Interactions with Hosts at Cool Temperatures, Not Cold Tolerance, Explain the Unique Epidemiology of *Ralstonia solanacearum* Race 3 Biovar 2

Annett Milling, Fanhong Meng, Timothy P. Denny, and Caitilyn Allen

First, second, and fourth authors: Department of Plant Pathology, University of Wisconsin-Madison, Madison 53706; and third author: Department of Plant Pathology, University of Georgia, Athens 30603.
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ABSTRACT

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Most strains of the bacterial wilt pathogen *Ralstonia solanacearum* are tropical, but race 3 biovar 2 (R3bv2) strains can attack plants in temperate zones and tropical highlands. The basis of this distinctive ecological trait is not understood. We compared the survival of tropical, R3bv2, and warm-temperate North American strains of *R. solanacearum* under different conditions. In water at 4°C, North American strains remained culturable the longest (up to 90 days), whereas tropical strains remained culturable for the shortest time (≈40 days). However, live/dead staining indicated that cells of representative strains remained viable for >160 days. In contrast, inside potato tubers, R3bv2 strain UW551 survived >4 months at 4°C, whereas North American strain K60 and tropical strain GMI1000 were undetectable after <70 days in tubers. GMI1000 and UW551 grew similarly in minimal medium at 20 and 28°C and, although both strains wilted tomato plants rapidly at 28°C, UW551 was much more virulent at 20°C, killing all inoculated plants under conditions where GMI100 killed just over half. Thus, differences among the strains in the absence of a plant host were not predictive of their behavior in planta at cooler temperatures. These data indicate that interaction with plants is required for expression of the temperate epidemiological trait of R3bv2.

The ability to persist in an environment is critical for pathogen success. Determining an exotic phytopathogen's potential to survive the conditions in a new region is one important step in predicting whether it could become established there and pose a long-term threat to crops. In addition, understanding the biological mechanisms underlying pathogen persistence in an environment can suggest effective methods of exclusion and control.

Brown rot of potato, also known as bacterial wilt, is caused by a subgroup of *Ralstonia solanacearum* known as race 3 biovar 2 (R3bv2) (1,23). Brown rot is among the most serious diseases of potato worldwide, causing an estimated $950 million in losses each year (12). The disease is a major constraint to production in the tropical highlands of South America, Africa, and Asia but it is also present in Europe, the Middle East, and Australia (12,35). In addition to potato, R3bv2 also infects tomato, eggplant, geranium, and many weeds and wild plants (13,26,34,49,55). The bacterium has been accidentally introduced to North America and Europe on infected geranium cuttings imported from the highland tropics where R3bv2 is endemic (26,30,42,44,56) but it does not appear to have become established in North America.

R. solanacearum R3bv2 is transmitted via infected plant material, soil, or surface water. The bacterium invades plants through wounds and colonizes the host xylem tissue, causing wilting, tuber rot, and plant death. R3bv2 often forms symptomless, or latent, infections that facilitate unknowing pathogen spread in seed potato tubers or plant cuttings (1,45). R3bv2 probably originated in the Andes with potato, and isolates from around the world are nearly genetically and phenotypically identical, suggesting that it was distributed from South America in potato tubers (14,24,41,50). Like other strains from the Americas, R3bv2 belongs to the phylotype II subspecies division of the *R. solanacearum* species complex, and is further subclassified in sequevar 1 (14).

Most strains of *R. solanacearum* are tropical or subtropical, and many of these can wilt potato. However, field experiments and observations have documented the ability of R3bv2 to wilt potato plants at cooler temperatures than tropical or subtropical *R. solanacearum* strains (6,7,47,48). R3bv2 has also frequently been described as cold tolerant (15,23,43). However, it is not known if these differences reflect a greater ability of R3bv2 to survive in the environment at cold temperatures or, alternatively, more effective infection and wilting of plants under cool conditions. Epidemiological studies are contradictory regarding the ability of R3bv2 to survive cool conditions away from plants. In Australia, R3bv2 survived >2 years in bare fallow soils with an average winter temperature of 4°C (40). In contrast, although R3bv2 cells remained viable up to a year in field soils in the Netherlands at 12°C, at 4°C, the pathogen became undetectable after only a month (52). Similarly, R3bv2 cells could not be detected after a month in Dutch irrigation water at 4°C (51). Nevertheless, it has proven difficult to eradicate R3bv2 from northern Europe, where the pathogen may survive and multiply in alternative hosts, especially the semiaquatic weed bittersweet nightshade, *Solanum dulcamara* (11).

R3bv2 is a quarantine pathogen in Europe and North America, and is listed as a Select Agent in the United States (31). Although non-R3bv2 strains of *R. solanacearum* that can infect and wilt potato are endemic and widespread in the southeastern United States, these native strains have never become established north of the mid-Atlantic states. Regulators consider that R3bv2 poses a potentially severe threat to the American potato industry because

Corresponding author: C. Allen; E-mail address: cza@plantpath.wisc.edu

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it may be able to overwinter and become established in soils and waterways of the primary potato-growing regions in the northern states.

Therefore, we designed experiments to determine whether R3bv2 strains have a unique ability to persist in the environment at cold temperatures relative to native North American and tropical *R. solanacearum* strains. We further sought to quantify the relative virulence and survival of tropical and R3bv2 strains in host plants at cool temperatures.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *R. solanacearum* strains used in these experiments are listed and described in Table 1. Strains not previously subclassified were subclassified into phylotype and sequevar using the multiplex-polymerase chain reaction (PCR) phylotyping method followed by amplification and sequencing of the 750-bp internal fragment of the *egl* locus as described (14). All *R. solanacearum* strains were streaked onto casamino acids-peptone-glucose (CPG) solid medium (25) from –80°C glycerol stocks or room-temperature water stocks and grown at 28°C for 48 h. If required to select a rifampin-resistant mutant strain, rifampicin was added at a final concentration of 25 mg/liter. Growth medium components were purchased from Difco Laboratories (Detroit). All other chemicals and antibiotics were purchased from Sigma-Aldrich (St. Louis).

Culturability and viability of bacterial cells in cold water. The effect of low temperatures on the survival of diverse *R. solanacearum* strains was determined in water microcosms maintained at 4°C. Bacterial strains were streaked onto CPG agar for 48 h to obtain single colonies which were transferred into CPG broth and grown at 28°C with shaking at 225 rpm for 14 to 18 h to exponential growth phase (optical density at 600 nm [OD_{600}] = 0.1). Cells were pelleted by centrifugation at 2,500 × *g* for 5 min and washed twice with room-temperature ultrapure water (>18.2 mOhm). Water microcosms were prepared by transferring exponentially grown and washed cells into duplicate glass tubes (18 by 150 mm) filled with 10 ml of ultrapure water, establishing final cell densities of 1 to 2 × 10^7 CFU/ml. After an initial incubation at room temperature for 48 h, tubes were stored at 4°C without shaking. Samples were taken weekly from each tube and enumerated by serial dilution plating on CPG agar plates. Colonies on plates were counted after 36 to 48 h of incubation. To determine the proportion of cells that grew only in the presence of catalase, strains K60, GMI1000, and UW551 were dilution plated on CPG plates either amended or not amended by spreading 100 units of catalase (MP Biomedicals, Solon, OH) per milliliter onto the agar surface shortly before use. The detection limit was 1 log CFU/ml and a value of 1 log CFU/ml was also used to report samples below the detection limit. Each experiment contained two independent water microcosms (replicates) per bacterial strain. These microcosms were sampled successively at each time point by dilution plating in triplicate (subsamples). The experiments were repeated three times.

In parallel, viable cells of *R. solanacearum* strains K60, GMI1000, and UW551 from the same water microcosms were enumerated with a live/dead bacterial viability kit (BacLight; Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The SYTO 9 stain labels all bacteria whether the cell membrane is intact or damaged, resulting in green fluorescent cells. In contrast, propidium iodide (PI) penetrates only bacteria with compromised membranes, reducing SYTO 9 stain fluorescence when both dyes are present and resulting in red fluorescent cells. Thus, green-fluorescing cells are considered viable and red-fluorescing cells are considered dead. Cells were collected on black polycarbonate membrane filters (0.22 μm; GE Water & Process Technologies, Trevose, PA) and enumerated by epifluorescence microscopy at a magnification of ×1,000. At least 10 random fields containing, in total, 500 to 1,000 cells per sample, were scored. Total numbers of culturable and viable cells were determined in triplicate experiments.

After 154 days at 4°C, water microcosms containing viable but nonculturable (VBNC) cells of K60, GMI1000, and UW551 (50 ml) were used to inoculate unwounded 21-day-old susceptible tomato plants of cv. Bonny Best via a soil-soak inoculation (46). Briefly, 14-day-old tomato plants were transplanted into 10-cm-diameter pots with 80 g of soil. Plants were inoculated by pouring a bacterial suspension onto the soil. Plants were monitored daily for disease progress by a rater who did not know which treatment each plant had been given, and symptoms were scored on a 0 to 4 disease index where 0 indicates no disease, 1 indicates 1 to 25% of leaves wilted, 2 indicates 25 to 50% of leaves wilted, 3 indicates 51 to 75% of leaves wilted, and 4 indicates 76 to 100% of leaves wilted. Twelve tomato plants were inoculated with water from individual microcosms for each strain. Plants were kept at 28°C and monitored daily for disease symptoms. Sixteen days after inoculation, tomato stem tissue samples were ground in sterile ultrapure water and tested with *R. solanacearum*-specific immunostrips (Agdia Inc., Elkhart, IN) for the presence of the pathogen in planta and were also plated on CPG agar. Plates were checked for the presence of colonies after 48 h of incubation at 28°C.

Survival in potato tubers. We measured the survival of *R. solanacearum* strains K60, GMI1000, and UW551 in susceptible potato minituber (Sklarczyk Seed Farm, Johannesburg, MI) cvs.

TABLE 1. *Ralstonia solanacearum* strains used in this research

Group[a]	Strain	Race	Biovar	Phylotype or sequevar[b]	Isolated from	Location	Source
A	K60[c]	1	1	II/7	Tomato	North Carolina, United States	29
A	AW1	1	1	II/7	Tomato	Alabama, United States	9
A	UW27	1	1	II/7	Tobacco	Florida, United States	C. Allen
A	UW576 (= Rs5)	1	1	II/7	Tomato	Florida, United States	27
B	UW551[c]	3	2	II/1	Geranium	Wisconsin, United States	46
B	IPO1609	3	2	II/1	Potato	The Netherlands	53
B	UW260	3	2	II/1	Potato	Cajamarca Chota, Peru	C. Allen
B	UW276	3	2	II/1	Potato	Mexico	C. Allen
B	UW492	3	2	II/1	Potato	San Ramon, Peru	C. Allen
C	UW582 (= Rs120)	1	1	II/5	Hydrangea	Florida, United States	27
C	GMI1000[c]	1	3	I/18	Tomato	French Guyana	4
C	UW585 (= Rs123)	1	3	I/13	Pepper	Florida, United States	27
C	UW568	1	3	I/14	Soil, tomato field	Guatemala	38

[a] Groups A, B, and C were distinguished based on relative survival in 4°C water.
[b] Phylotype was determined by multiplex polymerase chain reaction and sequevar was determined based on DNA sequence of a 750-bp fragment of the *egl* gene as described by Fegan and Prior (14).
[c] Spontaneous rifampicin-resistant variants K60-rif, UW551-rif, and GMI1000-rif were used in potato tuber survival studies.

Russet Norkotah (15 to 17 mm in diameter) and Shepody (18 to 24 mm in diameter) at 4°C. All three strains tested were capable of wilting potato plants (data not shown). Experiments were performed with rifampicin-resistant *R. solanacearum* strains to facilitate pathogen detection in the natural microbial background. Spontaneous rifampicin-resistant K60 (K60-rif) grows in culture and in planta and causes a wilt of tomato plants indistinguishable from that caused by the wild-type parent strain (45). Natural transformation (28) was used to transfer the rifampicin resistance from K60-rif into strains UW551 and GMI1000; briefly, washed cells of each recipient strain were incubated with total DNA from K60-rif for 24 h and transformants were selected on CPG plates containing rifampicin. The wild-type virulence of the resulting rifampicin-resistant strains was confirmed.

Cultures grown to exponential phase and washed as described above were directly injected into potato tubers. A needle was inserted ≈0.5 cm deep into the tuber and 2 μl of a 1×10^9 CFU/ml suspension were injected into each tuber with a long 10-μl pipette tip, generating final cell densities of 1 to 2×10^6 CFU/g tuber. Two inoculations per tuber were made and inoculation sites were marked. Tubers were stored at 4°C in the dark and sampled destructively at intervals with a no. 5 cork borer by removing a cylinder of tissue (0.5 to 1 cm long, ≈0.3 g) containing the inoculation site. Tissue was ground in sterile deionized water (1:10 dilution). Serial dilutions were plated onto CPG agar plates containing rifampin at 25 mg/liter and cycloheximide at 100 mg/liter and colonies were counted after 48 h of incubation at 28°C. Survival rates were calculated based on duplicate experiments for each potato cultivar using two to three individual tubers per sampling time point. The detection limit was 1 log CFU/g potato tissue and a value of 1 log CFU/g was also used to report samples below the detection limit.

To resuscitate and allow bacteria to multiply after culturable cells could no longer be detected on CPG agar plates, 0.5 g of potato tuber tissue was transferred to 4.5 ml of CPG broth supplemented with filter-sterilized solutions of polymyxin B sulfate at 100 mg/ml, bacitracin at 25 mg/ml, penicillin-G at 0.5 mg/ml, chloramphenicol at 5 mg/ml, 2,3,5,-triphenyl tetrazolium chloride, cycloheximide at 100 mg/ml, and rifampicin at 25 mg/ml and incubated with shaking at 28°C for 72 h. This enrichment broth was then tested with *R. solanacearum*-specific immunostrips for the presence of the pathogen. In addition, broth was plated on CPG agar plates amended with rifampicin and with or without catalase. Enrichment broth (10 μl) was also directly injected into the stem tissue of 21-day-old Bonny Best tomato plants between the cotyledons and the first true leaf. Plants were kept at 28°C and monitored daily for disease symptoms. Two weeks after inoculation, stem tissue samples from near the inoculation site were ground in sterile ultrapure water, serially diluted, and plated on CPG agar containing rifampicin. Plates were checked for the presence of colonies after 48 h of incubation at 28°C. Tissue from three tubers per treatment (strain) was cultured in enrichment broth after 128 and 197 days at 4°C; three tomato plants were stem-inoculated with broth from each enrichment tube. Dilution platings were conducted in triplicate for each subsample.

Growth curves. Growth rates of *R. solanacearum* strains UW551 and GMI1000 were compared in Boucher's minimal medium (BMM) (4) at 20°C, a typical temperature in cool temperate or highland tropical potato fields, and at 28°C, which is more typical of tropical agriculture. For each experiment, a single colony grown from a frozen stock was inoculated into CPG broth and grown at 28°C overnight with shaking (225 rpm). This overnight culture was pelleted and washed twice with sterile deionized water, then used to inoculate a 125-ml flask containing 30 ml of prechilled (20°C) or prewarmed (28°C) BMM plus 0.2% glucose to a starting OD_{600} of 0.02 for 28°C and 0.08 for 20°C. CFU counts were determined by dilution plating on CPG agar plates every 12 h for cells grown at 28°C and every 24 h for cells grown at 20°C. Each experiment contained three flasks per strain at each temperature, and experiments were repeated at least three times.

Virulence assays. To compare the virulence of *R. solanacearum* strains GMI1000 and UW551 at different temperatures, we used a naturalistic soil soak inoculation as described above (46), except that half the plants were moved from 28 to 20°C growth chambers 1 day after transplanting, and plants were inoculated with either UW551 or GMI1000 by pouring a bacterial suspension onto the soil to a final density of $\approx 1 \times 10^8$ CFU/g of soil. Each experiment contained a minimum of 20 plants per strain at each temperature, and experiments were repeated at least three times.

Statistical analysis. Data were tested for statistical significance of differences using the JMP 7.0.2 software package (SAS Institute, Cary, NC). For survival experiments in water microcosms, potato tubers, and the growth of the pathogen in liquid medium, log-transformed data of culturable *R. solanacearum* cells were analyzed by using mean values to perform an analysis of variance (ANOVA). For relative virulence of strains on tomato plants, mean disease indices from three independent replicates were analyzed. Because of the serial nature of sampling, we applied repeated measures ANOVA to analyze the results of experiments on survival in water, growth in culture, and disease progress. Factors considered for the analysis included bacterial strain, temperature, replicate, potato cultivar, and time of incubation. We assessed the significance of differences among strains and groups using *F* statistics.

RESULTS

Culturability and viability of bacterial cells in water microcosms. Based on relative cold tolerance, we distinguished three groups of strains (Fig. 1). There were significant differences ($P = 0.0054$) in survival at 4°C between all three groups. The clustering of groups A, B, and C was repeatable and independent of experimental conditions; we observed the same groupings in

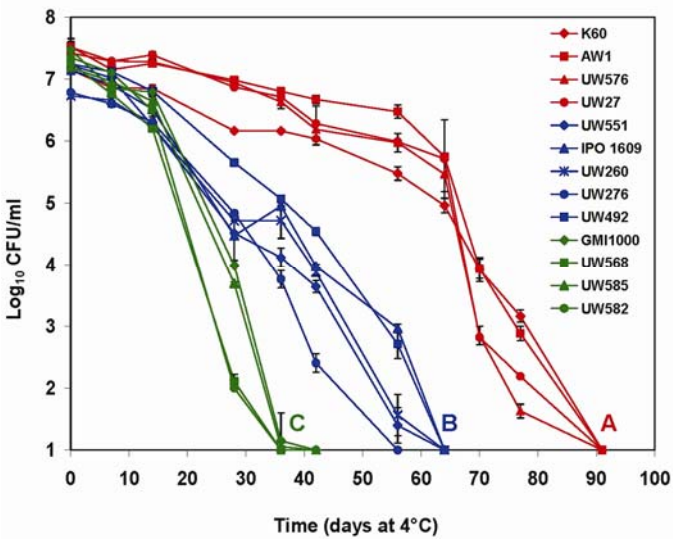

Fig. 1. Survival of diverse *Ralstonia solanacearum* strains in water at 4°C. Cells were starved for 2 days at room temperature before incubation at 4°C. Water microcosms were initially inoculated with 1×10^7 CFU/ml. Culturable cells were enumerated at regular intervals by dilution plating on casamino acids-peptone-glucose agar plates. Strains clustered into groups A, B, and C based on relative survival. This experiment was repeated three times; typical results are shown. Each experiment contained two replicates, each containing three subsamples. Points represent means of all subsamples; bars indicate standard error.

experiments using both log phase and stationary phase cells (data not shown). Group A, which consistently survived longest in water at 4°C compared with the other strains tested, included phylotype II, sequevar 7 (race 1, biovar 1 strains that originated in the southeastern United States). The viable cell counts of group A strains on unamended CPG plates declined to undetectable levels within 91 days. Group B, containing R3bv2 strains isolated from around the world, displayed an intermediate in vitro cold survival phenotype. Their culturable CFU counts dropped below the detection limit within 56 to 70 days of storage at 4°C. However, the group B R3bv2 strains survived cold water better than group C strains, which belong to phylogenetic groups that originated in tropical Asia and the Caribbean (Table 1). Group C strains were most sensitive to 4°C in water and their CFU counts dropped rapidly. No culturable group C bacteria could be recovered after 36 to 42 days. Subsequent experiments focused on one representative strain from each group: the *R. solanacearum* type strain K60 from group A; R3bv2 strain UW551 from group B, because it is well characterized and has been sequenced (16); and GMI1000 from group C, because its genome has also been sequenced (37).

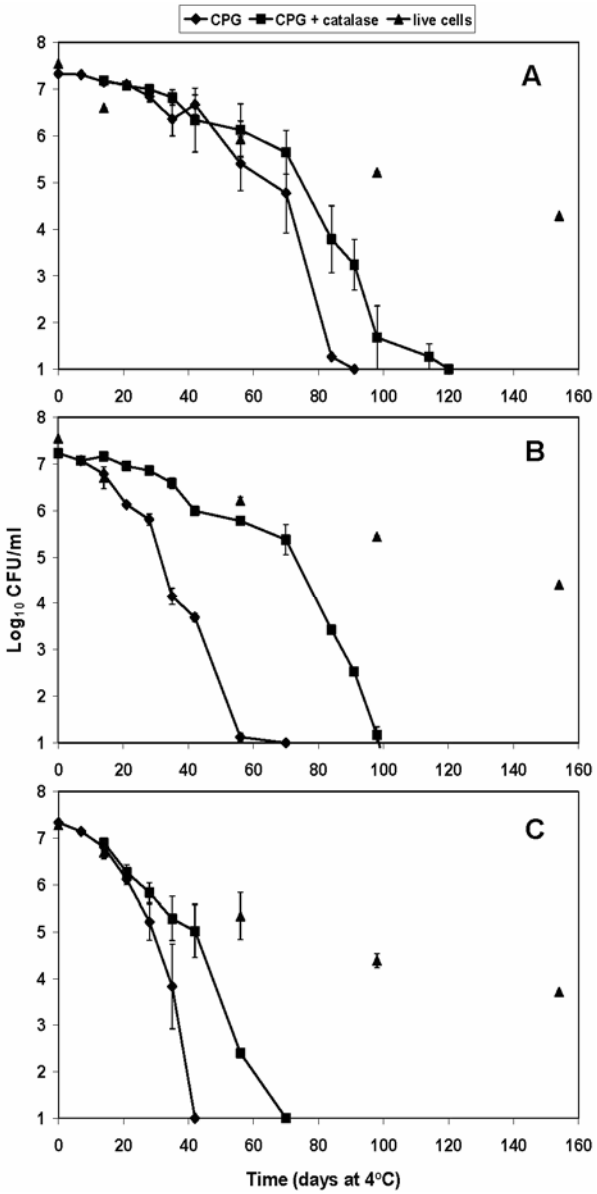

Fig. 2. *Ralstonia solanacearum* cells become viable but nonculturable over time in water at 4°C. Water microcosms were inoculated with **A,** endemic North American strain K60; **B,** race 3 biovar 2 strain UW551; or **C,** tropical strain GMI1000 at 1×10^7 CFU/ml. Cells were starved for 2 days at room temperature before incubation at 4°C. Culturable cells were enumerated at regular intervals by dilution plating on casamino acids-peptone-glucose agar plates with and without catalase. The live/dead stain was used to enumerate viable cells via epifluorescence microscopy. This experiment was repeated three times; typical results are shown. Each experiment contained two replicates, each containing three subsamples. Points represent means of all subsamples; bars indicate standard error.

Catalase amendment of plates dramatically increased the culturability of cold-treated *R. solanacearum* cells in all three groups (Fig. 2). At the beginning of each experiment, microcosm cell counts were the same on CPG plates with and without catalase but, as cells were stored at 4°C, colony counts on unsupplemented plates dropped faster than those on catalase-containing plates for all strains ($P < 0.0001$). After cell counts on CPG without catalase dropped below a threshold of log 1 CFU/ml, the CFU counts on catalase-containing plates were 10^3 to 10^5-fold greater, depending on the strain. Culturable K60 cells (group A) formed colonies on catalase-containing plates after up to 120 days at 4°C, although colonies were undetectable on unamended CPG media after 91 days. Similarly, cells of strain UW551 (group B) were culturable in the presence of catalase up to 98 days but for only 70 days without it. CFU counts for cold-sensitive tropical strain GMI1000 (group C) remained detectable on catalase-containing media until 70 days at 4°C storage but dropped below detection limits on CPG without catalase after just 42 days.

As expected, total cell numbers in water microcosms (live plus dead) remained stable (data not shown). Apparently, living cells of all three strains tested were present long after colonies no longer formed on plates. Even after 154 days in water at 4°C, water microcosms still contained between 2×10^4 and 3×10^5 cells with intact membranes per milliliter (Fig. 2). These results suggest that incubation at 4°C in water causes *R. solanacearum* cells from diverse strains to go into a VBNC state. The VBNC cells of K60, UW551, and GMI1000 that were present after 154 days at 4°C could not wilt tomato plants following soil-soak inoculation. Neither GMI1000 nor UW551 could be detected in inoculated tomato plants with immunostrips specific for *R. solanacearum*, which have a detection limit of 10^5 cells. However, the strip test did detect K60 in tomato midstem tissue from 7 of 12 inoculated plants 16 days after the tomato plants were inoculated with water containing VBNC cells, indicating that *R. solanacearum* cells had entered, spread, and multiplied in these plants. Nonetheless, no inoculated plant showed wilt symptoms. Furthermore, no CFU were recovered from any inoculated plants when ground stem tissue was plated on CPG with or without catalase, suggesting that, although the K60 cells could multiply in planta, they were in an irreversible state of nonculturability.

Survival in potato tubers. We also compared survival in potato minitubers of three representative *R. solanacearum* strains. At 4°C, a typical temperature for commercial seed potato storage, culturable cells of R3b2 strain UW551 could be detected by growth on plates significantly longer ($P < 0.0001$) than cells of either tropical strain GMI1000 (group C) or American strain K60 (group A) (Fig. 3). Four months after inoculation, we could still detect UW551 in potato tubers, whereas K60 and GMI1000 dropped to undetectable numbers less than 70 days after tuber inoculation. All three strains survived comparably in cvs. Russett Norkotah and Shepody, suggesting that potato cultivar did not influence survival of *R. solanacearum* at low temperatures.

Up to 6 months after tuber inoculation, cells of all three strains could still be recovered after but not before 72 h of enrichment culture of potato tissue as detected by *R. solanacearum*-specific immunostrips with a detection limit of 10^5 CFU (45). This result suggests that *R. solanacearum* cells had either moved into a reversible VBNC state during storage in potato tubers or had fallen below our detection threshold. After 128 days at 4°C, all three strains multiplied in tomato stems injected with enrichment broth from stored tubers. Strains K60 and GMI1000 reached populations in planta of 1×10^9 CFU/g of stem tissue but, al-

though culturable, these strains appeared to be nonvirulent because no inoculated tomato plants wilted. In contrast, UW551 reached average cell densities of 1×10^{10} CFU/g following inoculation and caused severe wilting symptoms in 60% of plants. However, after 197 days in tubers at 4°C, none of the three strains could be recovered by enrichment broth culture or subsequent plant inoculation.

Tropical and R3bv2 strains grew similarly in culture at 20 and 28°C. There was no difference in growth rate between the two strains at 28°C ($P = 0.293$) (Table 2). Both strains reached stationary phase after ≈48 h, with peak cell density at 60 h (Fig. 4A). The overall time course of growth for UW551 and GMI1000 were also quite similar at 20°C, where both strains grew more slowly even though the starting cell density was higher than for the 28°C cultures, reaching maximum cell density at 168 h (Fig. 4B). In the second half of the 20°C growth curve, after 72 h, UW551 cell density was approximately twofold higher than that of GMI1000. This difference, although small, was reproducible and significant ($P = 0.0034$). Maximum cell densities in minimal medium for UW551 at 20°C were comparable with those of UW551 and GMI1000 at 28°C ($P > 0.0001$).

R3bv2 was much more virulent at 20°C than a tropical strain. In a soil-soak inoculation of unwounded susceptible tomato plants, both GMI1000 and UW551 were highly virulent at 28°C (Fig. 5). In all, 94% of inoculated plants were dead by 16 days postinoculation and the strains had indistinguishable disease progress curves. At 20°C, UW551 remained highly virulent and killed all inoculated plants, although it took 3 weeks rather than 2 weeks to reach full mortality. In striking contrast, at 20°C, tropical strain GMI1000 killed barely half the plants 1 month after inoculation. Thus, there was no difference in virulence on tomato plants between GMI1000 and UW551 at a lowland tropical soil temperature ($P = 0.87$) (Table 2) while UW551 was much more virulent than GMI1000 ($P = 0.017$) at a temperature typical of potato culture in the highland tropics.

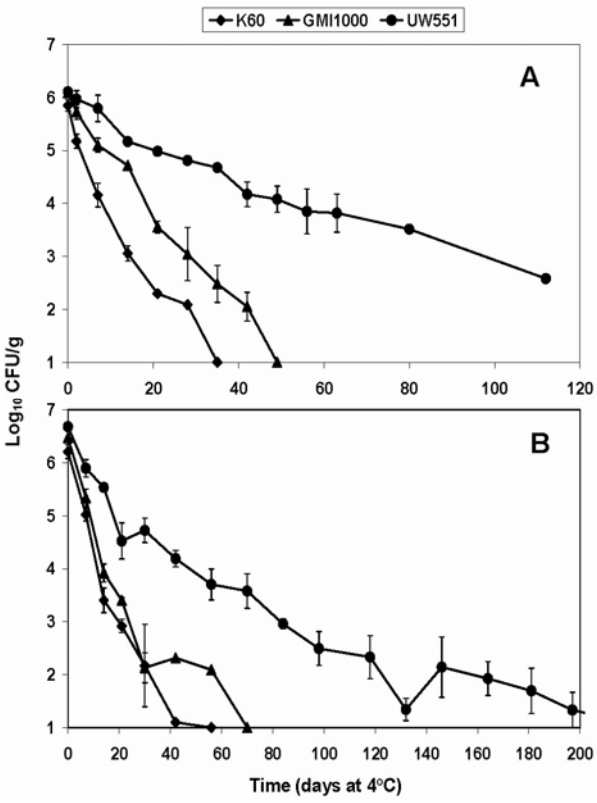

Fig. 3. Survival of *Ralstonia solanacearum* strains in potato tubers at 4°C. Race 3 biovar 2 strain UW551, tropical strain GMI1000, and endemic North American strain K60 were inoculated into potato minitubers **A,** cv. Russett Norkotah and **B,** cv. Shepody. Tubers were injected to an initial cell density of 1 to 2×10^6 CFU/tuber and were immediately stored at 4°C. Culturable cells were enumerated at regular intervals by dilution plating on casamino acids-peptone-glucose agar plates. Two or three tubers were sampled per strain per time point. Detection limit was 10 CFU/g of potato tissue. This experiment was repeated twice; typical results are shown. Points represent means of technical replicates; bars indicate standard error.

DISCUSSION

We undertook these experiments to better understand the ability of *R. solanacearum* R3bv2 strains to survive and cause disease in cool temperate conditions where other *R. solanacearum* strains do not threaten crops. Collectively, our data indicate that R3bv2 has no special adaptation to survive cold temperatures in water under controlled conditions, nor did it grow much faster than a tropical strain when cultured at the moderately cool temperature of 20°C. However, the presence of host plant tissue appears to mediate distinctive cold adaptation for R3bv2, both in terms of survival at near-freezing temperatures and ability to wilt plants at cool temperatures.

R. solanacearum is often spread through waterways by flooding and irrigation, and the bacterium can survive for years in pure water in the laboratory (8,23,39). It was possible that R3bv2 is a successful temperate pathogen because it is better able to persist in cold water than tropical strains. Therefore, we measured survival of diverse *R. solanacearum* strains over time in water microcosms maintained at 4°C. Although R3bv2 is supposed to be more cold tolerant than other *R. solanacearum* strains (15, 23,43), we found that endemic strains from the southeastern United States actually survived longer in water at 4°C than R3bv2 strains. The southeastern U.S. isolates used in this study, like all native North American strains characterized to date, belong to the sequevar 7 subgroup. These strains, historically known as race 1 biovar 1, behaved similarly and all remained culturable in cold water several weeks longer than the R3bv2 strains. Although the R3bv2 strains tested were isolated from four continents, they are all in sequevar 1 and had similar survival in water, in keeping with their likely clonal nature. The group C tropical isolates came from four different sequevars but had similarly brief survival in 4°C water. We conducted these experiments in pure water to isolate the effects of temperature on survival from those of microbial competition or predation in water. This limits the extent to which these results can be extrapolated to the field. However, previous studies with nonsterile natural agricultural drainage water suggest that R3bv2 populations decline even faster in the presence of other microbes at 4°C, becoming undetectable in only 30 days (51). This is consistent with field observations in rivers in the United Kingdom and with data showing that, in the absence of host plants, *R. solanacearum* strains are poor competitors in soil and water microbial communities (2,10,17,20,23). We did not

TABLE 2. Results from repeated-measures analyses of variance of *Ralstonia solanacearum* strain growth in culture and virulence on tomato at 20 and 28°C

Experiment	Effect	df[a]	*F* value	*P* value[b]
Growth in culture				
At 20°C	Strain	1	137.21	<0.0001***
	Rep (strain)	2	3.04	0.076 ns
	Time × strain	8	11.36	0.0007***
At 28°C	Strain	1	1.193	0.293 ns
	Rep (strain)	2	2.267	0.140 ns
	Time × strain	8	9.84	0.0034**
Virulence on tomato				
At 20°C	Strain	1	15.46	0.017**
At 28°C	Strain	1	0.030	0.87 ns

[a] df, degrees of freedom.
[b] Symbols: *** and ** = $P < 0.0001$ and 0.001, respectively; ns = not significant ($P > 0.05$).

measure the effects of temperature and plant tissue on soil survival but data from numerous previous studies suggest that *R. solanacearum*, especially R3bv2, survives long-term in natural soil primarily in association with plant debris (17–20,22,40,52).

We found that R3bv2 strain UW551, southeastern U.S. strain K60, and tropical strain GMI1000 all began to enter a VBNC state after 4 to 6 weeks in water at 4°C. These cells did not grow on unamended rich medium plates but some did grow on plates amended with catalase. It was previously observed that addition of catalase increased the number of colonies formed by *R. solanacearum* cells following cold storage (54), possibly because stressed cells are less able to detoxify the hydrogen peroxide present in autoclaved rich medium. Cells also had intact membranes when examined under a microscope following live/dead staining. Previous studies documented that several R3bv2 isolates became VBNC over time in 4°C water, and there is some evidence that exposure to copper can trigger VBNC in American sequevar 7 strain AW1, although copper did not have this effect on R3bv2 strain IPO1609 (3,21,51,54). VBNC has been described as a survival or resting state for bacteria, but it may also be a transition stage between life and death (32,36,53). VBNC cells of human pathogens such as *Escherichia coli* and *Vibrio cholerae* can be revived by passage through a host (36). The epidemiological significance of VBNC *R. solanacearum* cells is unknown, because it is unclear how frequently or even if VBNC cells can revive and attack plants (53). Our data confirm that cold triggers *R. solanacearum* cells to become VBNC in large numbers, with almost 10^5 live-staining R3bv2 cells per milliliter present after 114 days when no detectable CFU remained. We found that these cells are not likely to resuscitate and grow in plants, consistent with a previous report (54). Studies are needed to determine the prevalence of VBNC R3bv2 cells in naturally infested environments because these cells are invisible to the culture-dependent detection methods used in most previous studies. More extensive, carefully controlled experiments are also needed to conclusively determine whether VBNC *R. solanacearum* cells can revive and pose a risk to crops.

In contrast to our finding that endemic U.S. *R. solanacearum* strains survived longer than R3bv2 strains in water at 4°C, R3bv2 strain UW551 remained detectable significantly longer than tropical or native North American strains in potato tubers at 4°C. This result is epidemiologically relevant because potato seed tubers are typically stored at 4 to 5°C for ≈6 months, and latently infected seed tubers are a common source of potato brown rot outbreaks (1,35). However, although GMI1000 and K60 rapidly became unculturable in potato tubers, these strains could still be detected following prolonged enrichment culture in rich broth. This may be the result of surviving culturable cells that were too few to be detected by dilution plating (our detection limit was 10 cells/g of potato tissue). The enrichment method does not allow quantification of recovered cells. Alternatively, *R. solanacearum* cells may have entered a reversible VBNC state after months of storage in potato tubers at 4°C. These preliminary results suggest a potential risk of resuscitation of VBNC cells in potato tubers under favorable conditions. Experiments are needed to determine the probability that potato tubers containing VBNC cells could cause latent or active infections under field conditions.

The most direct explanation for the differential pathogenic aggressiveness of R3bv2 in temperate climates is that this subgroup can multiply faster at cooler temperatures than tropical strains. We tested this hypothesis by measuring the growth rate of tropical strain GMI1000 and R3bv2 strain UW551 in minimal medium under cool and warm conditions. There was no difference in doubling time or overall growth curve in liquid minimal medium between R3bv2 and tropical strain GMI1000 at 28°C and only a minor difference between the strains at 20°C. This result is consistent with a previous report that K60 and R3bv2 strain UW257 grew similarly in culture at 16°C, even though the R3bv2 strain was more virulent on potato at 16°C than K60 (6). This finding demonstrates that R3bv2 is not more virulent in cool climates simply because it grows faster than tropical strains at low

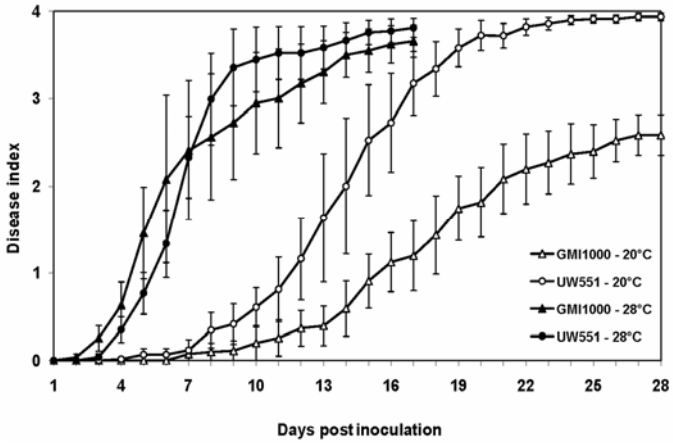

Fig. 5. Virulence of tropical and cool-temperate *Ralstonia solanacearum* strains on tomato plants at 20 and 28°C. Sixteen-day-old unwounded tomato plants (cv. Bonny Best) were inoculated by pouring bacteria onto the soil to a final concentration about 1×10^8 CFU/g soil. Plants were rated daily on a disease index scale of 0 to 4 where 0 indicated healthy and 4 indicated 100% wilted. Each point represents the mean disease index for three independent experiments containing 20 plants for each; bars indicate standard error.

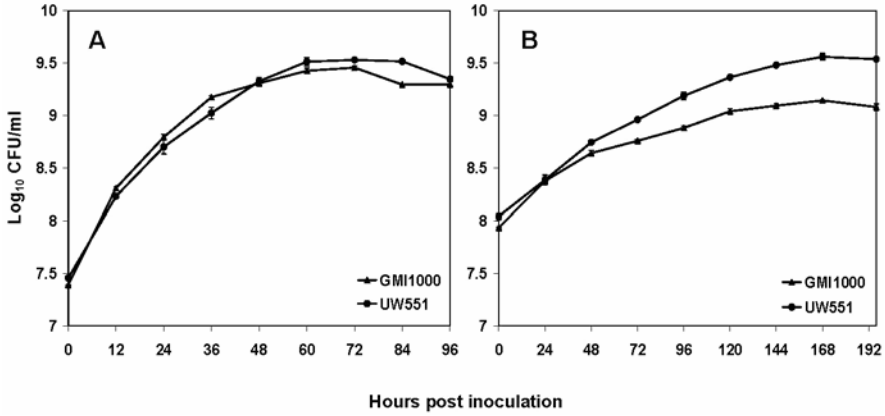

Fig. 4. Growth of *Ralstonia solanacearum* tropical strain GMI100 and cool temperate race 3 biovar 2 strain UW551 at **A,** 28°C or **B,** 20°C in minimal medium supplemented with 0.2% glucose. Cell counts were performed by dilution plating every 12 h for cells grown at 28°C and every 24 h for cells grown at 20°C. Data shown reflect the average of three experiments, each containing three cell count plates per strain per time point; bars indicate standard error of the mean.

temperatures. It has been suggested that R3bv2 strains have a lower optimal growth temperature in culture than other *R. solanacearum* strains (15) but we found that UW551, like GMI1000 and K60, grew faster at 28°C than at cooler temperatures (C. Allen, *unpublished data*). Collectively, these results show that differential growth rates at cooler temperatures are unlikely to fully explain the behavioral differences between the strains in the field.

Our experiments with tomato inoculated in a controlled environment are consistent with extensive field observations that R3bv2 is more virulent at cool temperatures than tropical strains like GMI1000 (23). Although both strains killed plants at similar rates at 28°C, UW551 was a much more effective pathogen at 20°C, a typical temperature on potato farms in the highland tropics. If R3bv2 is as virulent as a tropical strain at 28°C, why isn't R3bv2 a serious problem in the lowland tropics? Our growth-chamber experiments measured the effects of temperature in isolation, apart from other factors affecting pathogen success in natural agroecosystems. It is possible that R3bv2 strains do not compete well with tropical soil microflora or tolerate physical conditions of tropical soils. It would be interesting to measure the competitive ability of GMI1000 and UW551 to colonize tomato plants following dual inoculation under various physical conditions.

The special quarantine status of R3bv2 is based on concern that, if this strain becomes established in North America, it could cause serious crop losses in cool temperate agriculture. However, it is not clear whether the success of R3bv2 in the highland tropics, where temperatures are cool year-round but rarely fall below freezing for extended periods, translates to an ability to persist and cause disease in northern temperate zones. Additional studies are needed to determine whether R3bv2 can survive a typical winter in North American potato-growing regions, where average temperatures may remain below –10°C for weeks at a time. R3bv2 has survived the more moderately cold winters of northern Europe, although some evidence suggests that it does so by associating with host plants like *S. dulcamara* (13). Moreover, although R3bv2 strains are still present in soils and waterways of northern Europe 13 years after their initial discovery, bacterial wilt has not caused significant crop losses there (3,5,12).

Our finding that differences in virulence and survival between R3bv2 and tropical *R. solanacearum* strains can be measured only in planta suggests that the epidemiological difference between these two groups cannot be explained by typical bacterial cold adaptations such as increased membrane fluidity or chaperone proteins that stabilize proteins or nucleic acids at low temperatures (33), unless these adaptations were specifically triggered by a plant host signal. A more likely hypothesis is that plant-induced cold adaptation traits are encoded by some of the ≈400 genes that are uniquely present in the UW551 genome relative to that of GMI1000 (16). Alternatively, genes that are present in both strains may be induced under cold conditions only in UW551. In planta gene expression microarray experiments are currently underway to distinguish among these alternatives and to gain a more mechanistic understanding of how low temperatures affect bacterial wilt virulence in tropical and R3bv2 strains.

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