The putative differences in virulence on tomato between strains KZR-5 and 715 may have been minor, which may relate to the reasons outlined above. On the other hand, the survival of strain KZR-5 in water at low temperature was clearly enhanced compared to that of strain 715. Thus, strain KZR-5 may have adapted to conditions prevailing in temperate climate waters in relation to the reference potato strain.

In this study, we pinpointed the activity of IS elements (and Rhs elements) as the main

mechanism that facilitates genomic changes in *R. solanacearum* bv2 and thus its potential adaptation to selective pressures from the environment. Despite our increasing knowledge about the genetic content of whole bacterial genomes (Binnewies et al. 2006), we still largely rely on the examination of single strains, and their unique genetic make-up, to assess how a particular genetic context (including the presence or absence of genomic islands) correlates with strain behavior in the environment. Future work with bv2 strain KZR-5 will attempt to more precisely establish this correlation.

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References

Akopyants NS, Fradkov A, Diatchenko L, Hill JE, Siebert PD, Lukyanov SA et al (1998) PCR-based subtractive hybridization and differences in gene content among strains of *Helicobacter pylori*. Proc Natl Acad Sci USA 95:13108–13113

Álvarez B, López MM, Biosca EG (2008) Survival strategies and pathogenicity of *Ralstonia solanacearum* phylotype II subjected to prolonged starvation in environmental water microcosms. Microbiology 154:3590–3598

Battle SE, Rello J, Hauser AR (2009) Genomic islands of *Pseudomonas aeruginosa*. FEMS Microbiol Lett 290:70–78

Bertolla F, Frostegard A, Brito B, Nesme X, Simonet P (1999) During infection of its host, the plant pathogen *Ralstonia solanacearum* naturally develops a state of competence and exchanges genetic material. Mol Plant Microbe Interact 12:467–472

Binnewies TT, Motro Y, Hallin PF, Lund O, Dunn D, La T, Hampson DJ et al (2006) Ten years of bacterial genome sequencing: comparative-genomics-based discoveries. Funct Integr Genomics 6(3):165–185

Castillo JA, Greenberg JT (2007) Evolutionary dynamics of *Ralstonia solanacearum.* Appl Environ Microbiol 73:1225–1238

Chatterji D, Ojha AK (2001) Revisiting the stringent response, ppGpp and starvation signaling. Curr Opin Microbiol 4:160–165

Ciampi L, Sequeira L (1980) Influence of temperature on virulence of race 3 strains of *Pseudomonas solanacearum*. Am Potato J 57:307–317

Darling AE, Miklos I, Ragan MA (2008) Dynamics of genome rearrangement in bacterial populations. PLoS Gene 4:e1000128

Das B, Bhadra RK (2008) Molecular characterization of *Vibrio cholerae* ΔrelA ΔspoT double mutants. Arch Microbiol 189:227–238

Elphinstone JG, Hennessy J, Wilson JK, Stead DE (1996) Sensitivity of different methods for the detection of *Pseudomonas solanacearum* (Smith) Smith in potato tuber extracts. Bull OEPP 26:663–678

Elphinstone JG, Stanford HM, Stead DE (1998) Detection of *Ralstonia solanacearum* in potato tubers, *Solanum dulcamara* and associated irrigation water. In: Prior PH, Allen C, Elphinstone J (eds) Bacterial wilt disease: molecular and ecological aspects. Springer-Verlag, Berlin, pp 133–139

Fegan M, Prior P (2005) How complex is the *Ralstonia solanacearum* species complex? In: Allen C, Prior P, Hayward AC (eds) Bacterial wilt disease and the *Ralstonia solanacearum* species complex. American Phytopathological Society Press, St. Paul, pp 449–461

Gabriel DW, Allen C, Schell M, Denny TP, Greenberg JT, Duan YP et al (2006) Identification of open reading frames unique to a select agent: *Ralstonia solanacearum* race 3 biovar 2. Mol Plant Microbe Interact 19:69–79

Granada GA, Sequira L (1983) Survival of *Pseudomonas solanacearum* in soil, rhizosphere and plant roots. Can J Microbiol 29:433–440

Grey BE, Steck TR (2001) The viable but nonculturable state of *Ralstonia solanacearum* may be involved in long-term survival and plant infection. Appl Environ Microbiol 67:3866–3872

Guidot A, Prior P, Schoenfeld J, Carrere S, Genin S, Boucher C (2007) Genomic structure and phylogeny of the plant pathogen *Ralstonia solanacearum* inferred from gene distribution analysis. J Bacteriol 189:377–387

Guidot A, Coupat B, Fall S, Prior P, Bertolla F (2009a) Horizontal gene transfer between *Ralstonia solanacearum* strains detected by comparative genomic hybridization on microarrays. ISME J 3:549–562

Guidot A, Elbaz M, Carrere S, Siri MI, Pianzzola MJ, Prior P et al (2009b) Specific genes from the potato brown rot strains of *Ralstonia solanacearum* and their potential use for strain detection. Phytopathology 99:1105–1112

Hill CW (1999) Large genomic sequence repetitions in bacteria: lessons from rRNA operons and Rhs elements. Res Microbiol 150:665–674

Janke B, Dobrindt U, Hacker J, Blum-Oehler G (2001) A subtractive hybridisation analysis of genomic differences between the uropathogenic *E. coli* strain 536 and the *E. coli* K-12 strain MG1655. FEMS Microbiol Lett 199:61–66

Janse JD (1996) Potato brown rot in western Europe—history, present occurence and some remarks on possible origin, epidemiology and control strategies. Bull OEPP 26:679–695

Janse JD (1998) Experiences with potato brown rot *Ralstonia solanacearum* biovar 2, race 3 in the Netherlands. In: Prior PH, Allen C, Elphinstone J (eds) Bacterial wilt disease, molecular and ecological aspects. Springer-Verlag, Berlin, pp 146–152

Janse JD, van den Beld HE, Elphinstone J, Simpkins S, Tjou-Tam-Sin NNA, van Vaerenbergh J (2004) Introduction to Europe of *Ralstonia solanacearum* biovar 2, race 3 in *Pelargonium* zonale cuttings. J Plant Pathol 86:147–155

Lemos JA, Lin VK, Nascimento MM, Abranches J, Burne RA (2007) Three gene products govern (p)ppGpp production by *Streptococcus mutans*. Mol Microbiol 65:1568–1581

Liu H, Zhang S, Schell MA, Denny TP (2005) Pyramiding unmarked deletions in *Ralstonia solanacearum* shows that secreted proteins in addition to plant cell-wall-degrading enzymes contribute to virulence. Mol Plant Microbe Interact 18:1296–1305

Mittenhuber G (2001) Comparative genomics and evolution of genes encoding bacterial (p)ppGpp synthetases/hydrolases (the Rel, RelA and SpoT proteins). J Mol Microbiol Biotechnol 3:585–600

Nanamiya H, Kasai K, Nozawa A, Yun CS, Narisawa T, Murakami K et al (2008) Identification and functional analysis of novel (p)ppGpp synthetase genes in *Bacillus subtilis*. Mol Microbiol 67:291–304

Nierman WC, DeShazer D, Kim HS, Tettelin H, Nelson KE, Feldblyum T et al (2004) Structural flexibility in the *Burkholderia mallei* genome. Proc Natl Acad Sci USA 101:14246–14251

Parsons YN, Banasko R, Detsika MG, Duangsonk K, Rainbow L, Hart CA (2003) Suppression-subtractive hybridisation reveals variations in gene distribution amongst the *Burkholderia cepacia* complex, including the presence in some strains of a genomic island containing putative polysaccharide production genes. Arch Microbiol 179:214–223

Salanoubat M, Genin S, Artiguenave F, Gouzy J, Mangenot S, Arlat M et al. (2002) Genome sequence of the plant pathogen *Ralstonia solanacearum*. Nature 415:497–502

Stevens P, Van Elsas JD (2010) Genetic and phenotypic diversity of *Ralstonia solanacearum* biovar 2 strains obtained from Dutch waterways. Antonie van Leeuwenhoek 97(2):171–188

Swanepoel AE (1990) The effect of temperature on the development of wilting and on progeny tuber infection of potatoes inoculated with South-African strains of biovar-2 and biovar-3 of *Pseudomonas-solanacearum*. Potato Res 33:287–290

Terol J, Munoz-Soriano V, Arahal DR, Buades C, Perez-Perez A, Llop P et al (2006) Random genome sequencing of *Ralstonia solanacearum* strain IVIA 1602 and comparative analysis with strain GMI1000. J Phytopathol 154:556–564

Timms-Wilson TM, Bryant K, Bailey MJ (2001) Strain characterization and 16S–23S probe development for differentiating geographically dispersed isolates of the phytopathogen *Ralstonia solanacearum*. Environ Microbiol 3:785–797

Van der Wolf JM, Bonants PJM, Smith JJ, Hagenaar M, Nijhuis E, van Beckhoven JRCM (1998) Genetic diversity of *Ralstonia solancaeru*m Race 3 in Western Europe determined by AFLP, RC-PFGE and Rep-PCR. In: Prior PH, Allen C, Elphinstone J et al (eds) Bacterial wilt disease: molecular and ecological aspects. Springer-Verlag, Berlin, pp 44–49

Van Elsas JD, Kastelein P, van Bekkum P, van der Wolf JM, de Vries PM, van Overbeek LS (2000) Survival of *Ralstonia solanacearum* biovar 2, the causative agent of potato brown rot, in field and microcosm soils in temperate climates. Phytopathology 90:1358–1366

Van Elsas JD, Kastelein P, de Vries PM, van Overbeek LS (2001) Effects of ecological factors on the survival and physiology of *Ralstonia solanacearum* biovar 2 in irrigation water. Can J Microbiol 47:842–854

Van Overbeek LS, Bergervoet JHH, Jacobs FHH, van Elsas JD (2004) The low-temperature-induced viable-but-nonculturable state affects the virulence of *Ralstonia solanacearum* biovar 2. Phytopathology 94:463–469

Wang YD, Zhao S, Hill CW (1998) Rhs elements comprise three subfamilies which diverged prior to acquisition by *Escherichia coli*. J Bacteriol 180:4102–4110

Winstead NN, Kelman A (1952) Inoculation techniques for evaluating resistance to *Pseudomonas solanacearum*. Phytopathology 42:628–634

Zhang YL, Ong CT, Leung KY (2000) Molecular analysis of genetic differences between virulent and avirulent strains of *Aeromonas hydrophila* isolated from diseased fish. Microbiology 146:999–1009

Chapter IV

Comparative genomic hybridization of *R. solanacearum* biovar 2 strains

Patricia Stevens
Luciano Procopio
Jan Dirk van Elsas

Abstract

The genetic diversity of *R. solanacearum* biovar 2 strains is low compared to other strains of the *R. solanacearum* species complex. Comparative genome hybridization can be a powerful tool to detect differences in the genomes that are the result of rearrangements (inducing deletions). This chapter provides a list of 19 genes and one intergenic region that may be differentially present within *R. solanacearum* strains isolated from Dutch waterways. The list of genes was obtained by comparative genome hybridization of DNA of three environmental strains against a microarray containing *R. solanacearum* biovar 2 strain 1609 specific genes as well as the complete genome of biovar 3 strain GMI1000. In total, 12 genes and one intergene were identified that appeared to be present in strains 1609 and 715 but were absent from strain KZR-5. Of these, four genes plus the intergene localized to a previously identified genomic island, PGI-1, which is absent in strain KZR-5. The other eight genes represented a variety of functions. Based on PCR results, these genes turned out to be present in strain KZR-5. Moreover, the genome comparisons showed that two putative deletions were present in the genome of strain PA1 and one in that of strain KZR-1. These putative deletions in strains PA1 and KZR-1 localized to genes that encoded hypothetical proteins. Specifically, three genes and one intergene produced higher hybridization intensities in one or more environmental strains. Overall, we concluded that, next to the detection of clear deletions, some of the differential hybridizations resulted from putative gene duplications or, otherwise, rearrangements. However, further work needs to be done to confirm this conclusion.

Introduction

Comparative genome hybridization (CGH) has been shown to be a powerful tool to analyze the genome content of the *R. solanacearum* species complex. A DNA microarray representing the genes of strain GMI1000 has been developed (Occaialini et al., 2005). CGH using 18 strains that represented the whole spectrum of biodiversity of the *R. solanacearum* species complex has revealed that one third of the *R. solanacearum* genome consists of variable genes which are likely to be acquired through horizontal gene transfers (Guidot et al,. 2007). Based on the draft genome sequence of strain 1609, the GMI1000 representative microarray was expanded with oligonucleotides representing genes that were absent from strain GMI1000 (Guidot et al., 2009). Guidot et al. (2009) showed that using the GMI000/1609 microarray, biovar (bv)2 specific genes can be identified and a large proportion of these is organized in clusters in the 1609 genome, suggesting that many of the bv2 specific genes are located on islands and have potentially been acquired through horizontal gene transfers and, theoretically, can also be lost "*en bloc*" through deletions. Next to horizontal gene transfer, genome reduction (loss of genes or islands) may also contribute to increased fitness. This is most evident in organisms that are host dependent. Host dependency often results in loss, or inactivation, of genes that are no longer essential when living in the host. On the other hand, free-living organisms can also be subjected to genome reductions, especially if they have undergone a recent change of niche (Thomson et al., 2003). Genome adaptation by the acquisition or deletion of complete genomic regions (islands) has gained much attention of late and its importance in bacterial fitness becomes more and more clear.

Microarrays offer excellent possibilities to assess genomic differences between strains. However, as microarrays represent only 'known' genes, newly-acquired genes will remain undetected. Thus, the main disadvantage of this technique is that it will only provide information on the distribution of genes that are present on the microarray and that newly acquired genes or islands will remain undetected. The power of CGH thus lies in the detection of differences in the (known) gene content based on differences in hybridization intensity.

We previously detected a deletion of 17.6 Kb in environmental bv2 strain KZR-5 (chapter III, Stevens and van Elsas, in press) and we hypothesized that additional deletions may have occurred in the *R. solanacearum* population present in local waterways. As *R. solanacearum* bv2 has been introduced recently in Europe and belongs to the least diverse and most host range restricted type of all *R. solanacearum* strains, it might be adapting to specific conditions in water courses that result in a changed genomic make-up (i.e by deletions). When bacteria are - for prolonged periods of time - in close association with bittersweet plants, genome decay could be a means by which increased fitness can be gained. To better understand a possible role for genome change or reduction in the adaptation of *R. solanacearum* strains to temperate climate open environments, we compared the genome content of three novel Dutch environmental bv2 strains (KZR-5, KZR-1 and PA1) to that of potato strain 1609. As we identified a genomic deletion in strain KZR-5 (chapter III) by suppressive subtraction hybridization against potato strain 715, we also compared, as a control, the genomes of strains KZR-5 and 715, as well as strain 1609 against strain 715.

Material and Methods

Bacterial strains and growth conditions

The *R. solanacearum* strains used in this study were strains 1609, 715, KZR-1, KZR-5 and PA1. Bacterial strains were maintained at -80°C in 0.1 x TSBS media (10% strength Trypticase Soy Broth [Becton Dickinson and Company, Sparks, Md, USA], 0.1% sucrose; pH 7.2) containing 20% glycerol. All strains were grown at 28°C with shaking in liquid 0.1 x TSBS.

Microarray description

The DNA microarray used in these experiments was generated by Occhialini et al. (2005) and Guidot et al. (2009). The microarray initially encompassed 5,074 oligonucleotides (oligo's) representative of the 5,120 predicted genes of strain GMI1000 (Occhailini et al., 2005). To improve detection of genes present in the *R. solanacearum* species complex, the microarray was expanded by spotting an additional 660 oligo's specific for strain 1609 (representing 630 genes and 30 intergenic regions) which were absent, or divergent, in strain GMI1000. The microarray is composed of 70-or 65-mer oligo's and also includes some negative and "blank" controls.

DNA labeling and hybridization

Genomic DNA was extracted from the selected strains using the Ultraclean ™ microbial DNA extraction kit (MoBIO Laboratories Inc. Carlsbad, UK) and labeled with either Cy3 or Cy5 fluorescent dye (Amersham, Biosciences) by using the Bioprime DNA labeling system kit (Invitrogen) according to the manufacturer's instructions. For a 50µl reaction mixture, 2 µg of genomic DNA in 23 µl of sterile water was heated at 95°C for 10 min, combined with 20µl of 2.5 x random primers solution, heated again at 95°C for 5 min, and chilled on ice. Remaining components were added to the following final concentrations: 0.12 mM dATP, dGTP and dTTP; 0.06 mM dCTP;0.02 mM Cy3- or Cy5-dCTP (Amersham Biosciences); 1 mM Tris-HCl (pH 8.0); 0.1 mM EDTA; and 40 units of Klenow fragment (Invitrogen). The solution was incubated at 37°C for 2 h before the reaction was stopped by adding Tris-EDTA (pH 8.0) to a final concentration of 45 mM. The fluorescently-labeled DNA was purified using the QiaGen QiaQuick Nucleotide Removal Kit (Qiagen Benelux BV, Venlo, the Netherlands) and dissolved in 50 µl elution buffer. Hybridizations were carried out in an ISO20 hybridization incubator (Grant). Each experiment was run as a competitive hybridization by using Cy3-labeled DNA from the environmental strains KZR-1, KZR-5, PA1 and PA5 and Cy5-labeled DNA from strain 1609 (Table 1). For strain 715, Cy3- as well as Cy5-labeled DNA was used, depending on the comparator strain (Cy5 in combination with strain KZR-5 and Cy3 in combination with strain 1609)

Microarrays were prehybridized for 2 h at room temperature in Dig easy buffer (Roche) containing 25 mg/ml of salmon sperm DNA. Hybridization was done for 15 h at 42°C after 750 ng of Cy3 and Cy5-labeled DNA had been added. Following hybridization, microarrays were washed in 2×SSC, 0.5% SDS, at room temperature and subsequently for 5 min in 1× SSC, 0.25% SDS to remove non-specifically hybridized DNA.

Table 1. The strains, different combinations used for CGH and the cut-off values used for analysis of the CGH experiments are shown.

strain/label			cut off value used	
Cy5		Cy3	Cy5/Cy3	Cy3/Cy5
1609	x	KZR-5	2.0	5.0
715	x	KZR-5	2.0	5.0
1609	x	KZR-1	2.0	5.0
1609	x	PA1	2.0	5.0
1609	x	715	2.0	5.0

Array scanning and analysis

The hybridized slides were scanned in a GenePix Autoloader 4200AL (Axon Instruments) using GT LS (version 3.01; Genomics Solutions) with slide dimensions of 1500 x 3200 pixels, gain 55 / black 50 (Cy3; green), and gain 62 / black 30 (Cy5; red). Quantification of the signals from individual arrays was done using ArrayPro 4.5 (Media Cybernetics Inc., Silver Spring, MD). Prior to analysis, spots with impurities, high local background fluorescence, or weak intensity (5% of the weakest spots) were filtered out from all data sets. To determine which ratio of signals represented differences in genome content (i.e absence of genes) we calculated the ratio between the Cy5 and Cy3 signals. As at the chosen ratio (5.0), the sequences representing genes present on PGI-1 (known to be absent from strain KZR-5) were not differentially detected in the 1609:KZR-5 and 715:KZR-5 mixes, we set the cut-off value at 2.0 for these comparisons. The cutoff value was set at 5.0 when the hybridization signal was increased for environmental strains KZR-1, KZR-5 as well as PA1 when an increase in hybridization signal was found from the Cy3 channel (i.e the environmental strains and - in one case - strain 715, Table 1). The latter was because the autofluorensence produced by Cy3 was higher than that of Cy5 (van Hijum et al. 2005).

PCR analysis and DNA sequencing

PCR primers used to analyze the presence of genes identified as additional deletions in strain KZR-5 are listed in Table 2.

Table 2. Primers used to analyze the presence of identified sequences with CGH in strain KZR-5.

Target sequence	Primer name	Sequence of primer (5'-3')
RSIPO_00601	plpA-F	5' ATCGTGGTGGCGATCATCGG 3'
	plpA-R	5' CGAGCCAGCGTGCAAGTTAG 3'
RSIPO_03090	bfp-F	5' GCGTTGCATGAAGAGATCAG 3'
	bfp-R	5' AGTCCACCTCCGAGATGAAT 3'
RSIPO_03575	lysP-F	5' TACGCGGAGGAGTTCATTGG 3'
	lysP-R	5' TACCGCTGTTGCAGGATGAG 3'
RSIPO_04244	EPSph-F	5' ACCGTGACAACGAAGAACTC 3'
	EPSph-R	5' CAAGCGGACATAGTGATCCA 3'
RSIPO_04425	fliM-F	5' CCAACGAGGTCGATGAGGAA 3'
	fliM-R	5' CGTATTGGCCGTTGAAGGTG 3'

F, forward primer, R reverse primer

For PCR amplification, we used the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, USA). Standard PCR reaction mixtures contained 1x PCR buffer (1.5 mM MgCl, 10 mM Tris and 50 mM KCl, Roche Applied Science, Basel, Switserland), 2.5 mM $MgCl_2$, 10% DMSO, 200 μM each deoxynucleotide, 0.2 μM of each primer and 20 U/ml Taq DNA polymerase (Roche Applied Science, Basel, Switserland). The cycling program was as follows: denaturing at 96˚C for 5 min; 34 cycles (96˚C for 30 sec, 55˚C for 30 sec, 72˚C for 40 sec) and a final extension step at 72˚C for 5 min. Products were analyzed by electrophoresis on 1% agarose gels (Roche diagnostics, Mannheim, Germany) (Sambrook & Maniatis 1989). DNA sequencing was performed in Applied Biosystems 3730XL sequencers using the corresponding forward primer used for PCR amplification.

Results

CGH on the GMI1000/1609 microarray were performed to compare the gene content of three novel environmental bv2 strains, KZR-5, KZR-1 and PA1, to those of potato strain 1609. As a control, we also compared the gene content of strain 715 to that of strains 1609 and KZR-5. No differences in gene content were detected between strains 1609 and 715, which indicates the two strains are highly similar in gene content. The analysis identified a set of 19 genes and one intergene which appeared to be differentially present within this strain set (Tables 3, 5 and 6). Most of the identified genes indicated genomic differences between strains KZR-5 on the one hand and potato strains 1609 and 715 on the other hand, which suggested deletions in strain KZR-5 (Table 3). Of the 13 identified genomic regions, four genes (and one intergenic region) could be tentatively localized to genomic island PGI-1, which was previously shown to be absent from the genome of strain KZR-5 (grey box, see also chapter III). The other nine genes encoded by PGI-1 were not detected using CGH. Three of these were represented on the microarray by GMI1000 sequences and six of these genes had not been spotted on the microarray (A. Guidot, pers. comm) (Table 3).

Table 3. List of genes and intergenes that are putative specific for strains 1609 and/or 715 identified with CGH using strains 1609 and 715 against strain KZR-5.

spot microarray	ORF name	1609	715	KZR-5	decription locus	PCR
IPO1609_0376	RSIPO_00601	+	+	-	type 4 fimbrial pilin-like protein, plpA	+
IPO1609_0528	RSIPO_01206	+[2]	+	-	hypothetical protein	
IPO1609_0529	RSIPO_03090	+	+	-	bifunctional protein (mta/sah nucleosidase) (p46):	+
IPO1609_0527	RSIPO_03300	+	+[1]	-	hypothetical protein	+
IPO1609_0526	RSIPO_03301	+	+	-	transcription regulator protein	+
IPO1609_0524	RSIPO_03302	+	+	-	hypothetical protein	+
IPO1609_0522	intergene	+	+	-	non-coding between RSIPO_03304/03305	+
RS05157_AA	RSIPO_03304	+	+	-	hypothetical protein	+
IPO1609_0474	RSIPO_03575	+	+	-	lysine-specific permease protein, lysP	+
IPO1609_0523	RSIPO_03824	+	+	-	hypothetical protein	
IPO1609_0525	RSIPO_04244	+	+	-	exopolysaccharide phosphotransferase protein	+
IPO1609_0530	RSIPO_04425	+	+	-	flagellar motor switch protein fliM	+
RS03886_AA	Rsp0594	+	+[2]	-	hypothetical protein	

Positive (+) and negative (-) hybridizations on the microarray are reported for each gene. The grey box indicates the genes that localize to PGI-1 which is absent in strain KZR-5.

[1] The ratio between the signals was higher than 10

[2] No difference in hybridization signal was observed against strain KZR-5

Table 4. List of genes encoded by the genomic island PGI-1, their presence/absence on the microarray and detection of the PGI-1 deletion in strain KZR-5 through CGH. The strain used to design the corresponding oligonucleotide is also indicated

ORF	locus 1609	gene/function	presence/absence on micro array	CGH
1	RSIPO_03304	hypothetical protein	GMI1000 oligo	+
2	RSIPO_03303	hypothetical protein	no representative oligo on micro array	
3	RSIPO_03302	hypothetical protein	1609 oligo	+
4	RSIPO_04909	RelA/SpoT domain protein	no representative oligo on micro array	
5	NA	site specific integrase/recombinase	GMI1000 oligo	-
6	NA	bacteriophage related protein	GMI1000 oligo	-
7	RSIPO_04908	drug metabolite transporter (DMT) protein	no representative oligo on micro array	
8	RSIPO_03301	transcriptional regulator protein	1609 oligo	+
9	RSIPO_03300	hypothetical protein	1609 oligo	+
10	RSIPO_03299	hypothetical protein	no representative oligo on micro array	
11	RSIPO_04890	hypothetical protein	no representative oligo on micro array	
12	RSIPO_03298	*cbhA*, cellobiohydrolase	GMI1000 oligo	-
13	NA	hypothetical protein	no representative oligo on micro array	

NA, the gene is not annotated in strain 1609 (Stevens and van Elsas, 2010 in press)

Next to the genes localizing on PGI-1, another set of eight genes appeared to be differentially present between strain KZR-5 and 1609 as well as 715 (Table 3). However, PCR analysis of five of the thus identified genes indicated that they were all present in strain KZR-5 (data not shown). Sequence analysis of the amplicons obtained revealed that the sequences amplified indeed reflected the corresponding target sequences and no differences between the sequences of strain KZR-5 and those of strains 1609/715 were detected.

Furthermore, we identified one putative deletion in strain KZR-1 (containing an ORF with code IPO_03700) and two in strain PA1 (IPO_02168 and IPO_04016), all encoding hypothetical proteins (Table 4). Variations in hybridization intensities with the intergenic region probe IPO1609_0563 were consistently found using three different CGH combinations (Table 6), some of

them with signal ratio values exceeding 10 (up to 78). Two genes appeared to be specific for strain PA1 (one encoding a lytic transglycosylase [IPO_02168] and one encoding a dehydratase protein [IPO_04016]) whereas one was specific for strain KZR-1, encoding a vgr related protein [IPO_03700]).

Table 5. List of genes identified as putative deletions in strains KZR-1 and PA1

spot microarray	ORF name	1609	KZR-1	PA1	decription locus
RS00020_AA	RSc2699	+	-	+	hypothetical protein
RS05823_AA	RSp1167	+	-	+	hypothetical protein
RS05151_AA	RSc0768	+	+	-	hypothetical protein

Positive (+) and negative (-) hybridizations on the microarray are reported for each gene.

Table 6. List of genes and the intergene identified with CGH that gave increased hybridization values for strains KZR-5, KZR-1 and PA1

		combination CGH									
spot microarray	1609 nomenclature	1609	715	KZR-5	1609	KZR-1	1609	PA1	1609	715	description locus
IPO1609_0563 [1]	intergene	-	-	+[2]	-	+	-	+[2]	+	+	non-coding between type III effector and rhs related protein
IPO1609_0097	IPO_02168	+	+	+	+	+	-	+	+	+	lytic transglycosylase, catalytic protein
IPO1609_0362	IPO_04016	+	+	+	+	+	-	+[2]	+	+	dehydratase protein
IPO1609_0458	IPO_03700	+	+	+	-	+	+	+	+	+	vgr related protein

Positive (+) and negative (-) hybridizations on the microarray are reported for each gene.

[1] A single spot was detected for strain KZR-5 (against 1609, not 715) and KZR-1

[2] The ratio between the signals was higher than 10

Discussion

In this chapter, we described the results from comparison of the genome content of three *Ralstonia solanacearum* bv2 environmental strains to that of bv2 potato strain 1609 using CGH on a microarray representing bv2 specific genes of strain 1609 plus the complete gene repertoire of bv3 strain GMI1000.. Comparison of the genomes of Dutch environmental strains obtained from local waterways to those of strain 1609 (and in one case also 715) revealed a total of 20 genomic regions that were potentially differentially present within the strain set used. The PGI-1 region which is deleted from strain KZR-5 was consistently identified using two CGH combinations, i.e strain KZR-5 against strains 1609 and 715. Three of the four PGI-1 encoded genes represented by GMI1000 sequences on the micro array, i.e RSc0890 and RSc0891 and the *cbhA* gene, were however not detected by the CGH applied. Of these, genes RSc0890 and RSc0891 are much larger than the homologues genes present in the bv2 strains (Rsc0890: 498 vs 188 nucleotides and Rsc0891: 1383 vs 147 nucleotides). It is likely that the oligonucleotides used for the microarray construction corresponded to regions that are absent from the bv2 homologous genes, which would explain why they were not detected in this analysis. Although the overall nucleotide identity between the *cbhA* genes of GMI1000 and 1609 is 88%, the oligonucleotide representing the GMI1000 *cbhA* gene was also not detected in this analysis. We do not know the exact sequence of the oligonucelotide used for this gene, but it is likely that the sequence used for microarray design was too divergent between the GMI1000 and 1609 sequence to be detectable. Thus, these results are fully consistent with the contention that strain KZR-5 lacks the genomic island PGI-1.

Of the additional eight genes that appeared putatively deleted from strain KZR-5, at least five appeared to be present in all tested strains as determined by PCR analysis and DNA sequencing (Table 3). Why these genes were consistently found in both CGH experiments using strain KZR-5 as comparator (against 1609 as well as against strain 715) remains unsolved. Duplicate CGH experiments as well as dye swapping would probably decrease the change of identifying such "false" deletions, although dye swapping is not believed to have a significant impact on the results (Guidot et al., 2007). Other technical aspects could also have influenced the results obtained with CGH. The accessibility of different regions in the genome might differ between strains as a result of differences in purity of the DNA, the extent of denaturation prior to hybridization or rearrangements and duplications within the genome. The identified putative additional deletions in strain KZR-5 could thus be the result of such differences in DNA quality, rearrangements or duplications. The intergenic region represented by probe IPO1609_0563 is localized between the genes for a type III effector and an Rhs-related protein. Type III effectors are secreted in plant tissue in early stages of plant invasion, while Rhs and Vgr-related proteins are involved in recombination processes (Wang et al. 1998). Interestingly, the intergenic region was also identified as strain KZR-5 specific from suppressive hybridization between strains 715 and KZR-5 (chapter III), which strongly supports the notion that duplications (or possibly rearrangements) occurred in the environmental strains. In addition, the gene encoding a Vgr-related protein identified as specific for strain KZR-1 might also be involved in genome rearrangements or duplications.

To determine whether rearrangements or duplications were the actual mechanism behind the differences in hybridization signal intensities between the identified genes, PCR analysis and

Southern hybridizations with the corresponding sequences as DNA probe should be performed. A more in-dept analyses would thus provide the answers to these questions. Specifically, it would be intriguing to investigate whether the type III effector and the Rhs-related protein flanking the identified intergenic region show differences in copy numbers or genomic location between the strains and how this would affect the ecology/fitness of the Dutch environmental strains.

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References

Guidot A, Prior P, Schoenfeld J, Carrere S, Genin S, Boucher C (2007). Genomic structure and phylogeny of the plant pathogen *Ralstonia solanacearum* inferred from gene distribution analysis. J. Bacteriol 189:377-387.

Guidot A, Elbaz M, Carrère S, Siri MI, Pianzzola MJ, Prior P, Boucher C (2009) Specific genes from the potato brown rot strains of *Ralstonia solanacearum* and their potential use for strain detection. Phytopathol. 99:1105-12.

Occhialini A, Cunnac S, Reymond N, Genin S, Boucher C (2005). Genome-wide analysis of gene expression in *Ralstonia solanacearum* reveals that the *hrpB* gene acts as a regulatory switch controlling multiple virulence pathways. Mol Plant Microbe Interact.18:938-949.

Stevens P, van Elsas JD (2010) A putative genomic island, PGI-1, in *Ralstonia solanacearum* biovar 2 revealed by subtractive hybridization. Antonie van Leeuwenhoek. doi 10.1007/s10482-010-9450-4

Thomson N, Bently S, Holden M, Parkhill J (2003) Fitting the niche by genomic adaptation. Nature 1:92-93

Van Hijum SAFT, de Jong A, Baerends RJS, Karsens HA, Kramer NE, Larsen R, den Hengst CD, Albers CJ, Kok J, Kuipers OP (2005). A generally applicable validation scheme for the assessment of factors involved in reproducability and quality of DNA-microarray data. BMC Genomics 6:77

Wang YD, Zhao S, Hill CW (1998) Rhs elements comprise three subfamilies which diverged prior to acquisition by *Escherichia coli*. J Bacteriol 180:4102-4110

Chapter V

Ralstonia solanacearum ΔPGI-1 strain KZR-5 is affected in growth, response to cold stress and invasion of tomato

Patricia Stevens
Leonard Simon van Overbeek
Jan Dirk van Elsas

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Abstract

The survival and persistence of *Ralstonia solanacearum* biovar 2 in temperate climates is still poorly understood. To assess whether genomic variants of the organism show adaptation to local conditions, we compared the behavior of the environmental strain KZR-5, which underwent a deletion of a 17.6 Kb genomic island (denoted PGI-1), with that of environmental strain KZR-1 and two well-defined potato-derived strains, i.e Dutch strain 1609 and tropical strain 715. Although the exact set of functions harbored by PGI-1 is as yet unknown, the island encompasses two genes of potential ecological relevance, i.e. one encoding a hypothetical protein with a RelA/SpoT domain and a putative cellobiohydrolase. We thus assessed bacterial fate under conditions of amino acid starvation, during growth, upon incubation at low temperature and invasion of tomato plants.

In contrast to the other strains, environmental strain KZR-5 did not grow on media that induce amino acid starvation. In addition, its maximum growth rate at 28°C in rich medium was significantly reduced. On the other hand, long-term survival at 4°C was significantly enhanced as compared to that of strains 1609, 715 and KZR-1. Although strain KZR-5 showed growth rates (at 28°C) in 0.1 x TSBS and M63, which were similar to those of strains 1609 and 715, its ability to compete with these strains under these conditions was reduced. In singly-inoculated tomato plants, no significant differences in virulence were observed between strains KZR-5, 1609, 715 and KZR-1. However, reduced competitiveness of strain KZR-5 was found in experiments on tomato plant colonization and wilting when using 1:1 or 5:1 mixtures of strains. The potential role of PGI-1 in plant invasion, response to stress and growth in competition at high and moderate temperature is discussed.

Introduction

Ralstonia solanacearum biovar (bv) 2 is the causative agent of bacterial wilt of potato (*Solanum tuberosum*). In the Netherlands, it has appeared as from the mid 1990-ies and several outbreaks of the disease, potato brown rot, have been recorded in that period. Despite the strict quarantine measures that have been taken since then, the bacterium can still be found in Dutch local waterways, in surface water, ditch sediment as well as in the weed *Solanum dulcamara* (bittersweet) (Granada & Sequira 1983, van Elsas et al. 2000, Stevens & van Elsas 2010a). This strongly suggests the establishment of *R. solanacearum* biovar 2 in Dutch freshwater habitats. Because the organism is of tropical origin and supposedly non-endemic in Northwest Europe, it is generally assumed that the isolates found in these European ecosystems originate from the same ancestral (tropical) source. Indeed, amplified fragment length polymorphism (AFLP) analyses have revealed that the genomes of *R. solanacearum* biovar 2 strains obtained from different European countries (like France, the UK and the Netherlands) were very similar in comparison with those of isolates from elsewhere in the world (van der Wolf et al. 1998, Poussier et al. 1999, 2000) However, some degree of genetic variation among European strains was found to exist, as determined by pulsed field gel electrophoresis with rare-cutting restriction enzymes (RC-PFGE) and AFLP (van der Wolf et al. 1998, Stevens & van Elsas 2010a).

Studies on the survival of *R. solanacearum* biovar 2 under local (oligotrophic) conditions in field soils, ditches and canals have been published (van Elsas et al. 2000, 2001, Álvarez et al. 2008). From these studies, the conditions that affect the soil- and waterborne nature of *R. solanacearum* biovar 2 in the Netherlands, and its persistence in these open environments, have become evident. The survival strategies used by the organism might include physiological responses leading to reductions in cell size, entry of (part of) the population into the viable-but-non-culturable (VBNC) state and the formation of filamentous cells and cell aggregation (Álvarez et al. 2008). Although factors such as light, low or high pH, soil type, and the presence of an indigenous microbiota all can influence the survival of the organism, temperature seems to have an overriding impact on survival in temperate climates (van Elsas et al. 2000, 2001, Álvarez et al. 2007). Low temperatures can have a dual effect on the survival of *R. solanacearum* biovar 2. First, the organism might become injured upon this stress (van Elsas et al. 2005) and, secondly (on the positive side), predation or competition from the indigenous microorganisms may be reduced (Álvarez et al. 2007 We hypothesized that our environmental *R. solanacearum* biovar 2 strains might have adapted their genomic make-up to the selective forces prevailing in the novel habitat. Thus, key organismal properties that allow survival under stress conditions (low temperature, low nutrient availability) or plant host invasion may have changed. Thus, it is important to investigate to what extent the genomes of the environmental *R. solanacearum* strains did diverge and how this divergence affected their fitness under specific (temperate climate) conditions.

Previous work in our laboratory revealed genetic differences between several strains that were isolated from Dutch local waterways and *S. dulcamara* (bittersweet) plants growing at the edges of these waterways (Stevens & van Elsas 2010a) A thorough genetic analysis then showed that the genome of one of these strains, denoted KZR-5, harbors a deletion of 17.6Kb, denoted putative genomic island 1 (PGI-1) (Stevens & van Elsas 2010b) The island encodes a total of 13

open reading frames (ORF's). Among these ORF's was a putative cellobiohydrolase (*cbh*A) gene as well as a gene encoding a protein with a conserved RelA/SpoT domain.

In *Escherichia coli,* the functioning of two homologous enzymes, i.e. the RelA and SpoT proteins, mediate the synthesis of hyperphosphorylated guanosine nucleotide, (p)ppGpp, which acts as a nutritional alarmone (Cashel et al. 1996) The two proteins regulate the levels of (p)ppGpp in the cell in response to amino acid and/or carbon starvation, and are necessary in balancing the cell's nutritional capability and survival under stress conditions (Cashel et al. 1996) From a range of studies (Wendrich et al 1997, Calderon-Flores et al. 2005, Das & Bhadra 2008, Nanamiya et al 2008, Sun et al. 2009), it has become clear that (p)ppGpp acts as a global regulator during the adaptation of bacteria to different environmental conditions, including low temperature, different eukaryotic hosts (pathogenesis, symbiosis) and in respect of bacterial multicellular behavior (Braeken et al. 2006, Spira et al. 2008). In addition, the functioning of RelA and/or SpoT is often pleiotrophic, can differ between species and is dependent on the conditions used. Unlike Gram-negative bacteria such as *E. coli*, many bacteria harbor single genes for RelA/SpoT-like enzymes that function in both degradation as well as synthesis of (p)ppGpp Mittenbhuber 2001. Recently, another class of (p)ppGpp synthetases (small alarmone synthetases, SASs) has been identified in *Bacillus subtilis* (Nanamiya et al. 2008) and *Streptococcus mutans* (Lemos et al. 2007), but their exact function is unknown.

To understand the putative function of PGI-1 and the possible effect of the loss of the RelA/SpoT domain protein in ΔPGI-1 strain KZR-5, we investigated whether this strain is affected, as compared to potato strains 1609 and 715 and environmental strain KZR-1, in its response to amino acid starvation, to low temperature and upon growth in nutrient-poor and nutrient-rich media. Because PGI-1 also encodes a *cbh*A gene, we hypothesized that the PGI-1 deletion might also incur a change of behavior of KZR-5 in its invasion of susceptible host plants. We therefore looked at the strain's ability to degrade carboxymethylcellulose (CMC), which is typically performed by proteins with endoglucanase activity (EglI in *R. solanacearum*) and the ability to hydrolyze methylbelliferyl cellobiose (MUC), performed by proteins with exoglucanase activity (CbhA in *R. solanacearum*). Finally, to assess whether the lack of PGI-1 influenced the organism at any stage of interaction with the plant, we compared the virulence of strain KZR-5 to that of strains 1609, 715 and KZR-1 in single-strain- as well as joint- inoculation tomato invasion assays.

Materials and Methods

Bacterial strains and media

The bacterial strains used in this study are listed in Table 1. Strains 1609 and 715 were derived from diseased potato plants. Strains KZR-1 (PGI-1 +) and KZR-5 (ΔPGI-1) were isolated from bittersweet growing in a Dutch local waterway (Stevens & van Elsas 2010). All four *R. solanacearum* strains belong to bv2, which is a highly homogenous group of organisms. They produce similar BOX-PCR patterns and have identical 16S rRNA gene sequences (Stevens & van Elsas 2010). The *E. coli* BW 25113 wildtype and ΔrelA strain of the Keio collection (Datsenko & Wanner 2000, Baba et al. 2006) were kindly provided by Dr. Javier Pozueta Romero of the Agrobiotechnology Institute in Navarra, Spain. Bacterial strains were maintained at -80^{o}C in 0.1 x TSBS medium (10% strength Trypticase Soy Broth [Becton Dickinson and Company, Sparks, Md,

USA], 0.1% sucrose; pH 7.2) containing 20% glycerol. Strains were freshly grown prior to each experiment from these pure culture stocks. *E. coli* strains were routinely grown in Luria broth (LB) at 37°C. For routine analyses of *R. solanacearum* strains, the latter were grown at 28°C with shaking in liquid 0.1 x TSBS. For growth rate determinations, we also used BG (Bacto peptone 10 g, yeast extract 1 g, casamino acids 1 g, glucose 5 g; H_2O 1L; pH 7.2) and M63 (Amresco, Solon, USA) containing $(NH_4)2SO_4$, 2g; KH_2PO_4, 13.6 g; $FeSO_4$ x $7H_20$, 0.5 mg; MilliQ, 1L; pH, 7.0) media. These media were autoclaved for 15 min at 121°C, after which filter-sterilized $MgSO_4$ (1 mM) and glucose (0.1 %) were added. Solid growth media contained 1.5% (w/v) purified agar (Duchefa, Haarlem, The Netherlands) and were supplemented with 0.05% (w/v) 1,3,5 - tetrazolium chloride ([TZC] Sigma, St. Louis, USA).

Table 1 Bacterial strains used in this study

strain	relevant genotype	reference
R. solanacearum		
1609	pulsotype A	Van Elsas et al., 2000
715	pulsotype A	Timms-Wilson et al., 2001
KZR-1	pulsotype A	Stevens & van Elsas, 2010a
KZR-5	pulsotype C, deletion of 17.6 Kb putative genomic island, PGI-1	Stevens & van Elsas, 2010a/2010b
E. coli		
BW 25113 wildtype	wildtype K12-derivative	Baba et al. (2006)
BW 25113 ◊ relA	◊ relA from the Keio collection	Baba et al. (2006)

AT and SMG growth tests

Because (p)ppGpp-deficient mutants often exhibit multiple amino acid auxotrophy on minimal medium, we tested the growth of strains 1609, 715, KZR-1 and KZR-5 on plates containing 3-amino-1,2,4-triazole (AT), which blocks histidine biosynthesis, and on plates containing serine, methionine and glycine (SMG) as described (Uzan & Danchin 1976, Rudd et al. 1985. The *R. solanacearum* strains were streaked onto AT medium (M63 agar with 15 mM AT, thiamine 5 μg/ml, methionine 100 μg/ml) or SMG medium (M63 agar with 100 μg/ml each of serine, methionine and glycine) plates and incubated overnight at 28 & 37°C. As controls, *E. coli* strain BW25113 and its ΔrelA derivative were used (Table 1).

Qualitative enzyme assays

The production of exoglucanases (EC 3.2.1.9) by *R. solanacearum* strains was assessed by growing cells for 2-5 days at 28°C on M63 agar plates containing 0.4% glucose and 100 μM 4-methyl umbelliferyl-β-D-cellobiose (MUC, Sigma, St. Louis, USA), and subsequent examination of the growth under UV light. The appearance of fluorescence under UV light would indicate the cleavage of the cellobiose with the production of 4-methyl-umbelliferone (Tilbeurgh et al. 1984) Carboxymethyl cellulase activity of the type typically displayed by endo-1,4-β-D-glucanases (EC 3.2.1.4) was determined by growing cells for 2-7 days at 28°C on M9 agar plates containing 2.5 g/L carboxymethylcellulose (CMC [Sigma Aldrich, USA]). When sizeable colonies appeared, the plates were stained with 0.1% Congo red solution (Theater & Wood (1982) for 20 minutes and rinsed with 10 ml of 1M NaCl for 10 min. The appearance of a halo around the inoculation spot indicates endoglucanase activity.

Survival in sterile demineralized water

The effects of two temperatures, 4°C and 20°C, on the survival over time of *R. solanacearum* bv2 strains KZR-5, 1609 and 715 were assessed in sterile water microcosms. Exponentially-grown and washed cells (twice with MilliQ purified sterile water) were transferred to triplicate 100-ml bottles containing 20 ml of sterile ultrapure MilliQ water, establishing final population densities of between log 6.6 and log 7.1 cells per ml. Flasks were incubated without shaking, in the dark, at the appropriate temperatures, i.e 4°C and 20°C. At set times, 1-ml samples were aseptically removed from each flask. Aliquots were serially diluted in sterile Milli-Q water and plated (100 µl) onto 0.1 x TSBS agar plates. Plates were incubated for 2-5 days at 28°C, after which colonies were counted and the numbers of colony forming units per ml (CFU/ml) calculated.

Table 2 Growth of strains on SMG, AT and CMC containing media and decline rates of *R. solanacearum* strains upon incubation in sterile MilliQ water at 20°C and 4°C

strain	SMG	AT	CMC	decline rate 20°C	decline rate 4°C
E. coli WT	+	+	nd	nd	nd
E. coli ΔrelA	-	-	nd	nd	nd
R. solanacearum 1609	+	-	+	0.0029 [a]	0.051 [b]
R. solanacearum 715	+	-	+	0.0026 [a]	0.055 [b]
R. solanacearum KZR-1	+	-	+	0.0020 [a]	0.064 [b]
R. solanacearum KZR-5	-	-	+	0.0020 [a]	0.034 [c]

Statistical classes (a,b and c) are indicated. $P < 0.05$
nd, not determined. (+) growth on agar plates, (-), no growth on agar plates.

Determination of growth rates

The growth rates of strains KZR-5, 1609 and 715 were measured in cultures growing in 0.1 x TSBS, BG and M63 media. For growth in 0.1 x TSBS and M63 media, aliquots (20 µl) of overnight cultures grown at 28°C with shaking were aseptically added to flasks containing 20 ml of fresh medium, after which the resulting cultures were incubated at 28°C (as well as at 16°C for 0.1 x TSBS medium) until the OD_{660} values reached 0.8-1.2. For growth in BG medium, flasks were inoculated with cells from BGT plates using a sterile loop. Samples were taken at set times (depending on the medium used) and the OD_{660} values determined. In addition, in the growth experiments in BG and 0.1 x TSBS at 16°C, the number of colony forming units (CFU) was determined by dilution plating on BGT plates. The maximum growth rates for each strain and each growth medium were determined from the growth dynamics over the whole time course. Maximum growth rate was calculated using the formula $\mu_{max} = \ln(N_2/N_1)/(t_2-t_1)$, where N_2 is the cell concentration at t=2 and N_1 is the cell concentration at t=1.

Competition experiments

As subtle differences in the growth rates of strains KZR-5, 1609 and 715 might remain undetected when growing in single cultures, we tested the competitive abilities of strain KZR-5 against those of 1609 and 715 in 0.1 x TSBS at 16°C and 28°C in mixed cultures (KZR-5:1609 and KZR-5:715). The mixes initially received equal numbers of each strain. To prepare starter cultures for these

experiments, cells were grown overnight, with shaking, at the relevant temperature in 0.1 x TSBS until the OD_{660} values reached 0.7-1.1. They were then washed twice with sterile MilliQ-purified water. To obtain mixed cultures containing totals of 1.0 x 10^6 CFU/ml, appropriate volumes of washed cells were transferred to flasks containing 20 ml of 0.1 x TSBS (start OD_{660} ~ 0.05). These cultures were dilution-plated onto 0.1 x TSBS plates containing TZC to affirm the ratio between the inoculated strains (set at 1:1), as explained in the following.

To enable the discrimination of strains, we used colony morphology as the criterion in combination with PCR-based colony screens for confirmation. The colonies obtained for strains KZR-5, 1609 and 715 differed morphologically, and the colony morphologies were stable, on 0.1xTSBS plates containing TZC, as follows. Colonies of strains 1609 and 715 were distinguished from those of strain KZR-5 on the basis of their clearly reddish and dry colony morphology, as opposed to the whitish and glossy appearance of KZR-5 and KZR-1. In addition, the colonies of strain KZR-5 were ~ 1.5 fold larger than those of the two counterpart strains, as they visibly produced more extracellular polysaccharide. To support/confirm the colony-morphology-based screenings for strains KZR-5 and 1609, a colony PCR screen (system ps-6 (stevens& van Elsas 2010b) was used, which amplifies the PGI-1 genomic region that is present in strains 1609, 715 and KZR-5, but absent from strain KZR-5. Thus, the small reddish colonies typical for strains 1609 and 715 generated the predicted 559 bp fragment using this PCR system, while amplification on the basis of the large white colonies typical for strain KZR-5 yielded no product.

For the colony PCR analysis, colony material was picked with a sterilized toothpick and transferred to 100 µl MilliQ water, after which it was strongly mixed. One µl of this suspension, tenfold diluted or not, was then used as the template source for PCR analysis (25 µl volume). For PCR amplification, we used the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, USA). Standard PCR reaction mixtures contained 1x PCR buffer (1.5 mM MgCl, 10 mM Tris and 50 mM KCl, Roche Applied Science, Basel, Switserland), 2.5 mM $MgCl_2$, 10% DMSO, 200 µM each deoxynucleotide, 0.2 µM of each primer and 20 U/ml Taq DNA polymerase (Roche Applied Science, Basel, Switserland). The cycling program was as follows: a denaturation step at 96˚C for 7 min, followed by 34 cycles of: 96˚C, 40 sec; 60˚C, 45 s; 72˚C, 40 s, finalized by an extension step of 72˚C for 5 min. PCR products were analyzed by electrophoresis on 0.8 % agarose gels (Roche diagnostics, Mannheim, Germany).

To determine the competitive fitness of the strains at 28°C, the established mixed cultures were grown overnight or until reaching an OD_{660} of 0.9 (after approximately 7 generations), and dilution-plated at regular times onto BGT plates. To assess competition at 16°C, serial batch cultures were used. First cultures were established by transferring 10µl of each grown culture (OD_{660} between 0.5 and 0.8, after approximately two days) to flasks containing 20 ml fresh 0.1 x TSBS. After four transfers (approximately 40 generations), aliquots from the cultures were dilution-plated onto BGT plates, which was followed by quantification of the CFUs of competing strains as above.

Tomato invasion assays

To assess the behavior of environmental strain KZR-5 and potato strains 1609 and 715 when in association with tomato plants, at least twelve 3-4 week old tomato plants per strain/treatment in soil in pots were inoculated as describedStevens & van Elsas 2010a).

Single-strain inoculations: Strains KZR-5, 1609 and 715 were introduced into the soil adjacent to plants in replicated separate pots at cell densities of either 10^8 or 10^5 CFU/ml (estimated density per g soil was 5 x 10^6 or 5 x 10^3), after which plants were kept under a day/night regime of 26°C (day, 14h)/21°C (night, 10h). Control plants received sterile MilliQ water instead of bacterial cells. Plant disease development was then scored at 7, 14 and 21 days following inoculation using a disease matrix ranging from 0 (no wilting symptoms) to 4 (all leaves wilted) [34].
Mixed-strain inoculation: Mixed suspensions of strain KZR-5 with the comparator strains 1609, 715 as well as another environmental isolate, KZR-1 (all in 1:1 ratio's) were used to inoculate the plant/soil systems in a fashion similar to the foregoing. In addition, a ratio of 5:1 was tested for the mixture KZR-5:715. The strain mixtures were prepared by adding appropriate cell numbers of these strains to 250 ml Milli-Q water, establishing total cell densities of approximately 1 x 10^7 CFU/ml.

To enumerate the *R. solanacearum* cells from the soil and tomato plants, we used cell extraction/dlution plating procedures, using modified semi-selective SMSA plates (Elphinstone et al. 1996). Thus, two days after inoculation, 0.5 g soil samples were taken (in triplicate), shaken in 0.01M phosphate buffer (pH 7.0) for 1 h and dilution-plated on SMSA. At 10 and 21 days post-inoculation, cuttings of 1 cm of the lower stem parts (approximately 4 cm above the soil) were removed from three plants and shaken in 2 ml 0.01M phosphate buffer for up to two h at room temperature. After this, the resulting suspensions were dilution-plated onto modified SMSA plates. All plates were incubated at 28°C for 2-5 days, after which they were scored.

Following plate incubation, the ratio's between strains KZR-5 and 1609 as well as 715 were determined on the basis of the morphology of colonies, supported by PCR screens as described before. To determine the ratio's for the strain KZR-5:KZR-1 mixes, we applied the ps-6 PCR system (Stevens & van Elsas 2010b) consistently on 24 to 48 randomly-selected colonies for each replicate sample.

Statistical treatment of the data

All experiments were executed in triplicate, except the growth rate and long term survival (at 20°C) experiments which had duplicate systems. All CFU counts were log-transformed, after which mean values and standard deviations were calculated on the basis of log-transformed values. To describe the decline in CFU counts over time at 4°C, the log-transformed data were fitted to a modified logistic function by non-linear regression (Gauss-Newton method, Semenov et al. 2007) and the decline rate values were compared using Student's *t*-test (one-tailed distribution). For comparison of long-term survival values of the strains at 20°C, growth rates (μmax) and competition experiments, we used two-tailed Student's *t*-tests on the replicates. To determine the significance of the differences in long-term survival, the decline rate (slope) of each individual replicate was compared across strains. For the competition experiments, the ratio of strains at the beginning of the experiment was compared to that at the end of the experiment. Student *t*-tests were judged to be significant at $P < 0.05$.

Results and Discussion

Determination of the phenotype of the ΔPGI-1 mutant strain KZR-5

PGI-1 encodes a putative small alarmone synthetase. The genomic island PGI-1 was found to possess a putative gene (RSIPO_04909) that encodes a 486 amino acid (AA) long protein with a conserved (p)ppGpp synthetase catalytic domain (spanning AA 42 to 234, i.e. roughly 200 AA in size). This domain has also been found in the *E. coli* RelA and SpoT proteins. These proteins mediate the synthesis of the alarmone (p)ppGpp, which is relevant in the response of the organism to amino acid and/or carbon starvation. As many Gram-negative bacteria possess single copies of the *rel*A and *spoT* genes, we examined the *R. solanacearum* biovar 2 strain 1609 genome for it. This analysis revealed that one copy for each gene was present in strain 1609. These genes probably encoded proteins homologous to the *E. coli* RelA and SpoT proteins (RSIPO_01119, RelA and RSIPO_01943, SpoT). These proteins consistently possessed, next to the so-called RelA/SpoT domains, other conserved domains, namely HD (thought to be involved in (p)ppGpp degradation), TGS (conserved ATP/GTP-binding domain) and ACT (conserved ATP/GTP-binding and GTP-binding domain) (Fig. 1A) (Cashel et al. 1996). In contrast, the PGI-1-borne *R. solanacearum* RelA/SpoT domain protein was smaller than the RelA/ SpoT enzymes (486 versus 676-793 AA) and, clearly, did not possess the associated HD, TGS and ACT domains (Fig. 1A). BLASTP searches using the PGI-1 borne RelA/SpoT domain as the query revealed highest similarity to proteins of *Rhizobium etli* (identity 49%, E-value 2e-38), *Bacillus cereus* (identity 46%, E value 2e-31), *Streptomyces viridochromogenes* (identity 50%, E-value 8e-31) and *Exiguobacterium sp*. (43%, E-value 2e-30).

A subsequent comparison of the deduced amino acid sequence of the PGI-1-encoded protein (RSIPO_04909) with those of the homologues identified by NCBI BLASTP and of those (SAS proteins) of *Bacillus subtilis* (YjbM and YwaC) and *Streptococcus mutans* (RelP and RelQ) showed clear conservation of several amino acids at fixed positions (Fig. 1B). Specifically, ten residues were fully conserved between the aligned sequences (Fig. 1B). Of these, four had previously been shown to be functionally significant in *Streptococcus dysgalactiae* (Hogg et al. 2004). Another 11 residues were fully conserved among the SAS proteins of *B. subtilis* and *S. mutans*, the PGI-1-borne RelA/SpoT domain protein and its closest homologues, but not with the RelA and SpoT proteins of *E. col*, nor with their counterparts in *R. solanacearum*.

Surprisingly, phylogenetic analyses of the aligned (partial) amino acid sequences then revealed that the PGI-1 RelA/SpoT protein as well as the identified homologues of *R. etli, B. cereus, S. viridochromogenes* and *Exiguobacterium* were more closely related to the SAS proteins of *B. subtilis* and *S. mutans* than to either one of its own RelA or SpoT proteins (Fig. 1C). On this basis, we hypothesized that the PGI-1-encoded RelA/SpoT domain protein encodes a putative SAS-like protein, which – by analogy to the situation in *B. subtilis* – may be involved in the biosynthesis or level control of an alarmone with a mode of action distinct from that of the canonical RelA / SpoT homologues (Nanamiya et al 2008).

Moreover, the average G+C content of the *R. solanacearum* PGI-1-borne *relA*/*spoT* gene was lower than the average G+C content of its entire genome (i.e. 50 % versus 64%), actually being on the order of the G+C content of the genomes of the implicated Gram-positive species (40-50%).

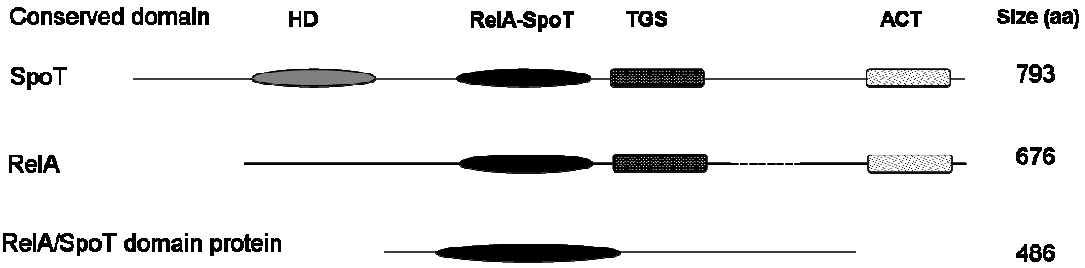
Taking into account that the gene encoding the respective RelA/SpoT domain protein of *R. solanacearum* localizes on genomic island PGI-1 (Stevens & van Elsas 2010b) and that the degree of homology with the SAS genes was rather low, the gene (or island) might have been anciently transferred from Gram-positive lineages to *R. solanacearum*. The presence of a SAS-like gene in *R. solanacearum* biovar 2 and its putative involvement in the physiology of this organism, e.g. the stringent response, has so far not been described.

SMG en AT tests. Comparison of the growth of *E. coli* BW25113 wildtype (WT), *E. coli* BW25113 ΔrelA and *R. solanacearum* strains KZR-5, 1609 and 715 on SMG-containing plates revealed a clear growth defect for environmental strain KZR-5 when compared to strains 1609 and 715 (Table 2). The *E. coli* BW25113 WT, and ΔrelA strains, which were used as controls, behaved as expected on SMG and AT, i.e. they showed fair growth of the WT strain and delayed growth of the ΔrelA strain on these media. None of the *R. solanacearum* strains grew on plates containing AT, not even after 15 days of incubation. Thus, environmental strain KZR-5 showed a phenotype similar to that of *E. coli* ΔrelA when grown on media containing excess 1-C metabolites, whereas potato strains 1609 and 715 did not.

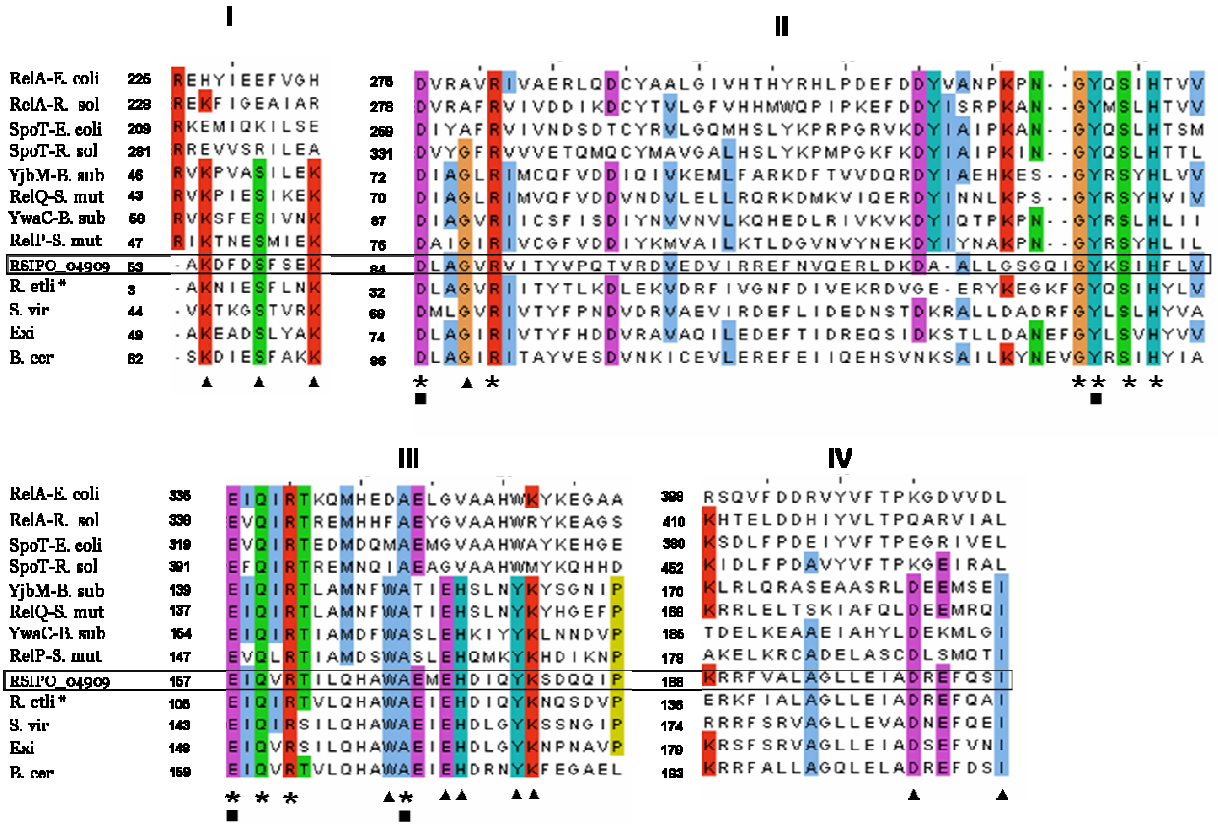
Exo-and endoglucanase activity. To assess whether strain KZR-5 is impaired in cellulose degradation, we tested its ability to utilize CMC and MUC. Overall, these tests, compared to those of strains 1609 and 715, revealed that strain KZR-5 exhibited "normal" endoglucanase activity. Specifically, no differences were observed between the three strains in the utilization of CMC, as judged by the sizes of the haloes of the corresponding colonies, following the procedure of Teather & Wood (1982), (Table 2). Unfortunately, we were not able to measure exoglucanase activity for any of the three *R. solanacearum* strains, using MUC as a substrate, in a reproducible manner. Although the CbhA protein is secreted by *R. solanacearum* biovar 3 strain GMI1000 (Liu et al. 2005), the substrate it hydrolyzes has never been demonstrated. It has been suggested that CbhA attacks the hemicellulose fraction of plant cell walls, and that *in vitro* enzyme activity measurements can be hampered (T. Denny, pers. comm.).

Figure 1

A


Conserved domain
HD
RelA-SpoT
TGS
ACT
Size (aa)
SpoT
793
RelA
676
RelA/SpoT domain protein
486

B


I
II
III
IV
RelA-E. coli
RelA-R. sol
SpoT-E. coli
SpoT-R. sol
YjbM-B. sub
RelQ-S. mut
YwaC-B. sub
RelP-S. mut
RSIPO_04909
R. etli
S. vir
Exi
B. cer

C

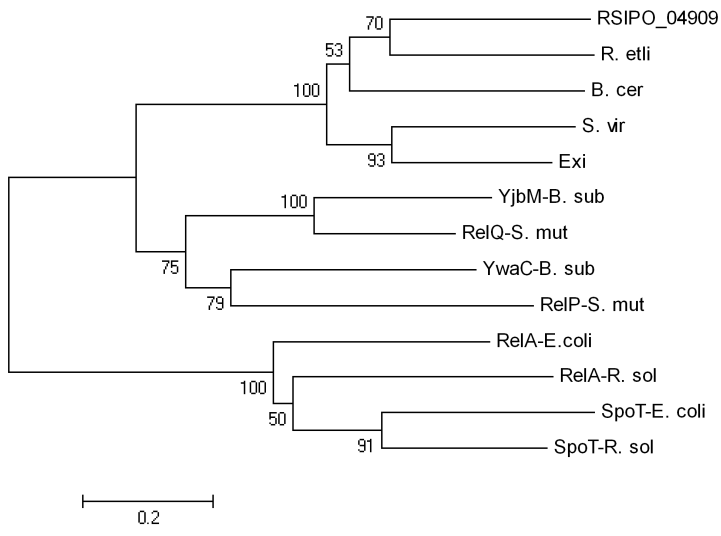

RSIPO_04909
R. etli
B. cer
S. vir
Exi
YjbM-B. sub
RelQ-S. mut
YwaC-B. sub
RelP-S. mut
RelA-E.coli
RelA-R. sol
SpoT-E. coli
SpoT-R. sol
70
53
100
93
100
75
79
100
50
91
0.2

Figure 1 Comparison between SpoT, RelA and RelA/SpoT domain containing proteins, (A) Conserved domains found in these proteins, (B) Partial alignment showing conservation of amino acid residues and (C) their phylogenetic relatedness. A) Comparison of conserved domains found in SpoT, RelA and RelA/SpoT domain containing proteins. HD domain (possessing a conserved histidine [H] and aspartate [D] residue): possibly involved in (p)ppGpp degradation, only present in SpoT. TGS and ACT domains: conserved ATP/GTP-binding and GTP-binding domains, respectively (Gentry & Cashel, 1996); found in SpoT as well as RelA. The RelA/SpoT domain is shared by all three types of the RelA/SpoT-like proteins. B) Alignment of the RelA/SpoT domain of SpoT and RelA of *E. coli*, *R. solanacearum* biovar 2, PGI-1 encoded RSIPO_04909 and eight related RelA/SpoT domain containing proteins. The position of the first amino acid of the four partial protein sequences (I through IV) is indicated and corresponds to the position of the first amino acid along the full length protein. Stars: residues that are fully conserved among all sequences; triangles: residues conserved among the SAS proteins of *B. subtilis* and *S. mutans*, the *R. solanacearum* RelA/SpoT domain protein and its closest homologues, but not with the RelA and SpoT proteins of *E. coli* and *R. solanacearum*. Squares: residues that were experimentally shown to be functionally significant in *Streptococcus dysgalactiae* (Hogg et al., 2004). Abbreviations: E. coli, *Eschericha coli*; R. sol, *Ralstonia solanacearum* biovar 2; B. sub, *Bacillus subtilis*; S. mut, *Streptococcus mutans*; R. etli, *Rhizobium etli*; S. vir, *Streptomyces viridochromogenes*; Exi, *Exiguobacteriu*m; B.cer, *Bacillus cereus*.* For *R. etli*, a partial protein sequence was used due to unavailability of the full length sequence. C) Phylogenetic analysis (neighbour-joining tree) of SpoT and RelA of *E. coli* and *R. solanacearum* biovar 2 together with the PGI-1 encoded RelA/SpoT domain protein, RSIPO_04909 and related RelA/SpoT domain containing proteins. The tree was conducted using Mega 4 software

Growth rates of strains 1609, 715 and KZR-5 in different media

The maximum exponential growth rates (u_{max}, h^{-1}) of strains KZR-5, 1609 and 715 were assessed in different liquid growth media (Figure 2). Overall, the growth rates of the three strains were lower when nutrient concentrations and/or temperature were lower (Figure 2). In 0.1 x TSBS at two temperatures, the three strains had similar growth rates, i.e. between 0.24 ± 0.014 and 0.27 ± 0.012[u h^{-1}] at 28°C and between 0.11 ± 0.05 and 0.12 ± 0.03 [u h^{-1}] at 16°C. The growth rates in M63 medium at 28°C were also similar between the three strains, i.e. between 0.043 ± 0.001 and 0.048 ± 0.001 (u, h^{-1}). In BG medium at 28°C, however, we found a significant difference in growth rate between strain KZR-5 and the two comparator strains. The maximum growth rates of strains 1609 and 715 were similar (between 0.63 ± 0.07 and 0.69 ± 0.07 (u, h^{-1}), while that of strain KZR-5 was significantly ($P<0.05$) lowered (0.39 ± 0.03 u, h^{-1} (Figure 2). Thus strain KZR-5 showed growth rates similar to those of strains 1609 and 715 in nutrient-poor M63 medium, while, on the other hand, it showed slower growth in nutrient-rich (BG) medium in comparison to the two other strains.

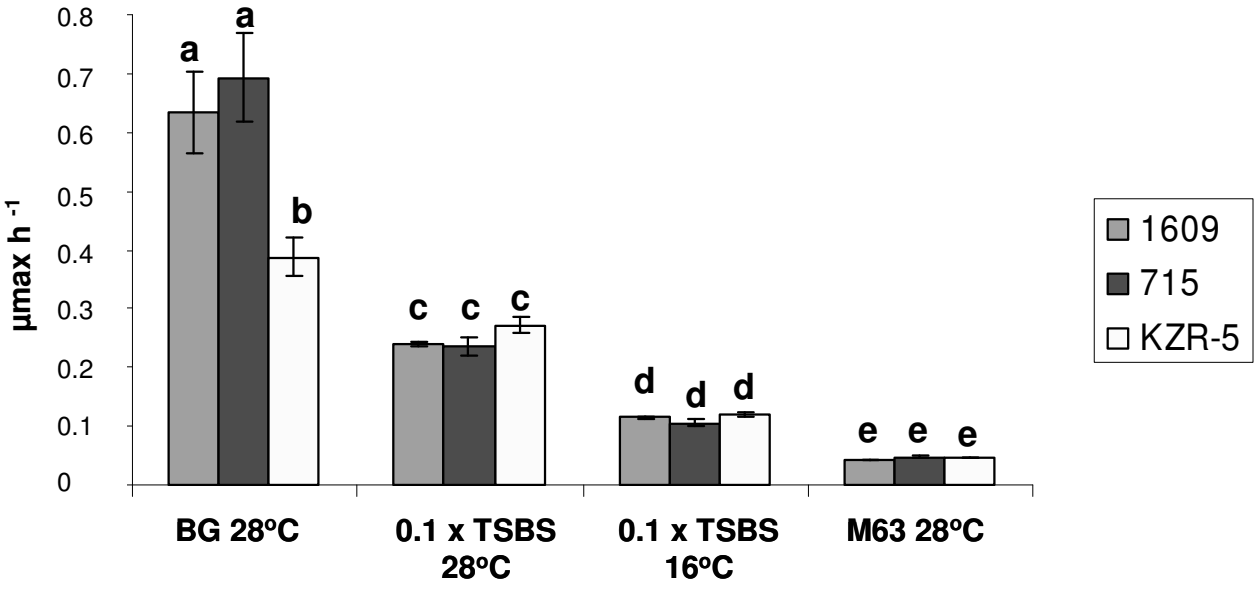


Figure 2 Maximum growth rate (μ_{max}; h^{-1}) of strains KZR-5, 1609 and 715 at 28°C in BG, 0.1 x TSBS and M63 and at 16°C in 0.1 x TSBS. Maximum growth rate was determined for exponentially growing cells. Values are the average of at least two independent experiments. Statistical classes (a, b, c, d, and e) are indicated ($P < 0.05$).

Competitiveness of *R. solanacearum* strain KZR-5 versus the comparator strains upon growth in 0.1xTSBS at 16°C and 28°C

As the growth rate of environmental strain KZR-5 in 0.1 x TSBS was similar to that of the potato strains 1609 and 715 (Fig. 2), we surmised there might be subtle differences in their behavior that can be evidenced in direct competition experiments. We thus used competition experiments in 0.1 x TSBS at 28°C as well as 16°C (Fig. 3). At both temperatures, the mixed cultures grew - as expected - in 16 h (28°C) and 50 h (16°C) to the maximum OD660 values of 0.8-1.2. In all replicates of the experiments with the mixture KZR-5:1609, we noted a decrease in the relative abundance of strain KZR-5, i.e. an average 8-fold decrease at 28°C and a 5-fold decrease at 16°C. Competition of strain KZR-5 against strain 715 showed a similar result, although the relative decrease in abundance of strain KZR-5 was lower (3-fold at 28°C and 2-fold at 16°C). The decreases in the relative abundances of strain KZR-5 versus strains 1609 and 715 were significant in both cases ($P < 0.05$).

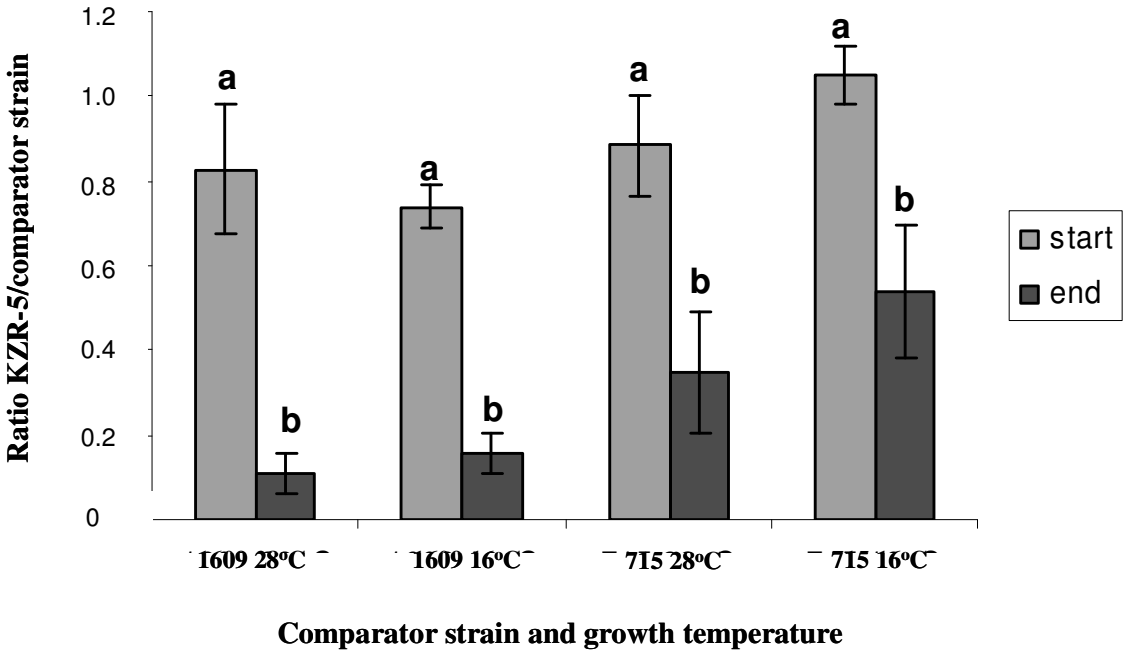


Figure 3 Competitiveness of *R. solanacearum* strain KZR-5 against strains 1609 and 715 in liquid 0.1 x TSBS. The ratio between ΔPGI-1 strain (KZR-5) and the comparator strains (1609 or 715) is shown at the beginning of the experiment (1:1) and after growth in mixed cultures. For competition at 28°C, the ratio between strains was determined from duplicate overnight cultures. For competition at 16°C, the ratio between the strains was determined from (duplicate) batch cultures after four serial transfers. Statistical classes (a and b) are indicated. $P < 0.05$

Survival of *R. solanacearum* strain KZR-5 versus the comparator strains in MilliQ water at 20°C and 4°C

The survival, as CFUs, of *R. solanacearum* biovar 2 strains 1609, 715, KZR-1 and KZR-5 in sterile MilliQ water in still vials at 20°C and 4°C was followed over time. Upon incubation at 20°C, the CFU numbers of strain KZR-5 showed an initial slight but progressive increase, from log 6.6 to maximally log 6.9 CFU/ml till day 34. These numbers then remained roughly stable until day 105. Upon extended incubation (28 months), the strain KZR-5 CFU numbers had decreased to between log 4.3 and log 6.3 CFU/ml.

The dynamics of strains 1609, 715 and KZR-1 resembled that of strain KZR-5, with strain KZR-5 surviving slightly, although not significantly, better than the three comparator strains. However, the long-term survival of strain KZR-5 was more erratic (P values between 0.3 and 0.9) than those of the other two strains (Table 2). When the four strains were incubated in water at 4°C, differences in population sizes started to emerge clearly after 30 days of incubation, with strain KZR-5 showing the highest survival upon further incubation. For all four strains, the CFU numbers declined until they became undetectable after 60 - 113 days (below the detection limit of 10 CFU/ml) (Fig. 4). The calculated decline rates over the whole time course were 0.034 ± 0.014 for strain KZR-5, 0.051 ± 0.009 for strain 1609, 0.055 ± 0.015 for strain 715 and 0.064 ± 0,005 for strain KZR-1. The difference in the decline rates between strain KZR-5 and the three comparator strains over time was at the border of significance. Specifically, the P value between KZR-5 and 1609 CFU numbers was 0.09 and between KZR-5 and 715 or KZR-1 0.05.

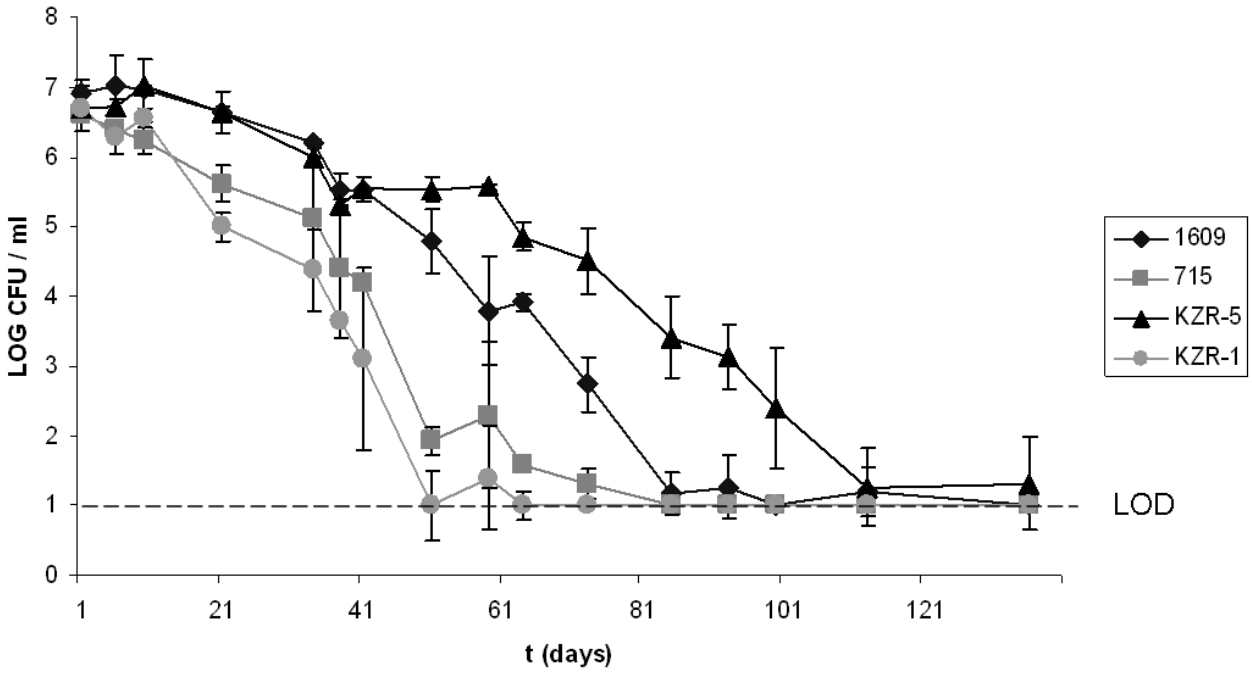


Figure 4 Survival of *R. solanacearum* strains 1609, 715, KZR-5 and KZR-1 at 4°C. The graphs show the colony forming unit enumerations on BG plates. Points represent the means of three replicate experiments. The limit of detection (LOD), 10 CFU/ml, is indicated

Strain KZR-5 thus showed enhanced survival in water at low temperature (4°C). On the other hand, we noted a decreased fitness upon growth in rich liquid medium. The rates of survival at low temperature and growth in rich media might indeed be related to cellular levels of (p)ppGpp, as shown for *E. coli* and *V. cholerae*, as in (Das & Bhadra 2008, Spira et al. 2008) This thus might support a possible link between the behavioral changes that were observed and the lack of the PGI-1-encoded putative RelA/SpoT domain protein. However, the mutants described in the aforementioned studies had increased basal levels of (p)ppGpp due to the complete absence of the SpoT protein, which is not likely to be the case in the ΔPGI-1 strain KZR-5.

Competitiveness of *R. solanacearum* strain KZR-5 upon invasion of tomato

In order to assess the virulence of environmental strain KZR-5 in comparison with those of potato strains 715 and 1609, we determined the effects of high (10^8 CFU/ml) as well as low (10^5 CFU/ml) inocula over time. First, control plants not receiving any inoculum remained 100% healthy over the course of the experiment. At both inoculum densities, strains KZR-5. KZR-1 and 715 induced wilting at similar rates. That is, full wilting was achieved in 100% of the plants within 14 days when using 10^8 CFU/ml, whereas between 67 - 72% of plants showed symptoms when using 10^5 CFU/ml. In this experiment, plants inoculated with strain 1609 (10^8 cells/ ml) wilted more slowly and symptoms were less severe (62% of the plants showed wilting at day 14 and 87% at day 21. Using low inoculum densities, strain 1609 caused signs of wilting in half of the plants after 21 days, thus appearing as less virulent than the two other strains.

Given the fact that strain KZR-5 did not show reduced virulence as compared to strain 715, we concluded their behaviour was grossly similar in uncompetitive situations. We then hypothesized that any differences in the rates of invasion and wilting might come out in

competitive situations, i.e. under strain-to-strain competitive set-ups. Hence, we decided to perform joint KZR-5: 715 and KZR-5:1609 inoculation experiments. Strain 1609 was included to shed light on its apparently lowered virulence. To support our assumptions, we also tested the competitive ability of strain KZR-5 against another environmental strain, i.e. KZR-1. Strain KZR-1 was, in contrast to strains 715 and 1609, indistinguishable on agar plates as judged from colony morphology. The results of all these experiments are shown in Fig. 5. First of all, plants not treated with any *R. solanacearum* strain developed into well-grown healthy plants (controls), indicating that no other pathogen or nutritional deficiency was present. At day 10, plants treated with all strain mixes showed the development of disease symptoms (as from wilting stage 1), beit that the plants treated with the KZR-5:KZR-1 mixes showed the highest wilting rates (wilting stages 1 through 3). At day 21, disease development had clearly progressed for all mixes used (wilting stages 3-4), but that with the KZR-5:1609 mix was less virulent than with the KZR-5:715 or KZR-5:KZR-1 mixes. Only 50% of the plants inoculated with the KZR-5:1609 mix showed wilting symptoms after 21 days, while 100% of the plants that had been inoculated with the KZR-5:715 and KZR-5:KZR-1 mixes showed disease symptoms . Analysis of the mixed *R. solanacearum* populations in the soil at day 2 revealed that strains 1609, 715, KZR-1 and KZR-5 survived fairly well in the soil compartments, i.e. between log 5.0 and log 6.0 CFU were measured per g soil for these partners in all strain mixes. Furthermore, for all mixes, the ratio between the two strains had remained roughly stable in the soil compartments, at 0.9 ± 0.45 (KZR-5:715) and 0.7 ± 0.26 (KZR-5:1609 as well as KZR-5:KZR-1) (Fig. 5).

In contrast, analysis of the ratio between the introduced strain KZR-5 and the competing strains 1609, 715 and KZR-1 10 and 21 days after inoculation of the tomato plants consistently showed progressive decreases of the prevalence of strain KZR-5 in the mixes (Fig. 5). Strikingly, this was also true for the KZR-5:1609 mix. Thus, although strain 1609 appeared as less virulent than strain KZR-5 in single inoculation experiments, it outcompeted strain KZR-5 in mixed-inoculation experiments. The plants inoculated with these two strains showed a reduced rate of wilting, which is remarkable.

To strengthen the notion that environmental strain KZR-5 was less competitive than its potato-derived competitor in these plant systems, we then tested its competitive ability against strain 715 using an initial excess of strain KZR-5, i.e. at an initial strain ratio of 5:1. Again, the relative proportion of strain KZR-5 in mixes was quite stable in the soil, whereas it decreased progressively upon colonization of the tomato stems (Fig. 5). The initial relative amount of strain KZR-5 was significantly higher than the relative amount found in the stems at day 21 ($P<0.05$). Overall, we conclude that the relative numbers of strain KZR-5 in mixes with three counterpart strains remained stable in the soil, whereas they decreased significantly during the process of invasion and colonization of tomato plants. This decrease was independent of the ratio between the strains at the onset of the experiment (Fig. 5).

The reduced ability of environmental strain KZR-5 to colonize tomato plants may be the result of a reduced capacity to regulate or perform one or more of the steps that are required in the intricate processes that are involved in successful plant colonization (Schell 2000). Overall, the plant invasion process is tightly regulated and requires a complex regulatory network of interacting gene products. Among the many genes that are up-and down-regulated are genes that encode cell-wall-degrading enzymes, such as *cbhA*. Although the gene product, CbhA, is not strictly essential for pathogenicity (the substrate it hydrolyzes was never demonstrated), it was

found to contribute to virulence on susceptible plants (Liu et al. 2005). Possibly, such cell-wall-degrading enzymes play roles in the initial stages of plant colonization as well as later, when cells have to penetrate the xylem vessels. For instance, EglI is required for efficient invasion of plant roots and colonization of stems, where it might function in the degradation of cellulosic glucan compounds Roberts et al. 1988, Denny et al. 1990). Thus, although we were not able to demonstrate the absence of cellobiohydrolase activity in strain KZR-5, we did observe a (subtle) drop in competitiveness upon plant colonization. It is tempting to speculate on the involvement of the PGI-1-located CbhA. However other factors might also play a role. For instance, PGI-1 encodes a transcriptional regulator which could affect numerous downstream processes. In addition, the function of the PGI-1-encoded hypothetical proteins is still unknown (Stevens & van Elsas 2010b). Furthermore, additional processes at the transcriptional or translational level might differ between the strains (regardless of PGI-1 being present or not).

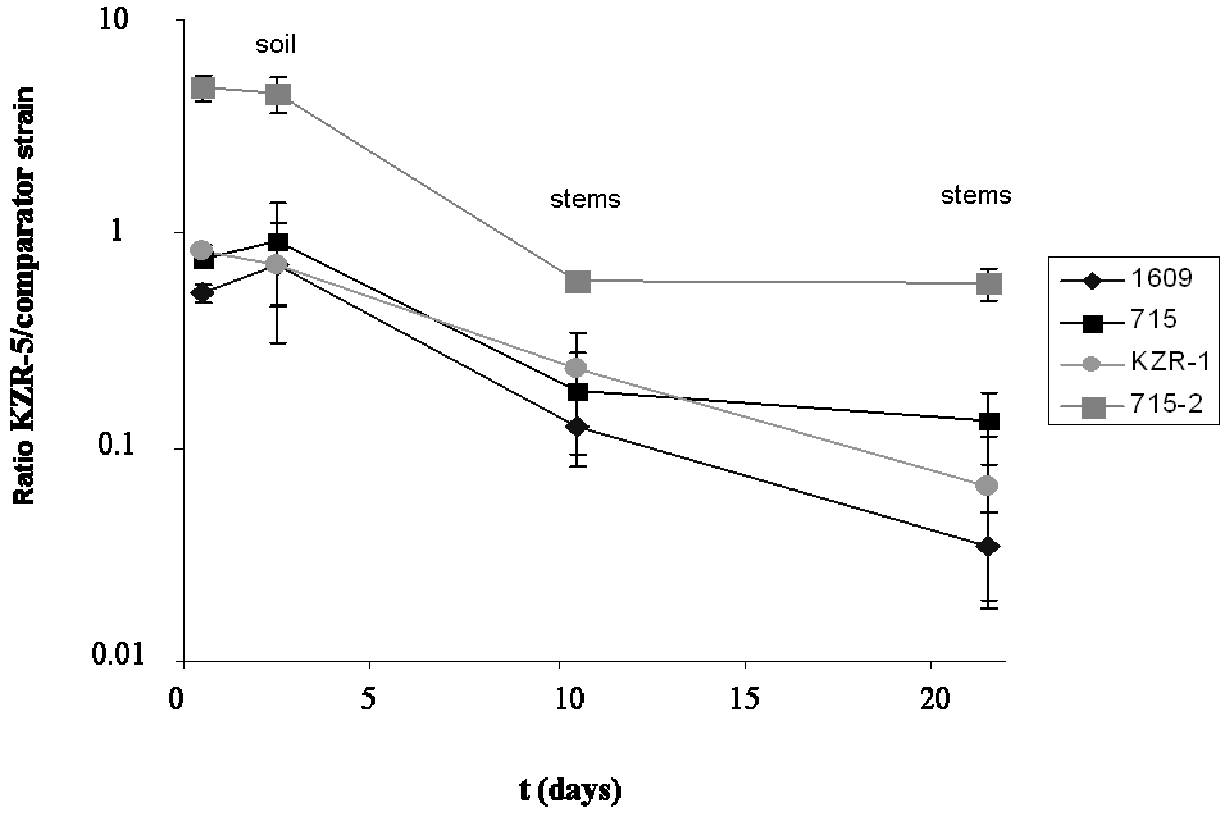


Figure 5 Competitiveness of *R. solanacearum* strain KZR-5 against strains 1609, 715 and KZR-1 upon invasion of tomato plants. The ratio between ΔPGI-1 strain KZR-5 and the comparator strains (1609, 715 and KZR-1) was determined by dilution plating on agar plates (BG or SMSA) at four time points. First, the ratio between strains was determined for the cell mixture used for inoculation of the plants on BG agar plates. Second, the ratio between strains in the soil was determined from CFUs on SMSA plates at day 2. Extraction of *R. solanacearum* from the tomato stems was done 10 and 21 days post-inoculation and the ratio between strains was determined from CFU counts on SMSA plates.

Temperature regime might also affect both growth and virulence of strains Yang & Ishiguro 2003, Milling et al. 2009. Milling et al (2009) showed that the virulence of *R. solanacearum* biovar 2 strain UW551was similar to that of GMI1000 (bv3) and K60 (bv1) at 28°C, while at 20°C strain UW551 revealed higher virulence (Milling et al. 2009). In addition, RelA mutations can lead to decreased

thermotolerance, as observed for *E. coli* (Yang & Ishiguro 2003). Our competition experiments in tomato, in which strain KZR-5 revealed to be less competitive than strains 1609 and 715, were performed at 26°C/21°C (day/night regime). On the basis of the foregoing, it is possible that strain KZR-5 has become less well equipped for plant invasion at the high-temperature regime used as opposed to lower temperatures.

Milling *et al*. (2009) suggested that the survival of *R. solanacearum* bv2 in temperate climates is related to its interaction with host plants rather than to its persistence at low temperature in the open environment. However, in our study, environmental strain KZR-5 showed clearly increased survival when compared to potato strains 1609 and 715. In contrast, its colonization efficiency on tomato host plants had decreased. However, whether the absence of genes for the SAS-like protein and the cellobiohydrolase indeed represents the key factor determining this altered phenotype of strain KZR-5 remained unsolved in this work and should be the target for future research. Thus, although we did not measure actual (p)ppGpp levels in this study, strain KZR-5 behaved like the *E. coli* (p)ppGpp mutant (ΔrelA). In *E. coli*, the effect seen on plates is due to isoleucine starvation. However, strain KZR-5 was able to grow normally (comparable to its siblings) on M63 minimal medium, which stood in contrast to the non-growth of *E. coli* ΔrelA. The overall (growth/survival) behavior of strain KZR-5 indicates that this strain is impaired in its response rate to specific nutritional conditions allowing growth, whereas it had enhanced tolerance to (low temperature) stress.

Acknowledgments

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References

Álvarez B, López MM, Biosca EG (2007) Influence of native microbiota on survival of *Ralstonia solanacearum* phylotype II in river water microcosms. Appl Environ Microbiol: 7210-7217

Álvarez B, López MM, Biosca EG (2008) Survival strategies and pathogenicity of *Ralstonia solanacearum* phylotype II subjected to prolonged starvation in environmental water microcosms. Microbiology 154:3590-3598

Baba T, Ara T, Hasegawa M, et al (2006) Construction of *Escherichia coli* K-12 in frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol 2

Uzan M, Danchin A (1976) Rapid test for RelA mutation in *Escherichia coli*. Biochem Biophys Res Comm 69: 751-758

Braeken K, Moris M, Daniels R, Vanderleyden J, Michiels J (2006) New horizons for (p)ppGpp in bacterial and plant physiology. Trends in Microbiology 14: 45-54.

Calderon-Flores A, Du Pont G, Huerta-Saquero A et al. (2005) The stringent response is required for amino acid and nitrate utilization, nod factor regulation, nodulation, and nitrogen fixation in *Rhizobium etli*. J Bacteriol 187:5075-5083

Cashel M, Gentry DR, Hernandez VJ et al. (1996) The stringent response In:Neidhardt FC, Curtiss R (eds) *Escherichia coli* and *Salmonella:* Cellular and Molecular Biology. Washington D.C: ASM Press, pp 1458-1496.

Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proceedings of the National* Academy of Sciences of the United States of America 97: 6640-6645

Das B, Bhadra RK (2008) Molecular characterization of *Vibrio cholerae* ΔrelA Δ spoT double mutants Archiv Microbiol 189: 227-238.

Denny TP, Carney BF, Schell MA (1990) Inactivation of multiple virulence genes. reduces the ability of *Pseudomonas solanacearum* to cause wilt symptoms. Mol Plant Microbe Interact 3: 293-300.

Elphinstone JG, J.Hennessy, J.K.Wilson, D.E.Stead (1996) Sensitivity of different methods for the detection of *Pseudomonas solanacearum* (Smith) Smith in potato tuber extracts. Bull. OEPP 26: 663-678.

Granada GA, Sequira L (1983) Survival of *Pseudomonas solanacearum* in soil, rhizosphere and plant roots. Canadian J Microbiol 29: 433-440

Hogg T, Mechold U, Malke H et al. (2004) Conformational antagonism between opposing active sites in a bifunctional RelA/SpoT homolog modulates (p)ppGpp metabolism during the stringent response. Cell 117: 57-68

Lemos JA, Lin VK, Nascimento MM, Abranches J, Burne RA (2007) Three gene products govern (p)ppGpp production by Streptococcus mutans. Mol Microbiol 65: 1568-1581

Liu H, Zhang S, Schell MA, Denny TP (2005) Pyramiding unmarked deletions in *Ralstonia solanacearum* shows that secreted proteins in addition to plant cell-wall-degrading enzymes contribute to virulence. Mol Plant Microbe Interact 18:1296-1305.

Milling A, Meng F, Denny TP, Allen C (2009) Interactions with hosts at cool temperatures, not cold tolerance, explain the unique epidemiology of *Ralstonia solanacearum* race 3 biovar 2. Phytopathology 99:1127-1134

Mittenhuber G (2001) Comparative genomics and evolution of genes encoding bacterial (p)ppGpp synthetases/hydrolases (the rel, RelA and SpoT proteins). J of Mol Microbiol Biotechn 3:585-600

Nanamiya H, Kasai K, Nozawa A, Yun CS, Narisawa T, Murakami K *et al.*(2008) Identification and functional analysis of novel (p)ppGpp synthetase genes in *Bacillus subtilis*. Mol Microbiol 67: 291-304

Poussier S, Trigalet-Démery D, Vandewalle P, Goffinet B, Luisetti J, Trigalet A (2000) Genetic diversity of *Ralstonia solanacearum* as assessed by PCR-RFLP of the *hrp* gene region, AFLP and 16S rRNA sequence analysis, and identification of an African subdivision. Microbiology 146:1679-1692.

Poussier S, Vandewalle P, Luisetti J (1999) Genetic diversity of African and worldwide strains of *Ralstonia solanacearum* as determined by PCR-restriction fragment length polymorphism analysis of the *hrp* gene region. App Environ Microbiol 65:2184-2194.

Roberts DP, Denny TP, Schell MA (1988) Cloning of the egl gene of *Pseudomonas solanacearum* and analysis of its role in phytopathogenicity. J Bacteriol 170:1445-1451

Rudd KE, Bochner BR, Cashel M, Roth JR (1985) Mutations in the *spoT* gene of *Salmonella typhimurium*: effects on his operon expression. J Bacteriol 163:534-542

Schell, MA (2000). Control of virulence and pathogenicity genes of *Ralstonia solanacearum* by an elaborate sensory network. Annu Rev Phytopathol 38:263-292.

Semenov AV, van Bruggen AHC, van Overbeek LS et al (2007) Influence of temperature fluctuations on *Escherichia* coli 0157:H7 and *Salmonella enterica serovar thyphimurium* in cow manure. Fems Microbial Ecol 60:419-428

Spira B, Hu X, Ferenci T (2008) Strain variation in ppGpp concentration and RpoS levels in laboratory strains of Escherichia coli K-12. Microbiology 154:2887-2895

Stevens P, Van Elsas JD (2010a) Genetic and phenotypic diversity of *Ralstonia solancearum* biovar 2 strains obtained from Dutch waterways. Antonie van Leeuwenhoek. 97(2):171-88

Stevens P, van Elsas JD (2010) A putative genomic island, PGI-1, in *Ralstonia solanacearum* biovar 2 revealed by subtractive hybridization. Antonie van Leeuwenhoek. doi 10.1007/s10482-010-9450-4

Sun W, Roland KL, Branger CG, Kuang X, Curtiss R (2009) The role of *relA* and *spoT* in *Yersinia pestis* KIM5+ pathogenicity. PLoS ONE 4: e6720

Teather RM, Wood PJ (1982) Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. Appl Environ Microbiol 43: 777-780

Tilbeurgh H, van Claeyssens M, De Bruyne CK (1984) The use of 4-methyl umbelliferyl and other chromophoric glycosides in the study of cellulolytic enzymes. FEBS Lett 149: 152-156

Van der Wolf JM, Bonants PJM, Smith JJ et al. (1998) Genetic diversity of *Ralstonia solancaerum* Race 3 in Western Europe determined by AFLP, RC-PFGE and Rep-PCR. In: Prior PH, Allen C, Elphinstone J (eds) Bacterial wilt disease: molecular and ecological aspects. Springer-Verslag, Berlin, Germany, pp 44-49.

Van Elsas JD, Kastelein P, de Vries PM, van Overbeek LS (2001) Effects of ecological factors on the survival and physiology of *Ralstonia solanacearum* biovar 2 in irrigation water. Canadian J Microbiol 47:842-854

Van Elsas JD, Kastelein P, van Bekkum P, van der Wolf JM, de Vries PM, van Overbeek LS (2000) Survival of *Ralstonia solanacearum* biovar 2, the causative agent of potato brown rot, in field and microcosm soils in temperate climates. Phytopathol 90:1358-1366

Van Elsas JD, van Overbeek LS, Trigalet A (2005a) The viable but non culturable state in *Ralstonia solanacearum*: Is there a realistic threat to our strategic concepts? In: Allen C, Prior P, Hayward AC (eds) Bacterial wilt disease and the *Ralstonia solanacearum* species complex. American Phytopathological Society Press, St. Paul, MN pp 103-119

Van Elsas JD, van Overbeek LS, Bailey MJ, Schönfeld J, Smalla K (2005b) Fate of *Ralstonia solanacearum* biovar 2 as affected by conditions and soil treatments in temperate climate zones In: Allen C, Prior P, Hayward AC (eds) Bacterial wilt disease and the *Ralstonia solanacearum* species complex. American Phytopathological Society Press, St. Paul, MN pp 39-49

Wendrich TM, Marahiel MA (1997) Cloning and characterization of a *relA*/*spoT* homologue from *Bacillus subtilis*. Mol Microbiol 26: 65-79

Winstead NN, Kelman A (1952) Inoculation techniques for evaluating resistance of *Pseudomonas solanacearum*. Phytopathol 1952, 42: 628-634

Yang XM, Ishiguro EE (2003) Temperature-sensitive growth and decreasedthermotolerance associated with relA mutations in Escherichia coli. J Bacteriol 185:5765-5771

Chapter VI

Ralstonia solanacearum biovar 2 shows enhanced tolerance to oxidative stress upon carbon starvation

Patricia Stevens
Leonard Simon van Overbeek
Jan Dirk van Elsas

Abstract

In this study, we investigated the ability of potato-derived *Ralstonia solanacearum* bv2 strains to respond to starvation conditions by enhancing their stress tolerance. In addition, we investigated whether newly-isolated environmental strains, exemplified by strain KZR-5, might show enhanced stress tolerance as compared to comparator strains obtained from potato. The data showed that *R. solanacearum* bv2 potato strain 1609 responds strongly to starvation by enhancing its tolerance to oxidative stress, whereas only minor responses were seen when other stressors, including high temperature, osmotic and ethanol stress, were applied. The responses to oxidative stress of tropical potato strain 715 were similar to that of strain 1609. Furthermore, upon both short-term (1 and 7 days) and long-term (28 months) carbon starvation, the environmental strain, KZR-5, which is devoid of a 17.6 Kb region encoding a putative RelA/SpoT domain protein, either showed enhanced or similar stress tolerance as compared to potato strains 1609 and 715. Surprisingly, KZR-5 revealed to be slightly more tolerant to oxidative stress when growing exponentially.

Introduction

Exponentially-growing bacterial cells that enter stationary phase are subjected to progressively increasing scarcity of essential nutrients, next to the accumulation of waste products. In the stationary phase, when cultures become starved for essential nutrients like carbon, cells of Gram-negative bacteria may undergo a range of tightly-regulated physiological changes that lead to a virtual growth arrest. This shift in metabolism may allow the cells to tolerate a range of otherwise stressful conditions, such as oxidative, temperature, osmotic and salt stresses (Jenkins et al. 1988, 1990, Storz and Hengge-Aronis 2000, Boor 2006). The basis of our current understanding of this so-called *stringent response* to starvation lies in the extensive work performed in the 1990-ies with *Escherichia coli* (Cashel et al. 1996, Storz and Hengge-Aronis, 2000). In this response, a so-called alarmone, (p)ppGpp, together with expressed RecA, incites a cascadal expression of a suite of regulons that collectively allow enhanced tolerance to diverse stressors to occur. Whereas this response has been convincingly shown to occur in *E. coli* as well as in several other Gram-negative bacteria (van Overbeek et al 1995, Storz and Hengge-Aronis 2000, Cashel et al. 1996, Boor 2006), it has by no means been revealed in all such bacteria. In our laboratory, we previously showed this typical physiological response in *Pseudomonas fluorescens*, and we pinpointed carbon-limited soil as a habitat where this response represents a key facet of bacterial life (van Overbeek et al. 1995, 1997b).

Ralstonia solanacearum biovar 2, the causative agent of bacterial wilt of potato (*Solanum tuberosum*), has appeared in the Netherlands as from the mid 1990-ies (Wenneker 1998, Stevens and van Elsas 2010a). Despite quarantine measures that have been taken since then, the organism can still be found in Dutch local waterways, in surface water, ditch sediment as well as in the weed *Solanum dulcamara* (bittersweet) (Stevens and van Elsas 2010a). Among the isolates obtained in our laboratory from Dutch waterways and *S. dulcamara* (bittersweet) plants, a limited degree of genetic variation was found to exist, as determined by pulsed field gel electrophoresis with rare-cutting restriction enzymes (RC-PFGE) and other molecular techniques (Stevens and Van Elsas 2010a). Subsequent genetic analyses then showed that the genomic island PGI-1, of 17.6 Kb, had been deleted from the genome of one of these strains, denoted KZR-5 (Stevens and van Elsas 2010b). The island was found to encode a total of 13 open reading frames (ORF's), among which possible genes with ecological relevance, such as a putative cellobiohydrolase (*cbh*A) gene as well as a gene encoding a protein with a conserved RelA/SpoT domain.

The survival and adaptation of *R. solanacearum* biovar 2 is obviously determined by the conditions that prevail in its habitat, in this case field soils, ditches and canals (Van Elsas et al 2000, 2005, Álvarez et al 2008). Next to seasonally-determined low temperature, a key prevailing condition in such habitats in Northwestern Europe is often low carbon availability. Physiological strategies that allow *R. solanacearum* biovar 2 to persist under these conditions might indeed encompass the changes in gene expression typical of the stringent response, which may allow the emergence of cells with enhanced stress tolerance (Boor 2006). Concomitantly, cell sizes, as well as the formation of filamentous cells and cell aggregation may play a role (Álvarez et al 2008). However, it is so far unknown whether *R. solanacearum* biovar 2 can exhibit a response which results in enhanced stress tolerance – possibly akin to what is seen in *E. coli* as well as *P. fluorescens* - and whether such a response is incited by (carbon) starvation.

In this study, we therefore hypothesized (1) that *R. solanacearum* biovar 2 [strain 1609] is able to survive conditions of starvation as viable CFUs, (2) responds to starvation conditions by enhancing its tolerance to particular stressors, and (3) particularly adapted environmental strains, exemplified by strain KZR-5, might show enhanced stress tolerance as compared to strains derived from potato. This enhanced stress tolerance might be advantageous during existence under local prevailing conditions.

Materials and methods

Strains

Ralstonia solanacearum strains 1609, 715 and KZR-5 were used throughout this study. Strains 1609 and 715 represent, respectively, a Dutch and a tropical potato-derived strain, as described (Stevens and van Elsas 2010a). Strain KZR-5 is a recent environmental isolate obtained in the Netherlands (Stevens and van Elsas 2010a), in which a 17.6 Kb deletion on the genome has been detected (Stevens and van Elsas 2010b). Bacterial strains were stored in 20% glycerol at –80°C. Prior to each experiment, cultures from this stock were grown in 0.1 x TSBS (10% strength Trypticase Soy Broth [Becton Dickinson and Company, MD, USA], 0.1% sucrose; pH 7.2) at 28°C with shaking at 180 rpm.

Growth and starvation

To obtain exponentially-growing and stationary phase cells, strains were grown in M9, 1/3 M63 or M63 medium supplemented with glucose (0.1%) at 28°C with shaking till OD_{660} was 0.3-0.4 or >0.8 respectively. M9 medium contained Na_2HPO_4.x H_2O, 6g; KH_2PO_4,3g; NH_4Cl, 1 g; NaCl, 0.5 g; $MgSO_4$ x $7H_2O$, 0.25 g; $CaCl_2.H_2O$, 0.0147 g; Millipore membrane-filtered H_2O (MilliQ), 1 L [pH 6.8]. M63 medium (Amresco, Solon, USA) contained $(NH_4)2SO_4$, 2g; KH_2PO_4, 13.6 g; $FeSO_4$ x $7H_20$, 0.5 mg; MilliQ, 1L; pH, 7.0). To obtain carbon-starved cells, cells from exponential cultures were spun down (8,000 x g, 15 min), washed twice in sterile milliQ and diluted in M9 or 1/3 M63 medium without C source until cell densities were between 10^3 ml^{-1} and 10^4 ml^{-1}. Flasks were kept at 28°C with shaking until the stress was applied. For long-term survival (and challenge with H_2O_2), cells from 0.1 x TSBS exponential cultures were washed, set at population densities of between 5 x 10^6 and 10^7 ml^{-1} in MilliQ and stored without shaking, in the dark, at room temperature.

Population dynamics and long-term persistence in water

We first tested the long-term persistence of strain 1609 in MilliQ microcosms. For this experiment, triplicate microcosms were set up with approximately 10^5 washed strain 1609 cells per ml and incubated at room temperature. The microcosms were periodically sampled and CFU counts as well as total microscopic cell counts determined in accordance with van Overbeek et al. (2004). Geometric averages were calculated and thus the population dynamics of the organism following starvation was monitored.

Determination of stress tolerance

The methods described by van Overbeek et al (1995) for *P. fluorescens* were used, with adaptations. Specifically, four stressors (heat, osmotic, ethanol and oxidative stress) were applied. Cells were heat-stressed by applying a short elevated temperature (47°C), whereas osmotic, ethanol and oxidative stresses were obtained by treating the cells with NaCl (2.7 M), ethanol (9% in water) and hydrogen peroxide (180 μM H_2O_2). The conditions, notably duration of treatment, under which the four stressors would reveal a response by *R. solanacearum* bv2 strain 1609 were investigated and fixed for this organism.

Statistical treatment of the data

All experiments were performed in triplicate (incidentally, in duplicate) systems, with repeats in time to test the results found. Data were considered to be significantly different between treatments at $P<0.05$ (Student's t test).

Results

***Ralstonia solanacearum* biovar 2 strain 1609 shows persistence of CFUs upon starvation in water**

Upon long-term incubation in ultrapure water at 20°C, *R. solanacearum* strain 1609 revealed considerable persistence of CFU numbers. Specifically, these cultures showed an initial slight increase of CFU numbers, followed by extended stability of the population size at approximately 1×10^6 ml^{-1}. At about 130 days of incubation, this population size was actually still larger than the inoculum density of about 5×10^5 ml^{-1}. Concomitantly, the microcopically-detected cell enumerations revealed very similar dynamics to the CFU-based organismal dynamics (Fig. 1).

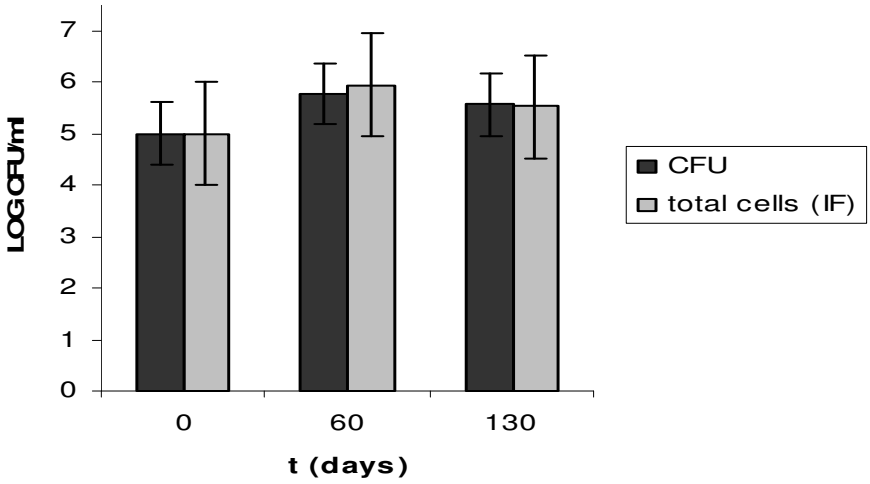

Figure 1 Assessment of the formation of viable-but-nonculturable cells of *R. solanacearum* strain 1609 upon incubation at 20°C. Total cells (IF) and CFUs on 0.1× TSBS agar plates at 0, 60 and 130 days. No significant difference was observed between CFUs on 0.1× TSBS and total cell numbers as determined by IF at 20°C.

These combined data indicated that (1) viable-but-nonculturable cells (VBNC) were not substantially formed under the starvation conditions applied, and (2) the initial population survived well and even grew, and then persisted as viable culturable cells. We thus investigated whether the process of starvation in water might incite a stringent response-dependent

enhancement of cellular tolerance to stresses akin to what has previously been found in *E. coli* and *P. fluorescens* (Jenkins et al. 1988, 1990, Storz and Hengge-Aronis 2000, van Overbeek et al 1995).

***R. solanacearum* biovar 2 strain 1609 shows enhanced tolerance to oxidative stress upon (prolonged) starvation in M9 minimal medium**

In an initial experiment performed in M9 medium devoid of carbon with washed *R. solanacearum biovar 2* strain 1609 cells, we tested the tolerance of,this strain after exponential growth or following starvation (1 and 7 d), to several stressors, including ethanol (9% in water), osmotic (2.7 M NaCl), temperature (47°C) and oxidative stress (180 μM H_2O_2). Repeated experiments showed that exponentially-grown strain 1609 cells were sensitive to all stresses applied, and starvation of cells for up to 7 d did not detectably enhance the resistance of this strain to the ethanol, osmotic and temperature stresses ($P<0.05$; data not shown). In contrast, following starvation, a clear enhancement of tolerance was noted to H_2O_2 (oxidative stress), and the enhancement grossly correlated to time of starvation (Fig. 2). Thus, it was clearly found, with one exception (see below; Fig. 2), that the stress tolerance of strain 1609 increased progressively with increasing time of starvation (Fig. 2). Surprisingly and breaking this trend, cells from late exponential phase appeared with a higher stress tolerance than cells taken later in starvation. In fact, their stress tolerance was akin to that of cells taken much later from starvation phase.

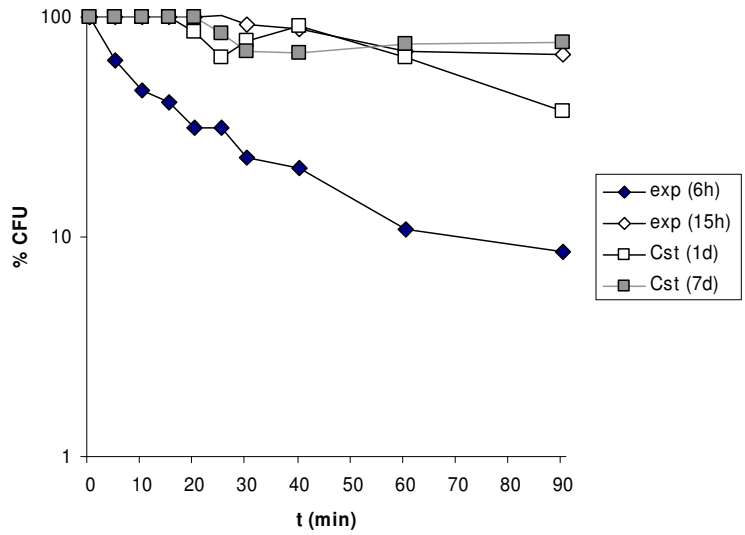

Figure 2 Survival of exponential, stationairy and carbon-starved strain 1609 cells, following a challenge in M9 with 180 μM H_2O_2. Late exponential cells were more resistant to H2O2 than cells incubated for one day under C starvation (water), because after introduction into water, *R. solanacearum* cells grew for several generations (also observed in van Elsas et al. 2005a) and later (7 days and longer) cells become starved. This is the reason for increased sensitivity for H_2O_2 in comparison with late exp cells.

In a follow-up experiment, we tested the oxidative stress tolerance of long-term starved strain 1609 cells. In this case, the surviving CFU counts were invariably very high, i.e. 60-90% of the population survived the challenge to up to 90 min after the challenge. The resulting population sizes of starved cells were significantly ($P<0.05$) higher than those of the control exponential cells

(which were extremely sensitive to the stressor), whereas untreated cultures revealed stable CFU counts.

Detection of oxidative stress tolerance in R. solanacearum biovar 2 strains 1609, 715 and KZR-5 in M63 as related to time of starvation

In experiments with *R. solanacearum* bv2 strains 1609, 715 and KZR-5 in full-strength or 1/3 M63 medium, we confirmed that, for all three strains, the tolerance to the oxidative stress applied was enhanced in cells examined 1 and 7 days following the onset of starvation, as compared to that in the corresponding early exponential cells (data not shown). Strikingly, the late-exponential cells revealed somewhat enhanced stress tolerance, much like observed for strain 1609 in the previous experiment in M9. For strain 1609, the effect of starvation was found to be smaller in M63 than in M9, although it was still significant. Specifically, in M63, the strain 1609 CFU counts dropped to 0.1% of the initial population for exponential cells and to approximately 10% for cells starved for 1 and 7d.

Comparative stress tolerance across the 1609, 715 and KZR-5 strains

Figure 3 shows the results of this experiment. Exponential cells growing in M63 or 1/3 M63 revealed CFU counts quickly dropping upon exposure to H_2O_2 for all three strains. However, we consistently found an enhancement of tolerance to the oxidative stress in strain KZR-5 cells (measured by the percentage survival 10 min after stress application; t=10) as compared to those of strains 1609 and 715 ($P<0.05$). Furthermore, cells of strains 1609, 715 and KZR-5, placed under starvation for 1 or 7 days also revealed progressive declines of CFU numbers with time following application of the stressor. In this case, the decline rates were invariably lower than those observed with exponential cells. For these cultures, we used the population sizes measured 30 (t=30) and 40 (t=40) min following the application of stress, to express the level of stress tolerance. Albeit not significant ($P>0.05$) in most cases, an overall trend towards an enhanced stress tolerance for environmental strain KZR-5 as compared to potato strains 1609 and 715 was observed. In one case, i.e. the comparison of 7-d starved KZR-5 cells to those of 1609 and 715 (t=40), the stress tolerance of strain KZR-5 was significantly enhanced.

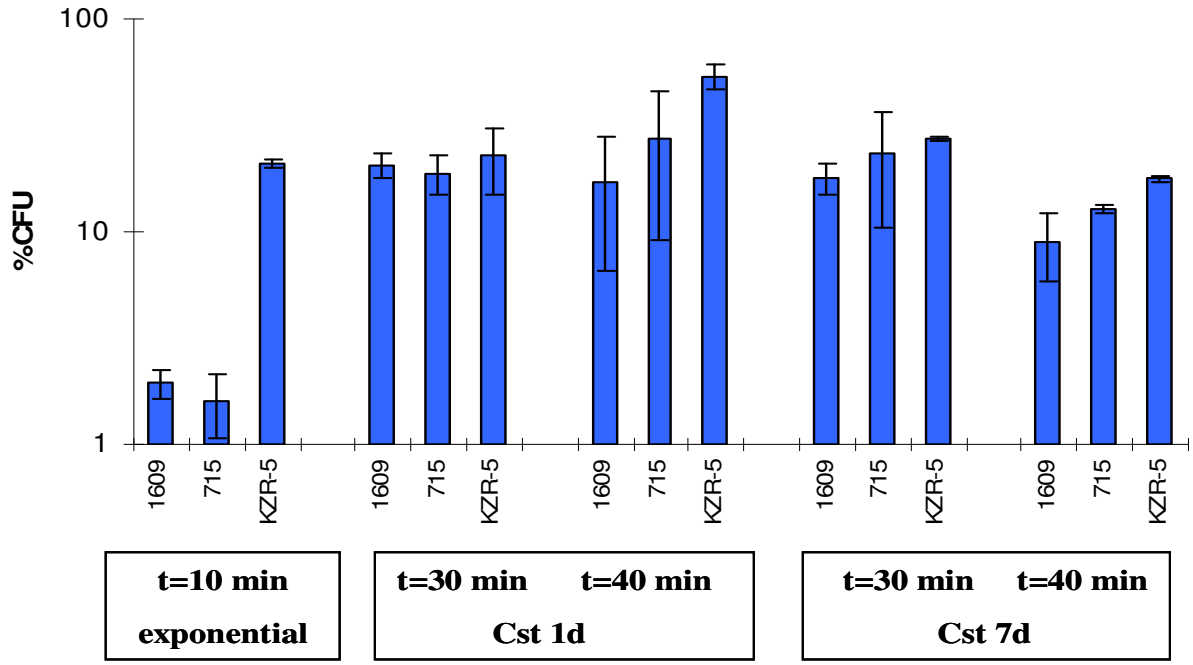

Figure 3. Comparative tolerances of strains 1609, 715 and KZR-5 of exponentially grown and C-starved cells to oxidative stress.

Long-term starvation strongly enhances tolerance to oxidative stress across R. solanacearum strains

Finally, we examined the stress tolerance of long-term (28 months) starved cells of strains 1609, 715 and KZR-5 in MilliQ. The data (Figure 4) revealed that all cultures had significantly enhanced their tolerance to stress when compared to cells maintained under starvation for up to 7 d ($P<0.05$). In fact, large parts of the three populations showed indifference to the stress, as they survived as viable and culturable cells even 90 min following the application of the stressor. Environmental strain KZR-5 again revealed to be somewhat more stress-tolerant than the two potato strains, especially at 10 minutes after the application of oxidative stress, albeit without significance at the end of the experiment ($P>0.05$; Figure 4).

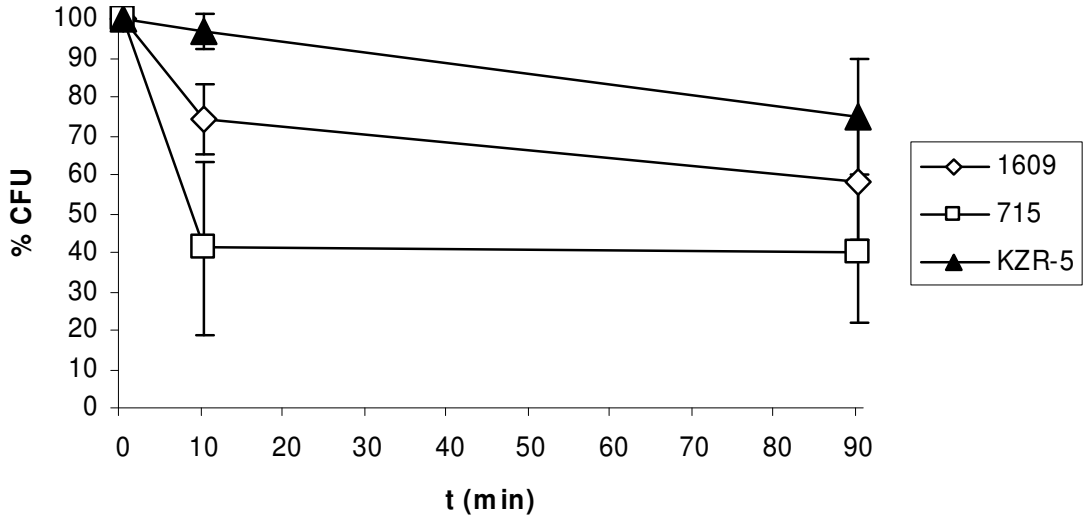

Figure 4 Survival of long-term starved *R. solanacearum* strains challenged with 180 µM H_2O_2 determined by CFU counts

Discussion

In this study, we clearly showed that the potato-derived *Ralstonia solanacearum* bv2 strain 1609 is able to survive for a long period of time (130 d) under carbon starvation conditions. Strikingly, the organisms survived as a virtually 100% culturable population, as evidenced by the fact that the CFU and total microscopic cell counts followed completely similar patterns over a period of approximately 130 days. This indicated (1) the absence of a response to starvation in the direction of debilitated or VBNC cells such as observed when *R. solanacearum* cells are exposed to low temperature (Van Overbeek et al. 2004, Van Elsas et al. 2005; Stevens et al. submitted) and (2) following from the foregoing, the possibility of the emergence of cells with enhanced stress tolerance as a result of the stringent response, much like in *E coli*.

Indeed, *R. solanacearum* bv2 strain 1609 was shown to yield cells that exhibit significantly enhanced tolerance to oxidative stress upon starvation. With one exception, the level of the stress tolerance tended to increase with increased time of starvation. On the other hand and contrary to the expectations, we could not discern any clear response to starvation in respect of the tolerance to the other stressors that were applied (heat, ethanol and osmotic stress) used. Possibly, the experimental conditions applied for *R. solanacearum* (which were derived from those that worked with *P. fluorescens*) did not allow for the ready detection of a response to these stressors. In other words, the adequate "window of opportunity" for the detection of a response had not yet been achieved.

The comparative assessment of the development of stress tolerance resulting from starvation across the environmental strain KZR-5 and the potato strains 1609 and 715 were revealing. Expectedly, all three strains revealed an enhancement of their tolerance of oxidative stress upon starvation, being starvation for 28 months the treatment that yielded the hardiest cells across the board. In addition, a trend towards the existence of a higher oxidative stress tolerance in strain KZR-5, as compared to that in both potato-derived strains, was discernible, albeit only incidentally in a significant manner. We cannot explain this potential phenotype of strain KZR-5, but the fact that the raise was already detectable in exponential KZR-5 cells was remarkable. Although we ignore the function of the putative RelA/SpoT domain protein carried by genomic island PGI-1, a possible relation of the absence of this protein in strain KZR-5 with the enhanced stress tolerance of this strain would be plausible, as such proteins might be (secondary) modulators of the fine regulation of the transition from exponential growth to virtual growth arrest. The absence of this system might make the "normal" stringent response leakier, in the sense that exponentially-growing cells or even cells with lowered growth rates might differentially express some of the pathways involved in the regulation of growth rate and the emergence of levels of tolerance to oxidative stress. It is clear that this response is complex, and that the current work requires confirmatory studies, possibly involving differential gene expression assays, to prove or disprove this key point.

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References

Álvarez B, López MM, Biosca EG (2008) Survival strategies and pathogenicity of *Ralstonia solanacearum* phylotype II subjected to prolonged starvation in environmental water microcosms. Microbiology 154:3590-3598

Boor K (2006) Bacterial stress responses: what doesn't kill them can make them stronger. PLOS Biology 4: 18-20.

Cashel M, Gentry DR, Hernandez VJ, Vinella D (1996) The stringent response. In:Neidhardt FC, Curtiss R (eds) *Escherichia coli* and *Salmonella:* Cellular and Molecular Biology. Washington D.C: ASM Press, pp 1458-1496.

Jenkins DE, Schultz JE, Matin A (1988) Starvation-induced cross protection against heat or H_2O_2 challenge in *Escherichia coli.* J Bacteriol 170:3910-3914.

Jenkins DE, Chaisson SA, Matin A (1990) Starvation-induced cross protection against osmotic challenge in *Escherichia coli*. J Bacteriol 172:2779-278 1.

Stevens P, Van Elsas JD (2010a) Genetic and phenotypic diversity of *Ralstonia solancearum* biovar 2 strains obtained from Dutch waterways. *Anthonie van Leeuwenhoek* 97(2):171-88.

Stevens P, van Elsas JD (2010) A putative genomic island, PGI-1, in *Ralstonia solanacearum* biovar 2 revealed by subtractive hybridization. Antonie van Leeuwenhoek. doi 10.1007/s10482-010-9450-4

Storz G, Hengge-Aronis R (2000) Bacterial stress responses. ASM Press, Washington DC.

Van Elsas JD, van Overbeek LS, Bailey MJ, Schönfeld J, Smalla K (2005a) Fate of *Ralstonia solanacearum* biovar 2 as affected by conditions and soil treatments in temperate climate zones In: Allen C, Prior P, Hayward AC (eds) Bacterial wilt disease and the *Ralstonia solanacearum* species complex. American Phytopathological Society Press, St. Paul, MN pp 39-49

Van Elsas JD, van Overbeek LS, Trigalet A (2005b) The viable-but-nonculturable state in *Ralstonia solanacearum:* is there a realistic threat to our strategic concepts? In: Allen C, Prior P, Hayward AC (eds) Bacterial wilt disease and the *Ralstonia solanacearum* species complex. American Phytopathological Society Press, St. Paul, pp 103-120

Van Elsas JD, Kastelein P, van Bekkum P, van der Wolf JM, de Vries PM, van Overbeek LS (2000) Survival of *Ralstonia solanacearum* biovar 2, the causative agent of potato brown rot, in field and microcosm soils in temperate climates. Phytopathol 90:1358-1366

Van Overbeek LS, Bergervoet JHH, Jacobs FHH, van Elsas JD (2004) The low-temperature-induced viable-but-not-culturable state affects the virulence of *Ralstonia solanacearum* biovar 2. Phytopathol 94:463-469

Van Overbeek LS, Eberl L, Givskov M et al. (1995) Survival of, and induced stress resistance in, carbon-starved *Pseudomonas fluorescens* cells residing in soil. Appl Environ Microbiol 61:4202-4208

Van Overbeek LS, van Elsas JD, van Veen JA (1997a) *Pseudomonas fluorescens* Tn5-B20 mutant RA92 responds to carbon limitation in soil. FEMS Microbiol Ecol 24: 57-71

Van Overbeek LS, van Veen Ja, van Elsas JD (1997b) Induced reporter gene activity, enhanced stress resistance, and competitive ability of a genetically modified *Pseudomonas fluorescens* strain released into a field plot planted with wheat. Appl Environ Microbiol 63:1965-1973

Van Veen JA, van Overbeek LS, van Elsas JD (1997) Fate and activity of micro-organisms following release into soil. Microbiol Mol Biol Rev 61:121-135

Chapter VII

General discussion and concluding remarks

Background

R. solanacearum bv2, the causative agent of potato brownrot, probably originates from the cradle of potato, the Andes and from there has incidentally spread to different regions of the world. In the Netherlands, bacterial wilt has been reported as from the mid-1990-ies (Janse 1998). Although - since then - potato brown rot has been reduced to less than <0.01% in the Netherlands (Breukers et al. 2005), the pathogen may persist in habitats such as the Dutch local waterways. Moreover, it is thought that bittersweet plants growing at the sides of such waterways have contributed to the establishment of the organism, given their capacity to serve as colonizable hosts (Elphinstone et al. 1998; Janse 1998). *R. solanacearum* bv2 has shown differential survival in soil, sediment and water environments (van Elsas et al. 2000, 2001). The survival in aquatic habitats may strongly depend on temperature (van Elsas et al. 2001), as 20°C turned out to be conducive, while 4°C was restrictive to survival (van Elsas et al. 2001, van Overbeek et al. 2004). Furthermore, the presence of sediment in water may have spurred the survival of *R. solanaceaum* bv2 (van Elsas et al., 2001). The levels of carbon that are readily available in the environments inhabited by *R. solanacearum* likely have played a key role as well.

Because *R. solanacearum* bv2 has persisted in Dutch local waterways for over 15 years, it may have experienced conditions that are strongly selective for particular genotypic and/or phenotypic adaptations. Selective forces that may be pinpointed as being most prominent are seasonally low temperatures and fluctuating but often low nutrient (carbon) availabilities (at least concerning the life outside of susceptible hosts). Hypothetically, these forces may have incited changes in the genome that enhance the survivability of the species. On the other hand, we ignore the number of cell divisions the Dutch environmental strains may have gone through and we also did not measure the precise selective forces that may have been operational in the environment. Nevertheless, the study presented in this thesis focussed on the genetic and phenotypic diversity of *R. solanacearum* bv2 populations obtained from Dutch environmental sources. We mainly focussed on the Dutch local waterways, as there were previous data indicating that the organism would be surviving in the habitats defined by these.

R. solanacearum bv2 survives in Dutch local waterways and retains virulence

To detect and obtain *R. solanacearum* bv2 strains in a region where major outbreaks of potato brown rot had occurred in the 1990s, a total of thirty samples were obtained from local waterways at four sampling events. Bulk water, sediment and bittersweet plants growing at the sides of waterways were sampled (chapter II). Screening of the samples for the presence of *R. solanacearum* bv2 was done using semi-selective medium SMSA and thus relied on the presence of culturable cells. Our data showed positive detection of *R. solanacearum* CFU in 20% of the samples. The success of isolation appeared to depend on the prevalent water temperature. In general, when the water temperature increased, the number of isolated strains increased too, a finding that corroborated that of Wenneker et al (1999). For instance, in June 2004, when the water temperature was highest (17°C), all samples analyzed were positive for the presence of *R. solanacearum*. In February 2005 (water temperature 6°C), no *R. solanacearum* colonies were detected. Nevertheless, it is possible that *R. solanacearum* cells were present in these samples in a

VBNC state, as VBNC cells remain undetected using the cultivation-based method used. In addition, no bittersweet plants could be analyzed in February 2005, for the simple reason that these plants occur below the surface of the water in winter (with only the roots and lower stem parts remaining). It is thus very well possible that these submerged plant parts, serving as refuges, contained culturable *R. solanacearum* cells. In fact, most of our isolates originated from bittersweet plants, thus confirming that these plants contribute significantly to the persistence of the organism in Dutch local waterways.

Phenotypic comparison of strains, in terms of growth rate at two temperatures, as well as the capacity to use a range of carbon sources did not allow clear conclusions in respect of ecological fitness or adaptation. Virulence testing of a total of fifteen environmental strains on tomato plants showed that these all caused extensive wilting of the inoculated plants. In contrast, van Overbeek et al. (2004) showed that - upon incubation at 4°C - *R. solanacearum* bv2 cells are no longer able to cause wilting, which might be related to the VBNC state these cells had presumably entered. It was further shown that several carbon sources used by the reference (potato-derived) strains (γ-aminobutyric acid, L-histidine, lactic acid, succinic acid, bromosuccinic acid, L-pyroglutamic acid and propionic acid), were not, or less readily, used by some of the environmental strains. These differences may be related to the ecological factors of the habitats the strains were isolated from (water environment for the environmental strains versus potato). The differences may be the result of either differences in gene expression or changes in the genome. From the results presented in chapter II, it is clear that the culturable *R. solanacearum* populations present in Dutch local waterways, as far as can be seen from the novel strains, maintained their ability to wilt host plants (tomato used as a reasonable model for potato) and thus remained a realistic threat to potato production in these areas.

What drives genome evolution in *R. solanacearum* bv2?

In this study, several molecular tools were employed to look at the genetic make-up of the 42 environmental strains, with a focus on (1) selected genomic regions involved in pathogenicity and (2) the overall genome (chapter II). The environmental strains were compared to two selected potato-derived strains (one from the Netherlands, 1609, and a tropical one, 715). Overall, the analyses revealed a high level of homogeneity across all strains. For instance, no differences were observed using several genomic fingerprinting techniques as well as OLST of six genomic regions. Based on these data, the new environmental strains had a roughly clonal appearance of the analyzed regions and were very similar to the two potato strains. Specifically, the OLST analysis of over 2,000 nucleotides showed a remarkable 100% homology between all strains, including the potato strains. This was rather surprising as one of the reference strains originated from Bangladesh and the occurrence of some SNPs was, at least for this strain, rather expected.

However, clear genomic differences were detected when using PFGE and IS*Rso3* fingerprintings. On the basis of these methods, five genomic groups could be identified. One such genomic group, pulsotype B/IS*Rso3* group 2 (consisting of 5 strains) was composed exclusively of strains obtained from a single location: a single bittersweet stem, its attached rhizosphere and the adjacent surface water. This might indicate an increased fitness of this particular population in the niche defined by the bittersweet/water interface. Next to the differences detected with PFGE and

ISRSo3 fingerprintings, a variable tandem repeat region, TR0578, was found which showed allelic variations in the strain set studied. Overall, the genomic differences that were revealed among the novel environmental strains, as compared to the potato strains, occurred at the level of genomic reshufflings as well as at the somewhat finer (TR0578) genetic level. This even extended to strains from single bittersweet plants. We selected environmental strain KZR-5 (pulsotype C) for further analyses, as it revealed a conspicuous difference in PFGE.

To perform a more in-depth analysis of the genetic make-up of strain KZR-5 (pulsotype C/IS*Rso3* group 1), as compared to tropical potato strain 715 (pulsotype A/IS*Rso3* group 2), an SSH library for each strain was constructed, which was assumed to represent putative strain-specific sequences (chapter III). Following extensive genetic analysis, this study revealed the excision of a 17.6 Kb genomic island, PGI-1, from strain KZR-5 (chapters III and IV). The PGI-1 island was present in all other strains examined, including the two potato-derived ones. The region showed the key features of a genomic island that is potentially mobile. An important observation was the presence in PGI-1 of identical composite IS elements at both flanks, which probably mediated the excision of the element from the genome of strain KZR-5. The island contained a total of 13 putative ORFs/genes. We could assign potential function to six ORFs, but the function of seven other putative ORFs that were identified on the genomic island remained largely unknown. Additional deletions are possibly present in the *R. solanacearum* populations that are present in current Dutch water environments (chapter IV). For instance, the putative deletions that were identified in strains KZR-1 and PA1 using CGH all encoded hypothetical proteins. The finding of suites of genes in *R. solanacearum* encoding hypothetical proteins is not unusual, as a large proportion of the *R. solanacearum* bv2 specific genes identified by Guidot et al. (2009) also encoded hypothetical proteins. Thus, due to the uncertainty about the function of many of these, identifying strain-specific sequences does not necessarily provide answers as to why strains behave differently under specific conditions. Comparison of the SSH-defined genomes of strain KZR-5 and 715 further indicated that a large proportion (20 %) of the putative strain-specific sequences were related to sequences involved in recombination processes (IS elements, Rhs elements). This, to the best of our knowledge, may indicate that the main drivers of the short-term diversification of *R. solanacearum* strains in the environment may well be recombination or shuffling processes. The importance of recombination and IS elements in shaping the *R. solanacearum* genome has recently become very evident from the work done by Guidot et al. (2007, 2009b). However, these studies addressed to what extent the underlying mechanisms contribute to genomic diversity among different biovars. One of the sequences found in our SSH libraries (an intergenic region that is localized between genes encoding a type III effector and a Rhs related protein) was also consistently detected in micro-array experiments using two additional environmental strains (chapter IV). The function of such intergenic regions is unfortunately not known, and so we can only speculate on it. A proportion of *R. solanacearum* bv2 specific sequences reported by Guidot et al. (2009b) were also found to represent intergenic regions. The fact that such intergenic regions were consistently found in different experiments might indicate that they are of ecological or evolutionary relevance.

In the work presented in chapter III, SSH was proven to be a powerful tool in detecting strain-specific sequences. The tool yielded one-sided results, as we did not detect genes, or islands, that had been newly acquired via HGT in strain KZR-5. Possibly, the relatively short time in the open (water) environment had not yet permitted successful HGT events. Given the fact that

we did not extent our analyses to these, it is also unclear whether newly-acquired genes might be present in the other environmental strains. Although the genomes of two additional environmental strains were compared to that of strain 1609, the method used (CGH using micro-arrays, chapter IV) by nature did not allow the detection of HGT acquired sequences.

Ecological relevance of PGI-1

To assess whether the deletion of genomic island PGI-1 in strain KZR-5 had resulted in adaptation to locally prevailing conditions, we compared the behavior of KZR-5 to that of strains 1609 and 715 under ecologically-relevant conditions. As the exact set of functions harbored by PGI-1 is as yet unknown, we focused on two putative genes of potential ecological relevance, i.e. the gene encoding a RelA/SpoT domain and CbhA. The resulting putative proteins are likely to be involved in stress response and/or interaction with the host plant, respectively (chapter V and VI)

A first observation was the significantly enhanced survival of strain KZR-5 at low temperature as compared to that of strain 715. Strain KZR-5 also showed (slightly) enhanced tolerance to oxidative stress when grown exponentially. It was, on the other hand, compromised in its growth in liquid culture (at 28 as well as 16^{o}C) and on media that induce amino acid starvation. It is possible, but this remained unconfirmed, that the loss of the gene that putatively encoded the RelA/SpoT domain protein was related to these phenotypic changes. Granted, the putative function of the RelA/SpoT domain protein was not elucidated in this research. However, as is the case for the SAS-like proteins, we speculate that it might be involved in a fine regulation (in respect of the effect on the cellular (p)ppGpp levels) of the growth at maximal growth rate versus growth arrest, and the respective shift between these two physiological states. This protein may serve a putative function that is related to, but different from, the classical RelA and SpoT proteins, in terms of how it is induced.

Strain KZR-5 showed, similar to *E. coli* RelA mutants, a clear phenotype (growth defect) on minimal medium that induced amino acid starvation. On the other hand, it was, unlike *E. coli* RelA mutants, able to grow normally on minimal medium and, on this medium, showed growth rates similar to strains 1609 and 715 (chapter V). Growth on rich medium was, however, reduced.

Nevertheless, we did not obtain conclusive evidence for the contention that the changed phenotype of KZR-5 was due to the absence of PGI-1 or whether other adaptive traits play a role. For instance, differences in gene expression rather than in genome content might have been at the basis of the changes. In any case, in terms of phenotypic changes, we did find support for the contention that strains from the open water environment indeed adapted to conditions that reigned in their habitat, including low temperatures that occur in winters.

Although no differences between the selected strains in pathogenicity on tomato were detected in single inoculation experiments, strain KZR-5 was clearly outcompeted in tomato invasion competition experiments by strains 715 as well as strain 1609. This was an unexpected result, since the latter strain had previously shown reduced pathogenicity in single inoculation experiments (chapters II and V). Concerning this (subtle) decrease in tomato plant colonization efficiency of strain KZR-5, we postulate that the lack of the *cbhA* gene may have played play a decisive role, as this gene contributes to wilting of the host plant in strain GMI1000 (Liu et al. 2005).

Response to oxidative stress

The data presented in this thesis showed that several *R. solanacearum* strains become tolerant to oxidative stress once they are starved for carbon (chapter VI). Tolerance to oxidative stress increased with prolonged (C) starvation, which was also seen for *P. fluorescens* (van Overbeek et al. 1995). Tolerance to other stressors that were applied (ethanol, salt and heat) did not increase in *R. solanacearum* upon carbon starvation. This stood in sharp contrast to the situation in *P. fluorescens* (van Overbeek et al. 1995). Probably a general stress response mechanism, which is maximally induced after long-term starvation, is the cause behind the observed "cross-tolerance" to oxidative stress. In addition, other (or overlapping) stress response mechanisms may be at the basis of the observed differences between *R. solanacearum* and *P. fluorescens* in the response to the other stressors applied.

Strikingly, strain KZR-5 showed a slightly enhanced tolerance to oxidative stress when grown exponentially, but differences were not significant when carbon-starved cells were subjected to the oxidative stress. It is possible that the increased tolerance for oxidative stress in exponential cells is related to the increased survival of KZR-5 at low temperature (chapter IV). When cold-stressed cells are relieved from oxidative stress by the addition of catalase, a temporary increase in culturability was observed (van Overbeek et al. 2004), which might indicate that enhanced tolerance to oxidative stress is the reason behind the enhanced tolerance to cold stress as well. Whether the SAS-like protein, which is absent from strain KZR-5, has anything to do with the potential tinkering of the fine-tuning of the starvation/oxidative stress response remained unclear (chapter VI). It would indeed be speculative at this point in time to assign a function to it in view of the data obtained so far.

Concluding remarks and outlook

The finding that most Dutch environmental strains showed a high degree of genetic homogeneity is in line with the early studies on the diversity of the mostly potato-derived *R. solanacearum* bv2 strains. It strengthens the idea that most, if not all, of these strains recently originated from a single source. However, we also demonstrated quite clearly, at a "higher" genetic level, that genetic differences between strains isolated from the open environment exist. Next to differences in their genetic make-up, phenotypic differences appeared that had to do with carbon source utilization, growth rate, survival at low temperature and colonization of tomato plants (when in competition).

In our study, so far no evidence was found for the acquisition of new traits by *R. solanacearum* bv2 via HGT. This could be due to the fact that the time course used to study genome evolution of *R. solanacearum* bv2 was rather short. Our Dutch potato strain, 1609, was isolated in the early 1990-ies (the year of isolation of tropical strain 715 is not known) and our environmental strains were obtained from Dutch water environments in 2004/2005. On the other hand, strong selective forces (i.e low temperature during winter periods, low carbon availability in many occupied niches), are likely to act on the *R. solanacearum* population present in the open environment. Next to these strong selective forces, the organism may have undergone growth in

association with bittersweet plants, which would allow genomic alterations to occur and become fixed in the population. It could be that there was simply not enough time (or cell-to-cell or DNA-to-cell contact) to allow the acquisition of genes or islands through HGT. On the other hand, the activity of IS elements present in the genome may have allowed the emergence of within-genome flexibility that eventually leads to adaptation to novel conditions. From several studies, it has become clear that the activity of IS elements as well as HGT contribute significantly to diversification of bacterial genomes (Mahillon & Chandler 1998, Gogarten & Townsend 2005, Darling et al. 2008,)). This includes that of *R. solanacearum* (Guidot et al. 2007, 2009). In the work described here, several lines of evidence were found for the contention that, indeed, the activity of IS elements is a major driving force that shapes the *R. solanacearum* bv2 genome in environmental populations.

To wrap up, the clearest evidence found for the involvement of DNA rearrangements in *R. solanacearum* bv2 were (1) differences in ISRso3 fingerprinting, (2) the finding of the genomic island PGI-1 and its structure (i.e IS elements at the flanks), and (3) the relative high proportion of strain-specific sequences involved in recombination processes (i.e the SSH libraries). As all previous studies have included mostly plant-derived strains (van der Wolf et al. 1998, Castillo et al.2007), these may have mainly ignored the "true" diversity of *R. solanacearum* bv2 populations in the open environment. Our data show the co-existence of different *R. solanacearum* bv2 types in the environment, which might reflect mechanisms that drive diversification and ensure enhanced adaptive capabilities.

Because cellular functioning is determined by gene expression as well, experiments that determine the gene expression profiles (i.e using micro-array technology or SSH) of different strains under low temperature/low nutrient conditions would also greatly enhance our knowledge on the genes involved in cold adaptation and survival.

An unexplored area for further research could also be on the potential role for the intergenic regions (in close vicinity of genes encoding effector proteins, IS elements or Rhs related proteins) in recombination processes and thus in ecological fitness.

The evidence provided in this thesis for a putative role of genomic island PGI-1 and IS elements as the main mechanisms in shaping the genome of *R .solanacarum* bv2 has to be seen as still preliminary. The function of PGI-1 (or single genes encoded on the island) in cold tolerance, plant invasion and the response to stress can only be conclusively proven by the directed introduction of such deletions in a parent (potato) strain. Such constructed strains should then be studied in experiments as described in this thesis. Unfortunately, using such genetically-modified *Ralstonia solanaceearum* strains, especially combined with plant experiments, is currently prohibited in the Netherlands. Nevertheless, such experiments with targeted *R. solanacearum* bv2 mutants would certainly provide direct insight in the function of PGI-1 and the genes it encodes. For instance, the exact mechanisms that underlie the differences observed in colonization efficiency of strain KZR-5 and the two comparator (potato) strains could be much better understood by performing direct competition experiments with wild-type using fluorescently-labeled (mutant) strains. This approach may clarify at which point of plant invasion, the differences between strains start to emerge as key traits and thus provide clues as to the exact role of CbhA in *R. solanacearum* bv2 plant colonization.

Unfortunately, we were not able to detect ΔPGI-1 strains in additional environmental strains, nor in experiments using bittersweet plants. Strain KZR-5 was isolated from a heavily-

colonized bittersweet stem, which might indicate that the deletion of PGI-1 was induced by high population density. Under such conditions, cells are likely to experience strong competition for nutrients and reduced growth. A "symbiotic" relationship with bittersweet, or other (endophytic) bacteria, might also have been of importance. We thus inoculated bittersweet plants with KZR-1 cell populations (isolated from the same bittersweet stem as strain KZR-5). Plants were kept in the greenhouse for 1.5 years and, after extraction from the stems, 150 colonies were screened by PCR for the presence of PGI-1. However, no KZR-5-"like" strains were found. Thus, the presence of bittersweet alone does not trigger deletion of the island at high rates. The environmental conditions in the greenhouse were also somewhat different from the open environment, as in our experiments the plants grew in soil systems. Furthermore, the community structure of other indigenous organisms was likely to be different from those in Dutch local waterways. When screening for the presecence of *R. solanacearum* in bittersweet, we repeatedly found the presence of another, morphologically similar bacterium, i.e *Phyllobacterium myrsinacearum.* This bacterium was found to be the second most abundant member of the bacterial communities of the rhizoplane of young sugar beet plants in Belgium and Spain (Lambert et al. 1990) Interestingly, it was able to use cellobiose (an intermediate in cellulose breakdown) very efficiently, as shown in BIOLOG assays (data not shown). It was also reported by Lambert et al. (1990) that *Phyllobacterium* strains are able to utilize aminobutyric acid and propionic acid, both substrates that strain KZR-5 was not able to use as sole carbon and energy sources. Possibly, the presence of *Phyllobacterium myrsinacearum* affects the survival of *R. solanacearum* in bittersweet and was even involved in the putatively changed nutritional requirements of strain KZR-5, thereby making CbhA, and possibly other hypothetical proteins, redundant. Hence, future experiments on the natural conditions that are propicious for PGI-1 deletion might include the presence of this organism.

Further, as bittersweet plants usually do not show disease symptoms, the possibility that *R. solanacearum* bv2 has evolved a commensalistic relationship with these plants and consequently behaves as a plant-associated organism, or even an endophyte in these hosts could be addressed. Plant colonization experiments using bittersweet might provide answers as to whether, for instance CbhA, may have become redundant under the local temperate climate conditions.

As indicated in the foregoing, to better understand the functioning of the SAS-like protein, and the other hypothetical proteins encoded by PGI-1, single knock-out mutants and comparisons should be made of strains during growth, starvation survival and low temperature. Measurements of the cellular levels of (p)ppGpp in such mutant strains, under different conditions, could give direction to future experiments to elucidate the role of SAS-like in fine-tuning of the cellular stress/cold response proteins in Gram-negative bacteria.

To obtain increased fundamental knowledge on the dynamics of genome evolution in *R. solanacearum* bv2, it would further be needed to investigate the extent to which the complete repertoire of the many IS elements present in the *R. solanacearum* genome contributes to genome diversification under different (stressful) conditions, such as (prolonged) persistence at low temperature and low nutrient availability. In this respect, the current strongly reduced cost of genome sequencing will spur genomic comparisons of suites of strains.

References

Castillo JA, Greenberg JT (2007) Evolutionary dynamics of *Ralstonia solanacearum* App Environ Microbiol 73**:**1225-1238

Elphinstone JG, Stanford HM, Stead DE (1998) Detection of *Ralstonia solancearum* in potato tubers, *Solanum dulcamara* and associated irrigation water. In: Prior PH, Allen C, Elphinstone J (eds) Bacterial wilt disease: molecular and ecological aspects. Springer-Verslag, Berlin, Germany, pp 133-139

Darling AE, Miklós I, Ragan MA (2008) Dynamics of genome rearrangement in bacterial populations PLoS Genet 4:1000128

Gogarten JP, Townsend JP (2005) Horizontal gene transfer, genome innovation and evolution..Nature reviews. Microbiology 3(9):679-87

Guidot A, Coupat B, Fall S, Prior P, Bertolla F (2009a) Horizontal gene transfer between *Ralstonia solanacearum* strains detected by comparative genomic hybridization on microarrays. Isme J 3:549-562

Guidot A, Elbaz M, Carrere S, Siri MI, Pianzzola MJ, Prior P *et al* (2009b**)** Specific genes from the potato brown rot strains of *Ralstonia solanacearum* and their potential use for strain detection. Phytopathol 99: 1105-1112.

Guidot A, Prior P, Schoenfeld J, Carrere S, Genin S, Boucher C (2007) Genomic structure and phylogeny of the plant pathogen *Ralstonia solanacearum* inferred from gene distribution analysis. J Bacteriol 189: 377-387

Janse JD (1998) Experiences with potato brown rot *Ralstonia solanacearum* biovar 2, race 3 in the Netherlands. In: Prior PH, Allen C, Elphinstone J (eds) Bacterial wilt disease: molecular and ecological aspects. Springer-Verslag, Berlin, Germany, pp 146-152

Lambert B, Joos H, Dierickx S et al. (1990) Identification and plant interaction of a Phyllobacterium sp., a predominant rhizobacterium of young sugar beet plants. Appl Environ Microbiol 56:1093-1102

Liu H, Zhang S, Schell MA, Denny TP (2005) Pyramiding unmarked deletions in *Ralstonia solanacearum* shows that secreted proteins in addition to plant cell-wall-degrading enzymes contribute to virulence. *Mol Plant Microbe Interact* 2005, 18: 1296-1305

Mahillon J, Chandler M (1998) Insertion sequences. Microbiol Mol Biol Rev 62:725-774.

Van der Wolf JM, Bonants PJM, Smith JJ, Hagenaar M, Nijhuis E, van Beckhoven JRCM, Saddler GS, Trigalet A, Feuillade R (1998) Genetic diversity of *Ralstonia solancaerum* Race 3 in Western Europe determined by AFLP, RC-PFGE and Rep-PCR. In: Prior PH, Allen C, Elphinstone J (eds) Bacterial wilt disease: molecular and ecological aspects. Springer-Verslag, Berlin, Germany, pp 44-49.

Van Elsas JD, Kastelein P, de Vries PM, van Overbeek LS (2001) Effects of ecological factors on the survival and physiology of *Ralstonia solanacearum* biovar 2 in irrigation water. Canadian J Microbiol 47:842-854

Van Elsas JD, Kastelein P, van Bekkum P, van der Wolf JM, de Vries PM, van Overbeek LS (2000b) Survival of *Ralstonia solanacearum* biovar 2, the causative agent of potato brown rot, in field and microcosm soils in temperate climates. Phytopathol 90:1358-1366

Wenneker M, Verdel MSW, Groeneveld RMW, Kempenaar C, van Beuningen AR, Janse JD (1999) *Ralstonia (Pseudomonas) solanacearum* race 3 (biovar 2) in surface water and natural weed hosts: First report on stinging nettle (*Urtica dioica*). Euro J Plant Pathol 105:307-315

Chapter VIII

Nederlandse samenvatting

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De bacterie *Ralstonia solanacearum* biovar 2 veroorzaakt bruinrot in aardappel. Deze bacterie komt oorspronkelijk uit tropische en warme gebieden, maar is in de negentiger jaren ook regelmatig gevonden in gematigde klimaatzones, waaronder Nederland. Aardappeltelers zijn sindsdien gebonden aan strenge regels ten aanzien van de irrigatie van het gewas op het veld. Het was bij de start van het onderzoek aannemelijk dat *R. solanacearum* nog steeds aanwezig was in het Nederlandse oppervlaktewater. Daarmee vormt deze bacterie een constante bedreiging voor de aardappelteelt.

Hoewel al langere tijd bekend is dat *R. solanacearum* slecht bestand is tegen lage temperaturen, zou de bacterie kunnen overleven in zogenaamde refuges in Nederland. Daarnaast is het mogelijk dat er genomische aanpassingen zijn opgetreden die tot verhoogde overleving hebben geleid. Dit onderzoek heeft zich dan ook gericht op de vraag hoe *Ralstonia solanacearum* biovar 2 in het Nederlandse klimaat overleeft. Heeft de bacterie zich aangepast? Zijn er nieuwe eigenschappen verkregen en zoja, welke? Bekend was dat voor de overleving in Nederlandse wateren de aanwezigheid van *Solanum dulcamara* (bitterzoet) een belangrijke factor is. Deze solanum soort groeit aan de oevers van kanalen en sloten en is een geschikte gastheerplant voor *R. solanacearum* biovar 2. De bacterie kan de wortels en onderste delen van de plant koloniseren. en, eenmaal in dit milieu, zou zij beter beschermd kunnen zijn tegen stressfactoren zoals lage temperaturen (bijvoorbeeld in de winter). Ook zijn er in de plant meer voedingstoffen beschikbaar dan in het open ecosysteem. Naast de aanwezigheid van bitterzoet-planten kan ook slootsediment een belangrijke refuge zijn voor *Ralstonia solanacearum* biovar 2. Het wordt algemeen aangenomen dat, wanneer de temperatuur in de zomer toeneemt, (een deel van) de *R. solanacearum* populatie dat aanwezig is in deze refuges, vrijkomt in het water, waardoor de planktonische populatiedichtheid weer toeneemt.

Naast de reservoirs in de refuges, is het ook mogelijk dat *R. solanacearum* in bulkwater door stress tijdelijk in een inactieve vorm, een "dormant" vorm is geraakt. Deze dormant vorm maakt dat *R. solanacearum* niet gemakkelijk op laboratoriummedia te kweken is en, als gevolg daarvan, ook niet gedetecteerd wordt. Een deel van deze cellen zou weer actief kunnen worden als de omstandigheden gunstiger zijn. Wij hypothetiseerden dat er in de winter selectie plaatsvindt van cellen die het best bestand zijn tegen lage temperaturen en andere (stress) factoren. Waarschijnlijk spelen beide processen (overleving in sediment en bitterzoet en tijdelijke overgang naar een "dormant" vorm) een rol bij de overleving van *R. solanacearum.*

Omdat de habitat van *R. solanacerum* biovar 2 in Nederland verschilt van die in tropische gebieden (het oorsprongsgebied), en met name lage temperaturen en de lage beschikbare hoeveelheid voedingstoffen een negatief effect kunnen hebben op de overleving, was onze belangrijkste onderzoeksvraag of de bacterie zich (genetisch) heeft aangepast, waardoor deze beter kan overleven leidend onder deze locale omstandigheden.

R. solanacearum biovar 2 staat bekend als een organisme dat wereldwijd weinig genetische variatie vertoont, hoewel de variatie in het oorsprongsgebied groter is dan elders. Dit kan verklaard worden doordat de aanwezigheid van *R. solanacearum* in gematigde klimaten het gevolg is van één (of enkele) bronnen van introductie, maar een alternatieve verklaring zou kunnen zijn dat er selectie plaatsvindt van een bepaalde genetische variant omdat deze

simpelweg het best uitgerust, het "fitst", is om op aardappel (en aanverwante soorten) te overleven. Met andere woorden, de waardplant als belangrijkste selector van de genetische constitutie van de bacterie. De aanwezigheid van eigenschappen die nodig zijn voor het ziekmaken (verwelken) van de gastheerplant lijkt hierbij van groot belang te zijn.

Om te bepalen of er, 15 jaar na de grootste uitbraken in Nederland, genetische variatie is opgetreden in de locale *R. solanacearum* biovar 2 populatie, werd de bacterie geisoleerd uit oppervlaktewater, sediment en de alternative gastheerplant bitterzoet in een regio waar het organisme grootschalige besmetting heeft veroorzaakt in de jaren 90. De isolaten (stammen), 42 in totaal, werden zowel genotypisch als fenotypisch geanalyseerd (hoofdstuk II). Hierbij is gekeken naar veranderingen in de capaciteit om verschilende koolstofbronnen te gebruiken, maar ook naar genen die betrokken zijn bij de interactie met de plant en pathogeniciteit (ziekte verwekkend). Alle stammen waren in staat tomaatplanten te verwelken en er werden geen verschillen gevonden in de onderzochte pathogeniciteits-genen. Zeer waarschijnlijk heeft *R. solanacearum* alle eigenschappen behouden om planten efficient te infecteren. Wel werden andere genetische verschillen gevonden, zelfs tussen stammen die afkomstig waren uit een en dezelfde bitterzoetplant. Dit was verrassend, vooral omdat *R. solanacearum* biovar 2 in gematigde klimaten wordt beschouwd als "klonaal" (onderling identiek). Mogelijk wijst dit op differentiatie (aanpassing) richting een variant die beter is uitgerust voor overleving in de nederlandse wateren (en dus "fitter" is).

In hoofdstuk III wordt een vergelijking gemaakt tussen het genetisch materiaal aanwezig in een tropische stam (stam 715), geisoleerd van aardappel, en dat van een Nederlandse stam (stam KZR-5), welke een afwijkend genetisch profiel liet zien. Uit dit werk bleek o.a dat in de Nederlandse stam een stuk genetisch materiaal, een genomisch eiland (PGI-1), is verwijderd (een deletie). Dit stuk DNA ter grootte van bijna 20,000 baseparen codeert voor ten minste dertien genen, hoewel de functie maar van een aantal bekend is. Het is bekend dat genomische eilanden onder bepaalde omstandigheden een fitnessvoordeel kunnen opleveren, maar onder andere omstandigheden juist een nadeel, afhankelijk van de ecologische condities waarin het organisme verkeert. Het stuk DNA is, vermoedelijk lang geleden, verkregen van een andere bacteriesoort via horizontale genoverdracht. Hoewel een duidelijke functie van dit stuk genetisch materiaal ontbreekt, bevatte het o.a een gen dat codeerde voor het enzym cellobiohydrolase (betrokken bij plant-interactie en de afbraak van cellulose). Een ander gen dat aanwezig is op dit stuk DNA vertoonde, op eiwit nivo, overeenkomst met eiwitten waarvan bekend is dat zij een rol spelen bij de cellulaire reactie op verschillende stressfactoren.

Naast het ontdekken van de deletie in stam KZR-5 werden ook aanwijzingen gevonden voor de activiteit van elementen die betrokken zouden kunnen zijn bij recombinatieprocessen, dwz interacties tussen delen van het aanwezige DNA die leiden tot een herindeling of verwijdering daarvan (een andere genvolgorde of een deletie). Het is bekend dat de activiteit van dit soort elementen effect kan hebben op een scala aan cellulaire processen.

In hoofdstukken V en VI wordt dieper ingegaan op de mogelijke effecten van de deletie van genomisch eiland PGI-1 op het ecologisch gedrag van stam KZR-5. De focus lag hierbij op het effect van verwijdering van het cellobiohydrolase-gen en het gen dat codeert voor een eiwit met een vermoedellijk functioneel stress-response domein, genaamd RelA/SpoT. Dit domein wordt gevonden in eiwitten die betrokken zijn bij de cellulaire response op stress.

Om meer inzicht te krijgen in de rol van de aanwezigheid van deze genen op de overleving van *R. solanacearum* werd een vergelijking gemaakt tussen het gedrag van stam KZR-5, afkomstig uit het open ecosysteem, en twee stammen die oosrponkelijk geïsoleerd werden van zieke aardappelplanten (de tropische stam 715 en een een Nederlandse stam 1609). Uit de verrichte experimenten bleek duidelijk dat stam KZR-5 langer kon overleven bij 4°C. Daarnaast bleek dat in experimenten waarbij oxidatieve stress werd toegepast door middel van het toevoegen van waterstofperoxide, stam KZR-5 beter bestand te zijn tegen deze stress dan 715 en 1609.

Tegelijkertijd vertoonde stam KZR-5 een verlaagde groeisnelheid op vloeibaar medium dat rijk was aan nutriënten. Het is mogelijk dat het specifieke gen aanwezig op PGI-1 dat codeert voor een eiwit met een RelA/SpoT domein, een rol speelt in het cel-metabolisme onder deze condities. Echter, de precieze rol van dit eiwit tijdens groei en/of stress condities is tijdens dit onderzoek helaas nog niet opgehelderd. Verder werd gevonden dat stam KZR-5 een verminderde fitness had bij kolonisatie van tomaat ten opzichte van stammen 1609 en 715. Deze verlaagde fitness kan mogelijk eveneens gecorreleerd worden aan de afwezigheid van PGI-1 en met name het cellobiohydrase-gen dat daarop ligt. In eerdere publicaties is duidelijk geworden dat het eiwit/enzym dat gecodeerd wordt door dit gen een rol zou kunnen spelen bij de afbraak van cellulose (en kolonisatie van de plant). Echter, er werden geen verschillen gevonden wanneer de stammen alleen bij de tomaatwortels werden aangebracht. Blijkbaar is het effect op kolonisatie van de tomaat plant, en vermoedelijk ook de functie van het cellobiohydrolase gen, subtiel en alleen in specifieke ecologische situaties waarneembaar. Vervolg-experimenten zijn nodig om het effect van (verwijdering van) PGI-1 op het ecologisch gedrag van *R. solanacearum* biovar 2 beter te kunnen begrijpen.

Over het geheel genomen bleek uit dit onderzoek dat vrijwel alle verschillen die werden gevonden in de *R. solanacearum* populaties te maken hadden met de activiteit van mechanismen waarvan, uit vele publicaties over werk met andere bacterieën, al bekend was dat zij een belangrijke rol spelen in genoom-flexibiliteit. Andere mechanismen die bijdragen aan bacteriele evolutie, zoals mutaties, leken in de door ons bestudeerde *R. solanacearum* stammen een ondergeschikte rol te spelen. Dit is mogelijk het gevolg van de - evolutionair gezien - relatief korte tijdsperiode dat *R. solanacearum* onderhevig is geweest aan locale omstandigheden met hoge selectieve druk.

Samenvattend vergroot deze studie het inzicht in de mechanismen die een rol spelen bij de overleving van *R. solanacearum* biovar 2 in watersystemen in een gematigde klimaatzone. De flexibiliteit van specifieke genomische elementen – zoals PGI-1 – kan leiden tot aanpassing en betere overleving van *R solanacearum* onder de locale condities. Met name effecten op het cellulaire vermogen om te gaan met stress spelen waarschijnlijk een rol.. *R. solanacearum* biovar 2 lijkt vooralsnog goed in staat tot overleving in watersystemen in ons klimaat en een voortgaande evolutie in de richting van verhoogde fitness onder locale omstandigheden is waarschijnlijk.

Publications

De Vries B, Todde V, Stevens P, Salomons F, van der Klei IJ, Veenhuis M (2006) **Pex14p is not required for N-starvation induced microautophagy and in catalytic amounts for macropexophagy in *Hansenula polymorpha*.** Autophagy 2:183-8

Stevens P, Monastyrska I, Leão-Helder AN, van der Klei IJ, Veenhuis M, Kiel JA (2005) ***Hansenula polymorpha* Vam7p is required for macropexophagy.** FEMS Yeast Res. 5:985-97

Stevens P, van Elsas JD (2010) **Genetic and phenotypic diversity of *Ralstonia solanacearum* biovar 2 strains obtained from Dutch waterways**. Antonie van Leeuwenhoek 97(2):171–188

Stevens P, van Elsas JD (2010) **A putative genomic island, PGI-1, in *Ralstonia solanacearum* biovar 2 revealed by subtractive hybridization.** Antonie van Leeuwenhoek. doi 10.1007/s10482-010-9450-4

Stevens P, van Overbeek LS, van Elsas JD (2010) ***Ralstonia solanacearum* ΔPGI-1 strain KZR-5 is affected in growth, response to cold stress and invasion of tomato.** Microb. Ecol. in press. Online: 18 Aug 2010

Van der Vossen E, Sikkema A, Hekkert BL, Gros J, Stevens P, Muskens M, Wouters D, Pereira A, Stiekema W, Allefs S (2003) **An ancient R gene from the wild potato species *Solanum bulbocastanum* confers broad-spectrum resistance to *Phytophthora infestans* in cultivated potato and tomato.** Plant J 36:867-82.