Adaptation of Ralstonia solanacearum biovar 2 to temperate climates

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Chapter II

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

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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.

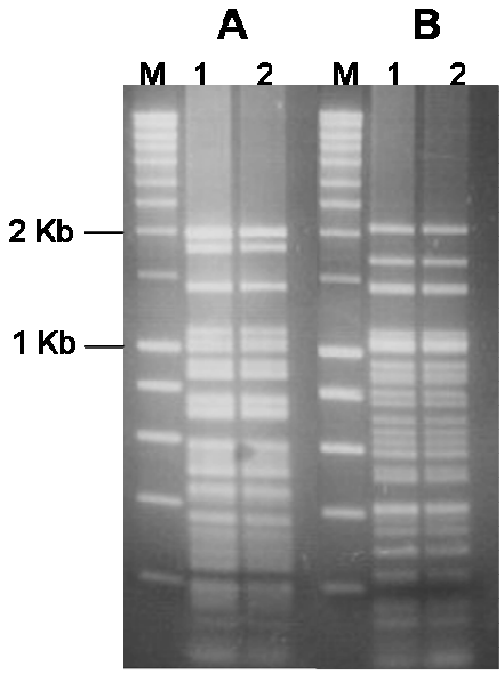

Figure 1. Agarose gel showing fingerprints of the *hrp* region (primerset pglA-F2 and hrcV-R2) after restriction with A) *HinfI* and B) *RsaI*. Lane M is Kb+ molecular size marker, lane 1 is strain 1609; lane 2 is strain KZR-5.

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).

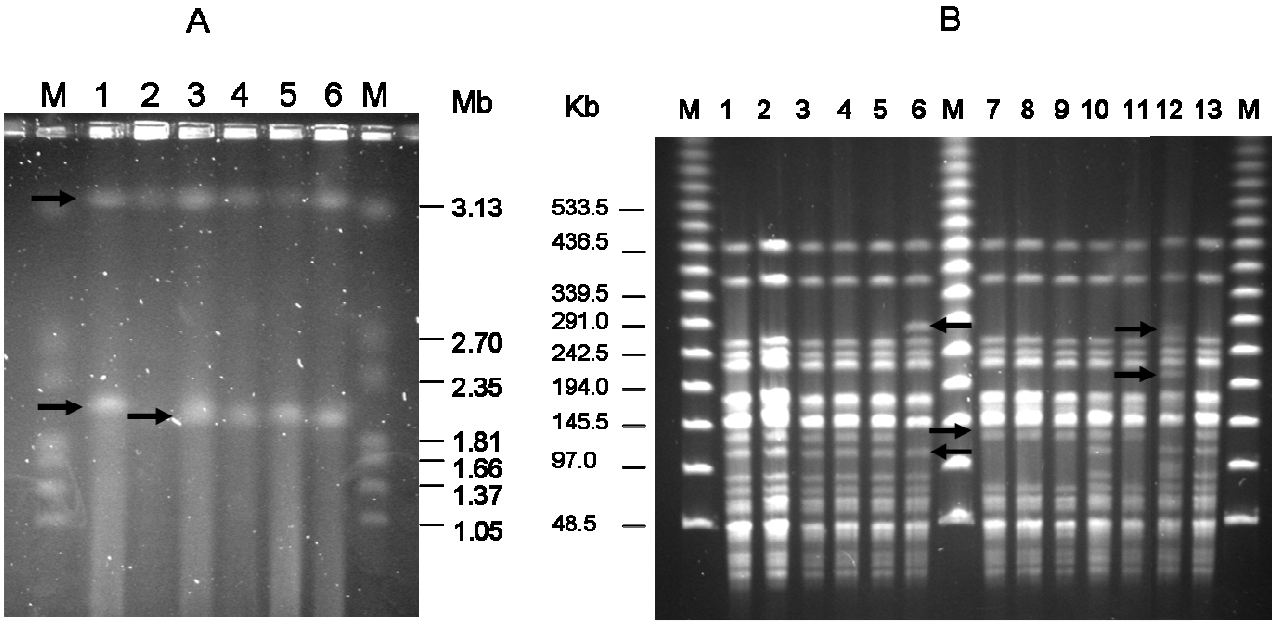

Figure 2 (A) Agarose gel of uncut genomic DNA of *R. solancearum* strains, showing the two circular replicons. Lane M is *H. wingei* chromosomal marker, lane 1: GMI1000 (bv3), lane 2: 1609, lane 3: 715 lane 4: KZR-5, lane 5: PA2, lane 6: PA5. Run conditions were: 0.8% chromosomal grade agarose (1 x TAE), 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.

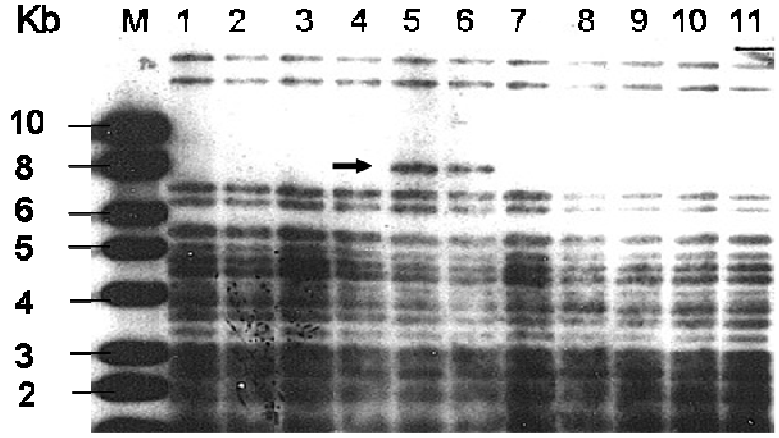

Figure 3. Southern blot analysis of *R. solanacearum* genomic DNA after restriction with *PstI* and hybridisation with an IS*Rso3-AvaI/RsaI* (610 bp) fragment as DNA probe. Lane M is Kb+ molecular size marker. Strains, lane 1: 1609, lane 2: KZR-2 lane 3: KZR-5, lane 4: KZR-1, lane 5: PA1, lane 6: PA2,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

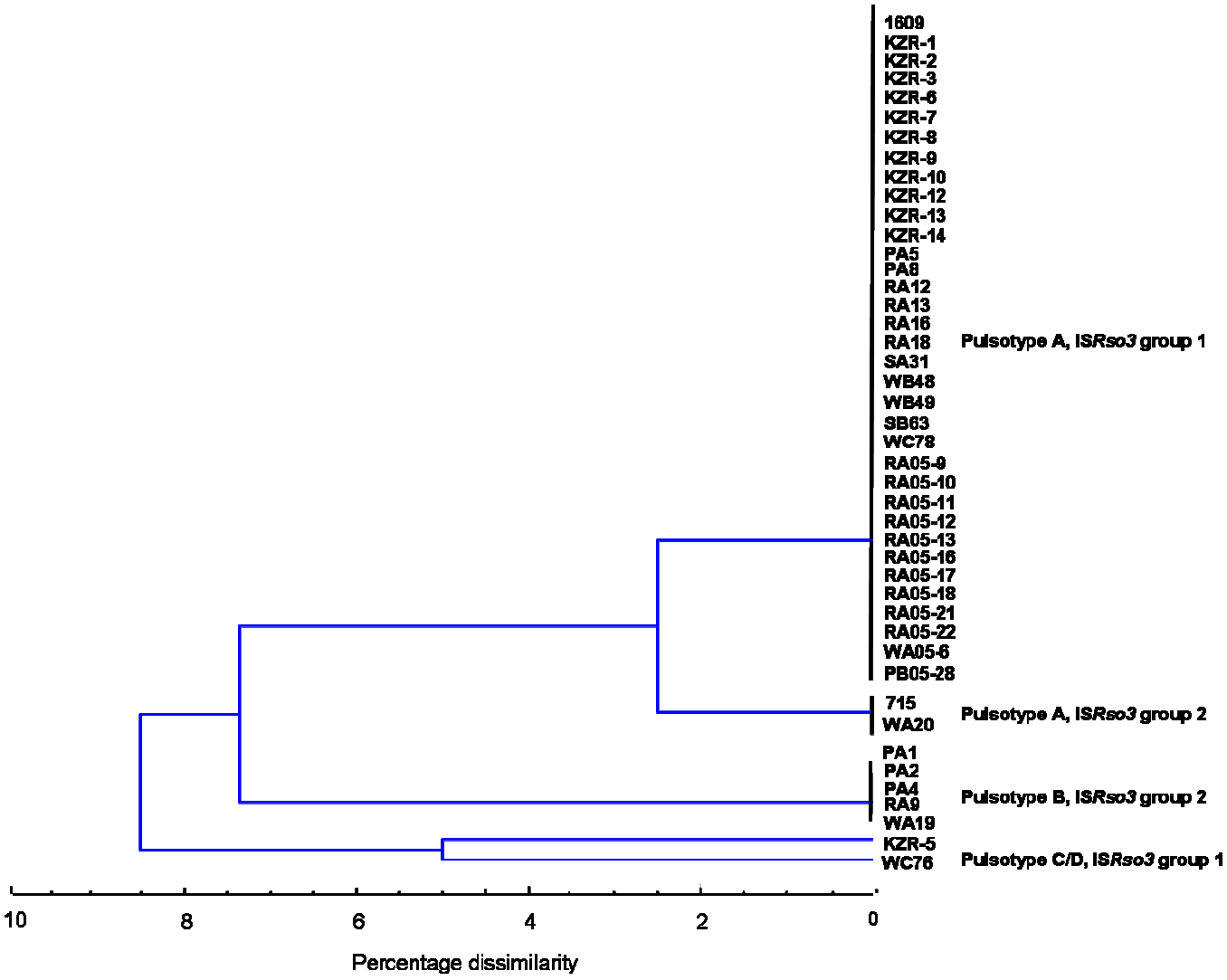

Figure 4. Dendogram obtained by cluster analysis with UPGA and Euclidean distance, using the PFGE and IS*Rso3* patterns of all *R. solanacearum* strains used in this study. Bands were scored as either present or absent. Genomotype defined by pulsotype (A-D) and IS*Rso3* group (1 or 2)

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.

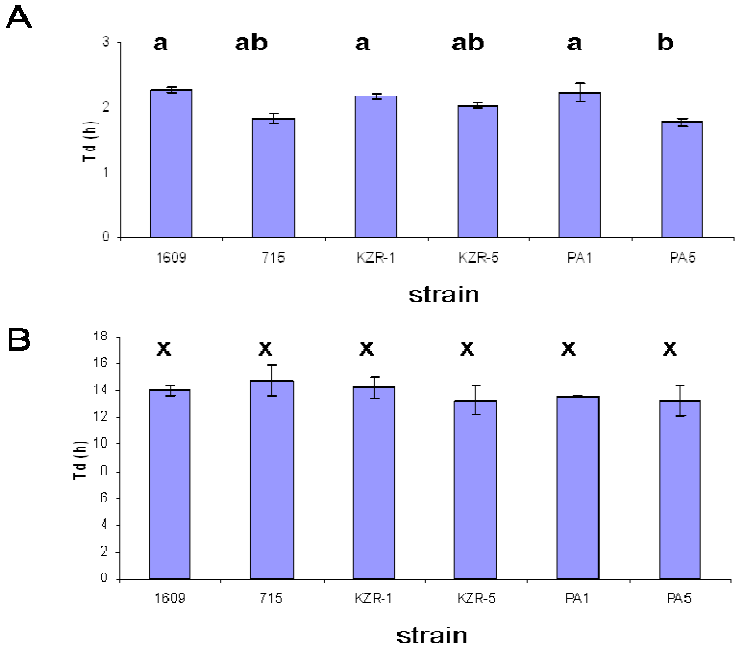

Figure 5. Doubling times in 0,1xTSBS of selected *R. solanacearum* strains in hours (h) at A) 28°C and B) 16°C. Statistical classes (a, ab, b and x) are indicated. P-values were < 0.05.

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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