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Ralstonia solanacearum Δ PGI-1 Strain KZR-5 Is Affected in Growth, Response to Cold Stress and Invasion of Tomato

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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 behaviour of environmental strain KZR-5, which underwent a deletion of the 17.6 kb genomic island PGI-1, with that of environmental strain KZR-1 and potato-derived strains 1609 and 715. PGI-1 harbours two genes of potential ecological relevance, i.e. one encoding a hypothetical protein with a RelA/SpoT domain and one 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 signifi-

cantly 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 two different media, 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 invasiveness were observed among strains KZR-5, KZR-1, 1609 and 715. However, reduced competitiveness of strain KZR-5 was found in experiments on tomato plant colonisation 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 temperatures 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-1990s 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) [11, 26, 35]. This strongly suggests the establishment of *R. solanacearum* bv2 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 originated from the same ancestral (tropical) source. Indeed, amplified fragment length polymorphism (AFLP) analyses have revealed that the genomes of *R. solanacearum* bv2 strains obtained from different European

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countries (like France, the UK and The Netherlands) were very similar in comparison with those of isolates from elsewhere in the world [18, 19, 33]. 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 and AFLP [26, 33]

Studies on the survival of *R. solanacearum* bv2 under local (oligotrophic) conditions in field soils, ditches and canals have been published [2, 34, 35]. From these studies, the conditions that affect the soil- and waterborne nature of *R. solanacearum* bv2 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 state and the formation of filamentous cells and cell aggregation [2]). 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 [1, 34, 35]. Low temperatures can have a dual effect on the survival of *R. solanacearum* bv2. First, the organism might become injured upon this stress [36], and second (on the positive side), predation or competition from the indigenous microorganisms may be reduced [1]. We hypothesised that our environmental *R. solanacearum* bv2 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 and 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.

A previous work in our laboratory revealed genetic differences between several strains that were isolated from Dutch local waterways and bittersweet plants growing at the edges of these waterways [26]. A thorough genetic analysis then showed that the genome of one of these strains, denoted KZR-5, harbors a deletion of 17.6 kb, denoted putative genomic island 1 (PGI-1) [25]. The island encodes a total of 13 open reading frames (ORFs). Among these ORFs was a putative cellobiohydrolase (*cbhA*) 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 [6]. 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 [6]. From a range of studies [5, 7, 17, 27, 37], 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 and symbiosis) and in respect of bacterial multicellular behaviour [4, 24]. 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 harbour single genes for RelA/SpoT-like enzymes that function in both degradation as well as synthesis of (p)ppGpp [16]. Recently, another class of (p)ppGpp synthetases (small alarmone synthetases, SASs) has been identified in *Bacillus subtilis* [17] and *Streptococcus mutans* [13], 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 Δ PGI-1 strain KZR-5, we investigated whether this strain is affected, as compared to potato strains 1609, 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 *cbhA* gene, we hypothesised that the PGI-1 deletion might also incur a change in behaviour 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 (EgII in *R. solanacearum*) and the ability to hydrolyse methylcellulose (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.

Methods

Bacterial Strains and Media

The bacterial strains used in this study are listed in Table 1. Strains 1609 (The Netherlands) and 715 (Bangladesh) were derived from diseased potato plants. Strains KZR-1 (PGI-1+) and KZR-5 (Δ PGI-1) were isolated from bittersweet growing in a Dutch local waterway [26]. 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 [26]. The *E. coli* BW 25113 wild-type and Δ relA strain of the Keio collection [3, 8] were kindly provided by Dr. Javier Pozueta Romero of the Agrobiotechnology

Table 1 Bacterial strains used in this study

Strain	Relevant genotype	Reference
<i>R. solanacearum</i>		
1609	Pulsotype A	[35]
715	Pulsotype A	[31]
KZR-1	Pulsotype A	[26]
KZR-5	Pulsotype C, deletion of 17.6 kb putative genomic island, PGI-1	[25, 26]
<i>E. coli</i>		
BW 25113 wild type	Wild-type K12 derivative	[3]
BW 25113 Δ relA	Δ relA from the Keio collection	[3]

Institute in Navarra, Spain. Bacterial strains were maintained at -80°C in $0.1\times$ TSBS medium [10% strength trypticase soy broth (Becton Dickinson and Company, Sparks, MD, USA), 0.1% sucrose; pH 7.2] containing 20% glycerol. *E. coli* strains were routinely grown in Luria broth at 37°C . For routine analyses of *R. solanacearum*, strains were grown at 28°C with shaking in liquid $0.1\times$ 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 , 1 L; pH 7.2) and M63 [Amresco, Solon, USA; containing $(\text{NH}_4)_2\text{SO}_4$, 2 g; KH_2PO_4 , 13.6 g; $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$, 0.5 mg; Milli-Q water, 1 L; pH 7.0] media. These media were autoclaved for 15 min at 121°C , after which filter-sterilised dissolved 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).

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 [21, 32]. The *R. solanacearum* strains were streaked onto AT medium (M63 agar with 15 mM AT, 5 $\mu\text{g}/\text{mL}$ thiamine and 100 $\mu\text{g}/\text{mL}$ methionine) or SMG medium (M63 agar with 100 $\mu\text{g}/\text{mL}$ each of serine, methionine and glycine) plates and incubated overnight at 28 and 37°C . As controls, *E. coli* strain BW25113 and its Δ relA 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-methylumbelliferone [30]. 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 of CMC (Sigma Aldrich, USA). When sizeable colonies appeared, the plates were stained with 0.1% Congo red solution [29] for 20 min and rinsed with 10 mL of 1 M NaCl for 10 min. The appearance of a halo around the inoculation spot indicated endoglucanase activity.

Survival in Sterile Demineralised Water

The effects of two temperatures, 4 and 20°C , on the survival over time of *R. solanacearum* bv2 strains 1609, 715, KZR-1 and KZR-5 were assessed in sterile water microcosms. Exponentially grown and washed cells (twice with Milli-Q purified sterile water) were transferred to triplicate 100-mL bottles containing 20 mL of sterile ultrapure Milli-Q water, establishing final population densities of between log 6.6 and log 7.1 cells per millilitre. Flasks were incubated without shaking, in the dark, at the appropriate temperatures, i.e. 4 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 $0.1\times$ 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 millilitre (CFU/mL) calculated.

Determination of Growth Rates

The growth rates of strains 1609, 715 and KZR-5 were measured in cultures growing in $0.1\times$ TSBS, BG and M63 media. For growth in $0.1\times$ 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 $0.1\times$ 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× TSBS at 16°C, the number of 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 1609, 715 and KZR-5 might remain undetected when growing in single cultures, we tested the competitive abilities of strain KZR-5 against 1609 and 715 in 0.1× TSBS at 16 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× TSBS until the OD₆₆₀ values reached 0.7–1.1. They were then washed twice with sterile Milli-Q water. To obtain mixed cultures containing totals of 1.0×10^6 CFU/mL, appropriate volumes of washed cells were transferred to flasks containing 20 mL of 0.1× TSBS (start OD₆₆₀, ~0.05). These cultures were dilution-plated onto 0.1× 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 1609, 715 and KZR-5/KZR-1 differed morphologically, and the colony morphologies were stable, on 0.1× TSBS 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. In addition, the colonies of strains KZR-5 and KZR-1 were ~1.5-fold larger than those of the other strains, as they visibly produced more extracellular polysaccharide. To support/confirm the colony-morphology-based screenings for strains KZR-5 and 1609/715, a colony PCR screen (system ps-6 [25]) was used, which amplifies the PGI-1 genomic region that is present in strains 1609 and 715 but absent from strain KZR-5. Thus, the small reddish colonies typical for strains 1609 and 715 (as well as the KZR-1 colonies) 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 sterilised toothpick and transferred to 100 µL Milli-Q water, after which it was strongly mixed. One microlitre of this suspension, 10-fold 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, CA, USA). Standard PCR reaction mixtures contained 1× PCR buffer (1.5 mM MgCl, 10 mM Tris and 50 mM KCl, Roche Applied Science, Basel, Switzerland), 2.5 mM MgCl₂, 10% dimethyl sulfoxide, 200 µM each deoxynucleotide, 0.2 µM of each primer and 20 U/mL Taq DNA polymerase (Roche Applied Science, Basel, Switzerland). The cycling program was as follows: a denaturation step at 96°C for 7 min, followed by 34 cycles of: 96°C, 40 s; 60°C, 45 s; 72°C, 40 s, finalised by an extension step of 72°C for 5 min. PCR products were analysed by electrophoresis on 0.8% agarose gels (Roche Diagnostics, Mannheim, Germany).

To determine the competitive fitness of the strains at 28°C, the mixed cultures were grown overnight or until reaching an OD₆₆₀ of 0.9 (after approximately seven generations) and dilution-plated at regular times onto BGT plates. To assess competition at 16°C, serial batch cultures were used. The first cultures were established by transferring 10 µl of each grown culture (OD₆₆₀ between 0.5 and 0.8, after approximately 2 days) to flasks containing 20 mL fresh 0.1× 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 the competing strains as above.

Tomato Invasion Assays

To assess the behaviour of environmental strains KZR-5 and KZR-1 and of potato strains 1609 and 715 when in association with tomato plants, at least 12 3–4-week-old tomato plants per strain/treatment in soil in pots were inoculated as described [26].

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 gram soil was 5×10^6 or 5×10^3), after which plants were kept under a day/night regime of 26°C (day, 14 h)/21°C (night, 10 h). Control plants received sterile Milli-Q 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) [38].

Mixed-Strain Inoculation

Mixed suspensions of strain KZR-5 with the comparator strains 1609, 715 and KZR-1 (all in 1:1 ratios) 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 10^7 CFU/mL.

To enumerate the *R. solanacearum* cells from the soil and tomato plants, we used cell extraction/dilution plating procedures, using modified semi-selective SMSA plates [10]. Thus, 2 days after inoculation, 0.5 g soil samples were taken (in triplicate), shaken in 0.01 M 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 0.01 M phosphate buffer for up to 2 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 ratios 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 [25] consistently on 24–48 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 [23]), 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 (μ_{\max}) 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's *t* tests were judged to be significant at $P < 0.05$.

Results and Discussion

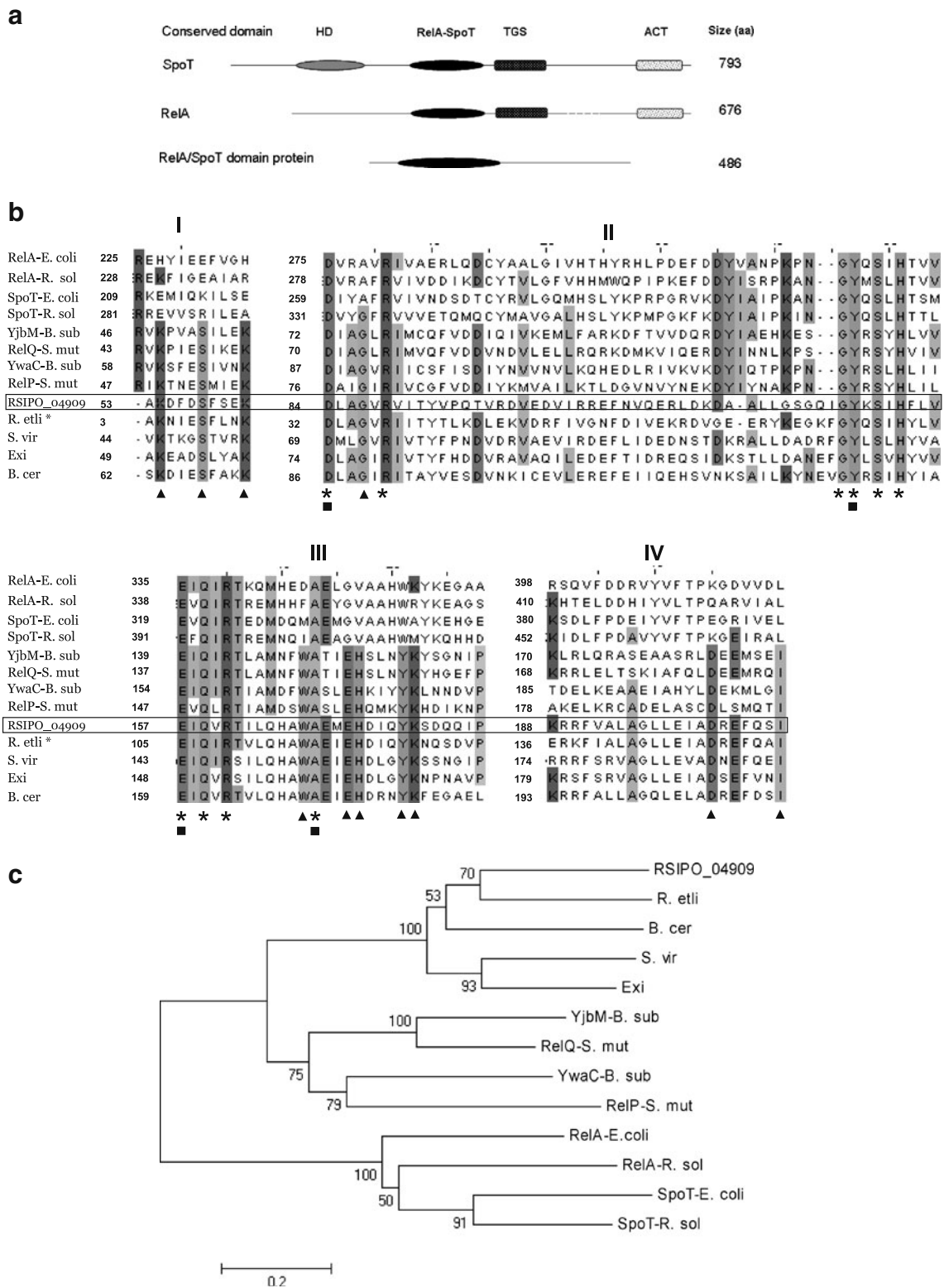
Determination of the Phenotype of the Δ PGI-1 Mutant Strain KZR-5

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 *relA* and *spoT* gene, we examined the *R. solanacearum* bv2 strain 1609 genome for it. This analysis revealed that one copy of each gene was present in strain 1609. These genes probably encode 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) [5]. 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).

BLAST-P 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 BLAST-P and those (SAS proteins) of *B. subtilis* (YjbM and YwaC) and *S. mutans* (RelP and RelQ) showed clear conservation of several amino acids at fixed positions (Fig. 1b). Specifically, 10 residues were fully conserved between the aligned sequences (Fig. 1b). Of these, four had previously been shown to be functionally significant in *Streptococcus dysgalactiae* [12]. 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. coli* or 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 hypothesised 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 [17].

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

Figure 1 Comparison between SpoT, RelA and RelA/SpoT domain containing proteins. **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 [6]; 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 using ClustalW. The position of the first amino acid of the four partial protein sequences (I–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* [12]. *E. coli*, *Escherichia coli*; *R. sol*, *Ralstonia solanacearum* biovar 2; *B. sub*, *Bacillus subtilis*; *S. mut*, *Streptococcus mutans*; *R. etli*, *Rhizobium etli*; *S. vir*, *Streptomyces viridochromogenes*; *Exi*, *Exiguobacterium*; *B. cer*, *Bacillus cereus*. *Asterisk* For *R. etli*, a partial protein sequence was used due to unavailability of the full-length sequence. **c** Phylogenetic analysis 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 Mega4 software [28], option neighbor joining, bootstrap 1,000 replicates and Poisson correction)

domain protein of *R. solanacearum* localises on genomic island PGI-1 [25] 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* bv2 and its putative involvement in the physiology of this organism, e.g. the stringent response, has so far not been described.

The PGI-1-Borne Putative RelA/SpoT Domain Protein May Confer a Phenotype on Strain KZR-5

SMG and AT tests Comparison of the growth of *E. coli* BW25113 wild type, *E. coli* BW25113 Δ relA and *R. solanacearum* strains 1609, 715, KZR-1 and KZR-5 on SMG-containing plates revealed a clear growth defect for environmental strain KZR-5 when compared to strains 1609, 715 and KZR-1 (Table 2). The *E. coli* BW25113 wild type and Δ relA strains, which were used as controls, behaved as expected on SMG and AT, i.e. they showed fair growth of the wild-type strain and delayed growth of the Δ relA 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* Δ relA when grown on media containing excess 1-C metabolites, whereas the other *R. solanacearum* strains did not.

Exo- and endoglucanase activity To assess whether strain KZR-5 is impaired in cellulose degradation, we tested its

ability to utilise CMC and MUC. Overall, these tests, compared to the other strains, revealed that strain KZR-5 exhibited “normal” endoglucanase activity. Specifically, no differences were observed between the strains in the utilisation of CMC, as judged by the sizes of the haloes of the corresponding colonies, following the procedure of Teather et al. [29] (Table 2). Unfortunately, we were not able to measure exoglucanase activity for any of the *R. solanacearum* strains, using MUC as a substrate, in a reproducible manner. Although the CbhA protein is secreted by *R. solanacearum* bv3 strain GMI1000 [14], the substrate it hydrolyses 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, personal communication).

Growth Rates of Strains 1609, 715 and KZR-5 in Different Media

The maximum exponential growth rates (μ_{\max} , h^{-1}) of strains 1609, 715 and KZR-5 were assessed in different liquid growth media (Fig. 3). Overall, the growth rates of the three strains were lower when nutrient concentrations and/or temperature were lower (Fig. 2). In $0.1\times$ TSBS at two temperatures, the three strains had similar growth rates, i.e. between 0.24 ± 0.014 and 0.27 ± 0.012 (μ , h^{-1}) at $28^{\circ}C$ and between 0.11 ± 0.05 and 0.12 ± 0.03 (μ , h^{-1}) at $16^{\circ}C$. The growth rates in M63 medium at $28^{\circ}C$ were also similar between the three strains, i.e. between 0.043 ± 0.001 and 0.048 ± 0.001 (μ , h^{-1}). In BG medium at $28^{\circ}C$, however, we found a significant difference in growth rate between strain KZR-5 and the comparator strains. The maximum growth rates of strains 1609 and 715 were similar (between 0.63 ± 0.07 and 0.69 ± 0.07 (μ , h^{-1})) while that of strain KZR-5 was significantly ($P<0.05$) lowered (0.39 ± 0.03 , μ , h^{-1} ; Fig. 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.

Competitiveness of *R. solanacearum* Strain KZR-5 Versus the Comparator Strains upon Growth in $0.1\times$ TSBS at 16 and $28^{\circ}C$

As the growth rate of environmental strain KZR-5 in $0.1\times$ TSBS was similar to that of the potato strains 1609 and 715 (Fig. 2), we surmised that there might be subtle differences in their behaviour that can be evidenced in direct competition experiments. We thus used competition experiments in $0.1\times$ TSBS at 28 as well as $16^{\circ}C$ (Fig. 3). At both temperatures, the mixed cultures grew—as expected—in

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

strain	SMG	AT	CMC	Decline rate, 20°C	Decline rate, 4°C
<i>E. coli</i> BW 25113 wild type	+	+	nd	nd	nd
<i>E. coli</i> BW 25113 Δ relA	-	-	nd	nd	nd
<i>R. solanacearum</i> 1609	+	-	+	0.0029 ^a	0.051 ^b
<i>R. solanacearum</i> 715	+	-	+	0.0026 ^a	0.055 ^b
<i>R. solanacearum</i> KZR-1	+	-	+	0.0020 ^a	0.064 ^b
<i>R. solanacearum</i> KZR-5	-	-	+	0.0020 ^a	0.034 ^c

Statistical classes (superscripted a, b and c) are indicated. $P < 0.05$

nd not determined, (+) growth on agar plates, (-) no growth on agar plates

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 ($P < 0.05$).

Survival of *R. solanacearum* Strain KZR-5 Versus the Comparator Strains in Sterile Water at 20 and 4°C

The survival, as CFUs, of strains 1609, 715, KZR-1 and KZR-5 in sterile Milli-Q water in still vials at 20 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 until 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

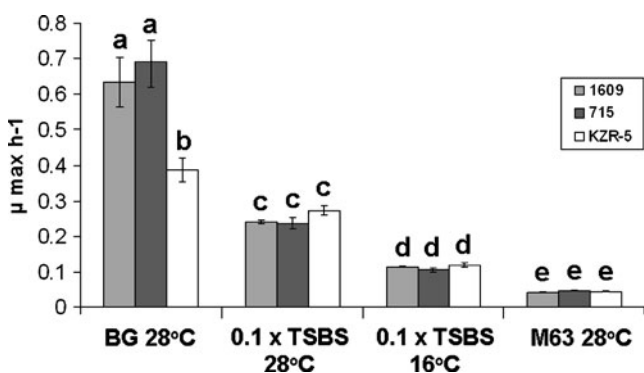


Figure 2 Maximum growth rate (μ_{\max} ; h^{-1}) of strains KZR-5, 1609 and 715 at 28°C in BG, 0.1× TSBS and M63 and at 16°C in 0.1× TSBS. Maximum growth rate was determined for exponentially growing cells. Values are the average of at least two independent experiments. Statistical classes (a, b, c,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

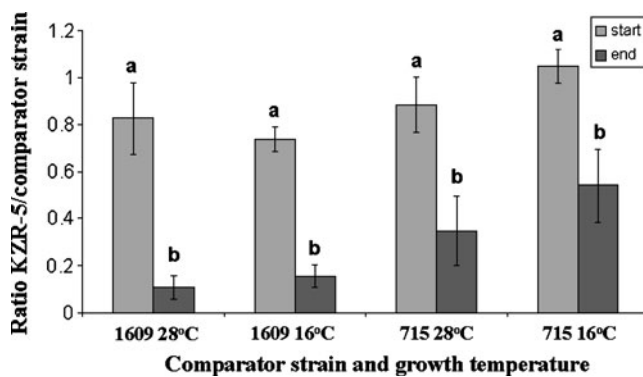


Figure 3 Competitiveness of *R. solanacearum* strain KZR-5 against strains 1609 and 715 in liquid 0.1× TSBS. The ratio between Δ PGI-1 strain (KZR-5) and the comparator strains (1609 or 715) is shown at the beginning of the experiment (1:1) and after growth in mixed cultures. For competition at 28°C, 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; $P < 0.05$

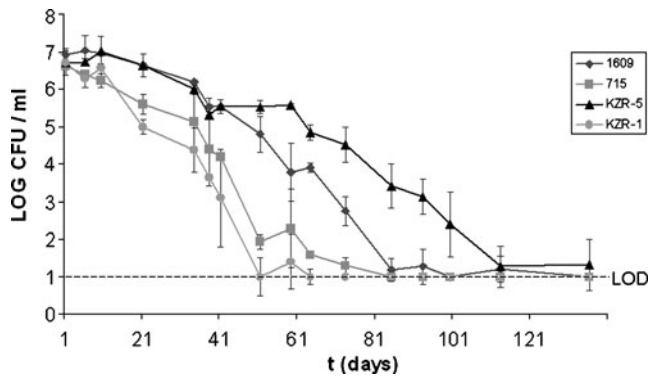


Figure 4 Survival of *R. solanacearum* strains 1609, 715, KZR-5 and KZR-1 at 4°C. The graph shows the colony forming unit enumerations on BG plates. Points represent the means of three replicate experiments. The limit of detection (LOD), 10 CFU/mL, is indicated

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 was 0.05.

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 *Vibrio cholerae* [7, 24]. This, thus, might provide support for a possible link between the behavioural 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 Δ PGI-1 strain KZR-5.

Competitiveness of *R. solanacearum* Strain KZR-5 upon Invasion of Tomato

To assess the virulence of environmental strain KZR-5 in comparison with those of 1609, 715 and KZR-1, we determined the effects of high (10^8 CFU/mL) as well as low (10^5 CFU/mL) inocula over time. First, 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% and 72% of the 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 hypothesised that any differences in the rates of invasion and wilting might become apparent 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 environmental strain KZR-1. The results of these experiments are shown in Fig. 5. At day 10, plants treated with all strain mixes showed the development of disease symptoms (as from wilting stage 1), being the KZR-5/KZR-1 mixes most virulent (wilting stages 1 through 3). At day 21, disease development had progressed to wilting stages 3–4 for all mixes used, being the KZR-5/1609 mix less virulent than 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.

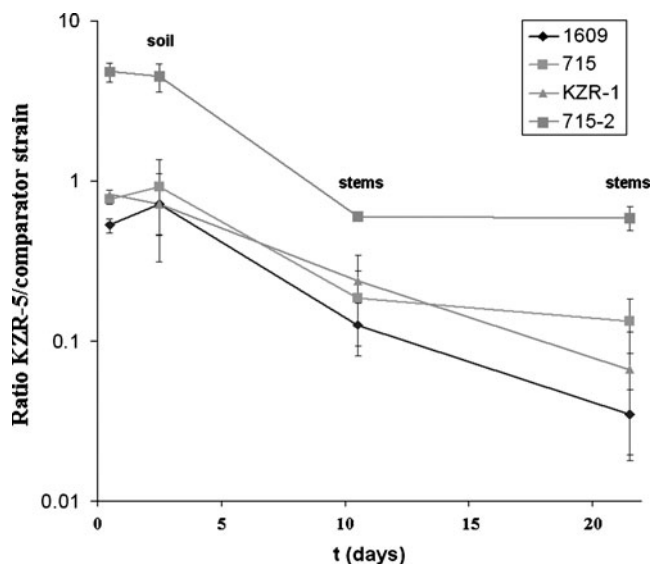


Figure 5 Competitiveness of *R. solanacearum* strain KZR-5 against strains 1609, 715 and KZR-1 upon invasion of tomato plants. The ratio between Δ PGI-1 strain KZR-5 and the comparator strains (1609, 715 and KZR-1) was determined by dilution plating on agar plates (BG or SMSA) at four time points. First, 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 (soil). 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 (stems)

Analysis of the mixed *R. solanacearum* populations in the soil at day 2 revealed that strains KZR-5, 1609, 715 and KZR-1 survived fairly well in the soil compartments, i.e. between log 5.0 and log 6.0 CFU were measured per gram soil for these 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 of strain KZR-5 versus competing strains 1609, 715 and KZR-1 at 10 and 21 days after inoculation of the tomato plants consistently showed progressive declines 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 an overall reduced rate of wilting, which is remarkable.

To strengthen the notion that environmental strain KZR-5 was less competitive than its competitors in these plant systems, we 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 the mixes was quite stable in the soil, whereas it decreased progressively upon colonisation 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 roughly stable in the soil, whereas they decreased significantly during the process of invasion and colonisation 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 colonise 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 colonisation [22]. 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 hydrolyses was never demonstrated), it was found to contribute to virulence on susceptible plants [14]. Possibly, such cell-wall-degrading enzymes play roles in the initial stages of plant colonisation as well as later, when cells have to penetrate the xylem vessels. For instance, EglI is required for efficient invasion of plant roots and colonisation of stems, where it might function in the degradation of cellulosic glucan compounds

[9, 20]. 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 colonisation. 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 [25]. Furthermore, additional processes at the transcriptional or translational level might differ between the strains (regardless of PGI-1 being present or not).

Temperature regime might also affect both the growth and virulence of strains [15, 39]. Milling et al. [13] showed that the virulence of *R. solanacearum* bv2 strain UW551 was similar to that of GMI1000 (bv3) and K60 (bv1) at 28°C, while at 20°C, strain UW551 revealed higher virulence [15]. In addition, RelA mutations can lead to decreased thermotolerance, as observed for *E. coli* [39]. Our competition experiments in tomato, in which strain KZR-5 revealed to be less competitive than strains 1609, 715 and KZR-1, were performed at 26/21°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. [15] 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 enhanced survival at low temperature when compared to potato strains 1609 and 715. In contrast, its colonisation 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 (Δ relA). 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* Δ relA. The overall (growth/survival) behaviour 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.

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