A *cbb₃*-Type Cytochrome C Oxidase Contributes to *Ralstonia solanacearum* R3bv2 Growth in Microaerobic Environments and to Bacterial Wilt Disease Development in Tomato

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Ralstonia solanacearum race 3 biovar 2 (R3bv2) is an economically important soilborne plant pathogen that causes bacterial wilt disease by infecting host plant roots and colonizing the xylem vessels. Little is known about R3bv2 behavior in the host rhizosphere and early in bacterial wilt pathogenesis. To explore this part of the disease cycle, we used a novel taxis-based promoter-trapping strategy to identify pathogen genes induced in the plant rhizosphere. This screen identified several *rex* (root exudate expressed) genes whose promoters were upregulated in the presence of tomato root exudates. One *rex* gene encodes an assembly protein for a high affinity *cbb₃*-type cytochrome *c* oxidase (*cbb₃-cco*) that enables respiration in low-oxygen conditions in other bacteria. R3bv2 *cbb₃-cco* gene expression increased under low-oxygen conditions, and a *cbb₃-cco* mutant strain grew more slowly in a microaerobic environment (0.5% O₂). Although the *cco* mutant could still wilt tomato plants, symptom onset was significantly delayed relative to the wild-type parent strain. Further, the *cco* mutant did not colonize host stems or adhere to roots as effectively as wild type. These results suggest that R3bv2 encounters low-oxygen environments during its interactions with host plants and that the pathogen depends on this oxidase to help it succeed in planta.

The root and its immediate surrounding area, the rhizosphere, are important for plant fitness. Under natural conditions, the rhizosphere contains vast numbers of microorganisms that interact not only with each other but with the plant root as well (Rainey 1999). Plants exude nutrients from roots, with up to 20% of available carbon in the rhizosphere coming from roots (Brencic and Winans 2005; Handelsman 1996). It is this relatively rich, fluctuating, and competitive environment that the pathogen *Ralstonia solanacearum* inhabits, growing and moving toward nutrient sources and signals from host plants that may be susceptible to disease.

R. solanacearum, the causal agent of bacterial wilt, is an economically important pathogen affecting crop production worldwide (Hayward 1991). The *R. solanacearum* species complex is a large heterogeneous group of motile, soilborne β-

proteobacteria distributed primarily in tropical and subtropical regions, although it is also problematic in temperate climates. *R. solanacearum* enters the vascular tissue of its host through natural openings or mechanical wounds. The pathogen multiplies in the root cortex and, then, rapidly invades and colonizes the xylem, ultimately disrupting water flow. Symptoms of bacterial wilt include stunting, chlorosis, and sudden wilting of the plant (Hayward 1991).

The *R. solanacearum* species complex includes a group historically known as race 3 biovar 2 (R3bv2) and now classified as phylotype II, sequevar 1 (Fegan and Prior 2005). R3bv2 causes bacterial wilt of tomato, southern wilt of geranium, and brown rot of potato, diseases that lead to tremendous losses, particularly in the tropics and subtropics (Allen et al. 2001). Because it poses a threat to temperate potato production, R3bv2 is a quarantine pest in Canada and Europe and a select agent pathogen in the U.S. (Lambert 2002).

R. solanacearum virulence is quantitative and complex, with many contributing factors, such as a suite of type III-secreted effectors, bacterial extracellular polysaccharide, several plant cell wall-degrading enzymes, motility, and energy taxis (González 2003; Saile et al. 1997; Schell 2000; Tans-Kersten et al. 2001; Yao and Allen 2006). Most known virulence factors were identified based on their roles in advanced wilt disease. We previously described >150 *R. solanacearum* genes specifically expressed in mid-phase bacterial wilt disease in tomato, which offered insight into the physiology of this pathogen during pathogenesis (Brown and Allen 2004). However, despite their epidemiological importance, little is known about the traits that this soilborne pathogen needs to survive in the rhizosphere and to begin colonizing host roots.

A significant proportion of the photosynthate that plants allocate to their roots is released into the rhizosphere (Marschner 1995). These root exudates consist of high-molecular weight mucilage and acid phosphatase as well as amino acids, phenolics, sugars, and organic acids, the latter two being the most abundant (Marschner 1995). The total amount of root exudate and its composition varies according to plant species, nutritional status, age, presence of microbes, and nitrogen amendments (Huber and Watson 1974; Lugtenberg; Marschner 1995). Root exudates form a nutrient source and a habitat signal for microorganisms (Marschner 1995). *R. solanacearum* chemotaxis toward host-plant root exudates by means of swimming motility guided by sensor proteins that direct movement (Yao and Allen 2006). Chemotaxis receptors transmit environmental signals to the flagellar motor via the core taxis signaling proteins CheA and CheW (Yao and Allen 2006). Chemotaxis is essen-

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tial for location and invasion of host roots in *R. solanacearum*; both *cheW* and *cheA* mutants are quantitatively reduced in virulence on tomato (Yao and Allen 2006). The observation that *R. solanacearum* can detect and respond to plant root exudates led to a series of questions. What R3bv2 genes are induced in response to host root exudates? What is their contribution to life in the rhizosphere and early host-pathogen interactions?

We sought to identify and characterize R3bv2 genes that affect bacterial behavior in the rhizosphere and early in pathogenesis. To accomplish this, we used a taxis-based expression technology strategy to identify pathogen genes induced by tomato root exudates (TRE). This in vitro approach was fashioned after in vivo expression technology (IVET), a population-to-genes approach that takes the ecological context of the organism into consideration and identifies genes based on gene induction under specific conditions (Rainey and Preston 2000). Originally developed for an in planta application (Osbourne et al. 1987), IVET uses a promoter-probe plasmid containing genomic DNA fragments that are cloned directly in front of a marker gene and a reporter gene, both of which are promoterless, to create a transcriptional fusion (Rainey and Preston 2000). These constructs are introduced into a mutant strain, which cannot succeed in a specific selective environment (usually the eukaryotic host). If a DNA fragment cloned upstream of the marker gene contains a promoter that is induced in the selective environment, the wild-type bacterial phenotype is restored to the mutant and it will be selected in the differentiating environment. The sequence of the cloned promoter suggests the function of one or more corresponding upregulated genes. This strategy has successfully identified genes of plant-associated bacteria induced under various conditions, including *R. solanacearum* genes involved in virulence and adaptation to the host in mid-bacterial wilt disease of tomato (Boch et al. 2002; Brown and Allen 2004; Dunn et al. 2003; Gal et al. 2003; Marco et al. 2005; Oke and Long 1999).

Traits that improve survival in the rhizosphere environment are undoubtedly important for *R. solanacearum*'s success as a pathogen. We hypothesized that, in addition to acting as chemoattractants, host root exudates also induce expression of bacterial *rex* (root exudate expressed) genes, whose products may facilitate root invasion and colonization or act directly as early virulence factors. We identified R3bv2 strain UW551 genes that were upregulated in the presence of chemicals found in the tomato rhizosphere. These included a locus encoding a high-affinity *cbb₃*-type cytochrome *c* oxidase (*cbb₃-cco*) that was required for full virulence and for growth of R3bv2 under the low-oxygen conditions found in the rhizosphere and plant xylem vessels.

RESULTS

A taxis-based expression technology strategy identified *R. solanacearum* R3bv2 genes induced by host root exudates.

Our modified IVET screen selected pathogen promoters that were active in the presence of TRE. We created a nonchemotactic deletion mutant, UW551Δ*cheW*, and used it to evaluate chemotaxis as a selectable trait for this screen. UW551Δ*cheW* did not form a halo on taxis motility agar, but it did retain swimming ability as observed under a microscope (data not shown). Chemotaxis was restored to UW551Δ*cheW* by complementation with a plasmid-borne copy of the *cheW* locus.

To determine if we could select a few tactic cells from a large pool of nontactic bacteria, we mixed 100 cells of wild-type UW551 with 10⁵ cells of UW551Δ*cheW*, and this mixture was spotted onto taxis agar containing TRE. In response to the nutrient gradient created by bacterial growth, the small tactic

subpopulation migrated away from the nontactic majority and formed an easily distinguishable halo (data not shown). This demonstrated that restoration of taxis was a selectable trait under our conditions.

To select promoters active in the presence of TRE, we constructed a promoter-trapping integrative plasmid vector, pJC*cheW*, which contains the promoterless *cheW* open reading frame (ORF) cloned upstream from a promoterless β-glucuronidase (*gus*) reporter gene. Cloning wild-type genomic UW551 DNA fragments into the *Xho*I site upstream of the promoterless *cheW* created transcriptional fusions with the potential to restore chemotaxis and express GUS activity (Fig. 1A).

To confirm that introduced promoters could restore taxis to the *cheW* mutant, we cloned various well-characterized promoters into the *Xho*I site of pJC*cheW*, transformed the constructs into UW551Δ*cheW*, and measured taxis of the resulting

Fig. 1. Overview of the *rex* (root exudate expressed genes) screen used to identify *Ralstonia solanacearum* race 3 biovar 2 (R3bv2) genes induced in response to tomato exudate. **A**, The *rex* screen vector pJC*cheW* was constructed by cloning the promoterless *cheW* from *Ralstonia solanacearum* UW551 into pVO155. pJC*cheW* consists of promoterless *cheW* and *uidA* (*gus*) genes, each with its own translational start (RBS). Random UW551 genomic DNA fragments were cloned into the *Xho*I restriction site, which is preceded by a transcriptional terminator (stem loop) and stop codons in all three reading frames. The *oriT* allowed for conjugation of the vector into *R. solanacearum* and *neo* (kanamycin resistant) provided a selectable marker. **B**, For the *rex* screen, random fragments of UW551 chromosomal DNA were cloned into the promoter-trapping vector to create a library. Strains with restored chemotactic response (active promoters) were selected by growth on taxis agar supplemented with 1% root exudate. Chemotactic strains were isolated, and the *rex* screen vector was recovered by retro-transfer. Chromosomal DNA inserts were sequenced for bioinformatic analysis.

strains. We used the *pilA* promoter, which drives transcription of the abundant type IV fimbrial protein (Kang et al. 2002), the *flhDC* promoter, which mediates low-level expression of a flagellar regulator (Tans-Kersten et al. 2004), and *cheW*'s own native promoter (Yao and Allen 2006). These three promoters all restored chemotaxis to UW551Δ*cheW*, but the *cheW* promoter did not restore taxis when cloned in the reverse orientation (data not shown). Thus both native and heterologous promoters could drive expression of the promoterless *cheW* ORF in pJC*cheW* and thereby identify genes in the *rex* screen.

An overview of the *rex* screen is shown in Figure 1B. A library of UW551 DNA fragments was cloned into pJC*cheW* and introduced into UW551Δ*cheW* by triparental mating. At least 80,000 strains were screened for the restoration of chemotaxis, more than the 68,000 required for 95% coverage of *R. solanacearum*'s 5.7-Mb genome (Ausubel et al. 1995). Eight fusions had taxis halo diameters as large or larger than the parental control strains, with diameters ranging from 0.5 to 3.5 cm larger than wild type. The presence of TRE increased the expression of these fusions by two- to fivefold (Table 1).

One *rex* insertion was in a gene cluster encoding synthesis and assembly of a high oxygen affinity cco.

The sequence adjacent to the *rex4* insertion resembled that encoding a 4Fe4S ferredoxin involved in electron transport in *ccb3*, a high affinity oxidase that contributes to aerobic respiration under microaerobic conditions (RRSL_01397; Table 1). RRSL_01397 encodes a protein with 37 and 35% identity at the amino acid level to CcoG (also referred to as FixG) from *Bradyrhizobium japonicum* and *Rhizobium etli*, respectively. CcoG is required for *ccb3*-Cco expression under oxygen-limiting conditions in *B. japonicum* (Preisig et al. 1996; Zufferey et al. 1996). ClustalW alignment of the UW551 CcoG homolog revealed the conserved ferredoxin motif Cys-X₁-X₁-Cys-X₃-X₄-Cys-X₅-X₆-X₇-Cys-Pro at positions 284 and 308 without the terminal proline, similar to motifs found in *Rhodobacter sphaeroides* RdxA (Neidle and Kaplan 1992), FixG in *B. japonicum*, and FixG in *Sinorhizobium meliloti*. Genes encoding Cbb₃-Cco are typically arranged in two operons, *ccoNOQP* (*fixNOQP*), which encodes structural proteins and is always found very close to *ccoGHIS* (*fixGHIS*), which encodes the assembly proteins. Both operons are essential for respiration under microaerobic conditions and symbiosis in *B. japonicum* (Preisig et al. 1996).

All genes required for Cbb₃-Cco synthesis and assembly appear to be present in UW551. We hypothesized that the region tagged by the *rex4* insertion encodes a high-affinity Cco that helps *R. solanacearum* succeed in hypoxic environments, such as the rhizosphere and xylem of its host plants. Such an operon is likely to be upregulated under low-oxygen conditions. We measured expression of the *rex4::gus* fusion at three oxygen levels, 21, 5, and 0.5%, corresponding to atmospheric, slightly hypoxic, and microaerobic conditions, respectively (Loesche 1969). After 24 h, *rex4* expression increased sevenfold in strains grown under 5% oxygen and more than 30-fold in strains grown under 0.5% oxygen relative to the strain grown at 21% oxygen. This induction of gene expression in hypoxic conditions was consistent with its putative function as a high-affinity cco.

To further explore the biological role of Cbb₃-Cco in *R. solanacearum*, we mutated the first gene in the operon, which encodes the CcoN structural protein in the Cbb₃-Cco complex (Fig. 2). Mutation of this ORF was expected to eliminate Cbb₃-Cco function, since the *cco* genes are coregulated in other systems and CcoN is necessary for Cco function (Mandon et al. 1994; Marchal et al. 1998; Preisig et al. 1996; Zufferey et al. 1998). The *ccoN* ORF was disrupted by a site-directed insertion of a gentamicin (Gm)-resistance cassette, and the expected mutation in strain UW551cco was confirmed by polymerase chain reaction (PCR) and Southern blot analysis (data not shown).

A *ccb3*-cco mutant grew more slowly in microaerobic environments.

We measured the growth rate of UW551cco, its wild-type parent strain, and a complemented mutant in culture in the presence of 21 (atmospheric) or 0.5% oxygen. After 24 h, all three strains grew similarly at 21% oxygen, but at 0.5% oxygen, UW551cco grew on average 53% slower than either UW551 or the complemented mutant ($P = 0.013$; Fig. 3A). After 5 days, the trend remained the same, with UW551cco at approximately 73% the population size of the wild-type and complemented strains ($P = 0.012$; Fig. 3B). We observed similar differences under semi-aerobic conditions in which bacterial cultures were grown in large volumes at low rpm to provoke hypoxia (data not shown). These results demonstrated that a defined *ccoN* mutant was quantitatively reduced in ability to grow in low-oxygen environments and constituted phenotypic evidence that this region encodes a high-affinity oxidase.

Table 1. *Ralstonia solanacearum* UW551 genes identified in the *rex* (root exudate expressed genes) screen

<i>rex</i> ID	Gene ^a	Predicted protein	<i>E</i> value	Closest match outside <i>Ralstonia</i>	Fold induction ^b
<i>rex1</i>	RRSL_00505 (<i>hrpG</i>)	Transcriptional regulator	7e-40	<i>Burkholderia graminis</i>	5 ± 0.9
<i>rex2</i>	RRSL_01880 (<i>pilP</i>)	Type IV pili assembly	e-32	<i>Acidovorax</i> sp.	2 ± 0.2
<i>rex3</i>	RRSL_02649 (<i>pehR</i>)	Transcriptional regulator	0.0	<i>Janthinobacterium</i> sp.	5 ± 1.2
<i>rex4</i>	RRSL_01397	Iron-sulfur 4Fe-4S/ferredoxin	0.0	<i>Bordetella bronchiseptica</i>	4 ± 1.1
<i>rex5</i>	RRSL_03240 (<i>dps</i>)	Nonspecific DNA binding	6e-71	<i>Burkholderia oklahomensis</i>	4 ± 2.8
<i>rex6</i>	RRSL_03587	Oxidoreductase, glyoxalate metabolism	6e-77	<i>Dechloromonas aromatica</i>	2 ± 1.0
<i>rex7</i>	RRSL_01657 (<i>vsrA</i>)	Sensory transduction, histidine kinase	0.0	<i>Cupriavidus taiwanensis</i>	3 ± 1.1

^a Gene names are based on annotations in the UW551 or GMI1000 genome sequences.

^b Fold induction of the *rex* gene plus or minus standard error, based on comparison of β-glucuronidase (*gus*) activity in minimal media with 1% tomato root exudate versus unamended minimal media.

Fig 2. Genomic context of genes encoding structural and assembly proteins of *cbb3*-type cytochrome *c* oxidase (*cbb3*-Cco) in the *Ralstonia solanacearum* UW551 genome. Gene annotations are based on conserved *cbb3*-cco loci in other plant-associated soilborne bacteria. *ccoN* was disrupted by inserting *aacC1*, encoding gentamicin resistance (open triangle), into a unique restriction site. Arrowheads represent predicted open reading frames.

The Cbb₃-cco is required for full virulence on tomato.

Tomato plants soil-soak inoculated with UW551cco developed bacterial wilt symptoms more slowly than did plants inoculated with the wild-type parental strain ($P < 0.0001$; Fig. 4A). Beginning 4 days after inoculation, disease development in UW551cco-inoculated plants lagged behind that induced by the wild-type strain, although, by 13 days after inoculation, both strains caused full mortality. A similar difference in disease progress was observed when approximately 200 bacteria were introduced directly into plant vasculature through a cut petiole ($P = 0.010$; Fig. 4B).

UW551cco had reduced ability to colonize tomato stems.

To quantify the cell density of *R. solanacearum* strains in plant stems, we inoculated cut tomato petioles with approximately 200 cells of either UW551 or UW551cco and measured bacterial population sizes in stems over time. By day 4, the wild-type strain had colonized 53% of inoculated plants with a mean population of 4.3×10^9 CFU per gram of stem, while the *cco* mutant colonized 40% of plants with a mean population of 2.8×10^8 CFU per gram of stem. At 6 days

after inoculation, 89% of stems from plants inoculated with either strain contained detectable bacteria. However, mean cell populations of UW551 were 4.3×10^{10} CFU per gram of stem, while those for UW551cco were more than 30-fold lower at 1.3×10^9 CFU/g. The median bacterial population size differed significantly between plants inoculated with UW551 (1.1×10^9 CFU/g) and UW551cco (8.2×10^7 CFU/g) ($P = 0.027$; Fig. 5A). There were no significant differences between strain stem populations by 8 days after inoculation, but 80% of tomato plants inoculated with wild type had died, while only 40% of plants inoculated with the *cco* mutant had died (data not shown). These results indicate that *R. solanacearum* needs a functional Cbb₃-Cco for rapid colonization of tomato vasculature.

The *cco* mutant had reduced competitive fitness on tomato plants.

We measured the ability of the *cco* mutant to invade and colonize tomato plants in competition with its wild-type parent strain. Plants were soil-soak inoculated with a 1:1 mixture of wild-type UW551-rif and the UW551cco mutant; when wilt-

Fig. 3. UW551 cytochrome *c* oxidase (*cco*) mutant grew more slowly than wild type in oxygen-limiting conditions. **A**, Bars represent mean population sizes after 24 and **B**, 120 h of growth in buffered minimal medium supplemented with 0.2% glucose at either 21% (atmospheric) or 0.5% (microaerobic) O₂. At both timepoints, growth of UW551cco (gray bars) was significantly reduced at 0.5% O₂ when compared to the wild-type parent (black bars) and the complemented mutant strain UW551cco(pUFIcco) (white bars). Averages are of five independent assays for the 24-h timepoint and three independent assays for the 120-h timepoint. Asterisks represent significantly different means ($P < 0.05$). Bars represent standard error of the mean.

Fig. 4. *Ralstonia solanacearum* UW551cco caused delayed bacterial wilt disease on tomato. **A**, ‘Bonny Best’ plants (21 to 22 days old) were inoculated in a naturalistic soil-soak inoculation or **B**, directly into tomato stems via cut petioles. Plants were rated daily on a disease index scale of 0 to 4. Disease development in plants inoculated with UW551cco mutant (squares) lagged significantly relative to its wild-type parent (circles). Results shown are the means of three independent assays with 16 plants per treatment. Bars represent standard error of the mean.

ing symptoms appeared, bacterial populations in the upper crown were quantified. The median population of wild-type cells in tomato stems was 2.2×10^9 CFU/g, while that of the *cco* mutant strain was 120-fold lower at 1.0×10^8 CFU/g ($P = 0.075$; Fig. 5B). Mean population sizes of UW551cco in tomato stems also trended lower than those of UW551-rif.

The *cco* mutant had reduced ability to adhere to tomato roots.

To determine the contribution of *cbb3-cco* to root attachment and colonization, we measured the relative ability of the wild-type and *cco* mutant strains to adhere to and colonize tomato seedling roots. Roots incubated 1.5 h with a suspension of UW551-GFP (green fluorescent protein) carried adhering populations 10-fold greater than those on roots inoculated with UW551cco-GFP ($P = 0.042$; Fig. 5C). After 4 h of incubation, UW551cco adhering populations increased slightly, but the average adhering population of the wild-type strain remained more than twice as large ($P = 0.158$; Fig. 5C). There was no visible difference between strains in root colonization, as assessed by fluorescence microscopy (data not shown).

DISCUSSION

We developed a novel strategy that identified seven *R. solanacearum* genes that are upregulated in response to TRE. Several of these *rex* genes were previously known to be plant-induced, providing a reassuring internal control that the screen functioned as expected. For example, *HrpG*, which activates *hrp* gene expression in response to a plant signal (Brito et al. 1999), is required for successful colonization of tomato roots and for bacterial wilt virulence (Vasse et al. 2000). The two-component regulator PehSR positively controls expression of the extracellular plant cell wall-degrading enzyme polygalacturonase and twitching and swimming motility, which are all virulence factors (Allen et al. 1997; Kang et al. 2002). PehSR expression increases when the bacterium grows in tobacco leaves, suggesting it responds to one or more undefined plant signals (Allen et al. 1997). VsrA is the sensor kinase of the two-component regulator

VsrAD, which controls diverse components of *R. solanacearum* virulence, including motility, exopolysaccharide production, and the type three secretion system (Schell et al. 1994) (J. Yao and C. Allen unpublished results). PilP, which is involved in assembly of type IV pili, is important in *R. solanacearum* twitching motility and virulence (Kang et al. 2002; Liu et al. 2001).

Other *rex* genes apparently contribute to bacterial fitness and environmental survival. The *rex5* insertion identified a locus that encodes a homolog of Dps, a nonspecific DNA binding protein widely present in prokaryotes. Dps protects *Escherichia coli* from nutrient and oxidative stress, especially in stationary phase (Almirón et al. 1992; Martinez 1997). The *rex6* insertion identified a gene annotated as a 2-hydroxy-3-oxopropionate reductase involved in glyoxylate and dicarboxylate metabolism. This enzyme may have been induced by a compound in TRE such as glycerate, which can be shuttled into the glycolytic pathway (White 2000). Interestingly, the operon immediately upstream encodes proteins involved in sulfur assimilation; both these loci may be important for rhizosphere nutrient uptake and catabolism.

The *rex4* insertion created a fusion with a gene encoding a 4Fe4S ferredoxin that mediates electron transport to O_2 as part of a *cbb3*-type Cco. Cbb3-Cco belongs to a family of proton-pumping, heme-copper terminal oxidases with a high affinity for oxygen, and it is associated with oxygen use in microaerobic environments. Cbb3-Cco functions in aerobic respiration; it is the terminal oxidase in the electron transport chain that runs from cytochrome *c* to O_2 , creating ATP in the process. Cbb3-cco is an evolutionarily ancient enzyme, well-suited for the low levels of oxygen in the earlier earth's atmosphere and is more closely related to nitric oxide reductase than to other Cco (Sakamoto and Sone 2004; van der Oost et al. 1994). This high-affinity Cco may be important in the rhizosphere, where oxygen levels can be quite low, depending on soil type, moisture levels, competitive consumption by the soil community, and oxygen consumption or output by the plant roots. For example, in the rhizosphere of *Juncus effusus* L. grown in a flooded rhizotron, oxygen levels were 10 to 70 μ M per liter around root tips and 0.9 μ M per liter in bulk soil (Blossfeld

Fig. 5. The *Ralstonia solanacearum* race 3 biovar 2 (R3bv2) cytochrome *c* oxidase (*cco*) mutant strain does not colonize tomato stems as well as the wild-type strain. **A**, Cut petioles of 21- or 22-day-old tomato plants were inoculated with approximately 200 CFU of either strain. At 6 days postinoculation, plant stems were ground and dilution-plated to determine bacterial population sizes. Colony counts were normalized to CFU per gram of tissue, and data were log-transformed for analysis. Colonization differed significantly when median populations were analyzed using the Mann-Whitney comparison ($P = 0.026$). Nineteen plants from four assays were analyzed per strain, and bars represent the standard error of the means. **B**, Wild-type R3bv2 was generally more effective than the *cco* mutant at invading and colonizing tomato plants. Tomato plants (21 or 22 days old) were soil-soak inoculated with a 1:1 mixture of UW551-rif and the UW551cco mutant in a competition assay. A stem section was dilution-plated onto selective medium when plants showed symptoms. A scatter plot shows the log-transformed populations of each strain from individual plants. The boxed 'X' indicates median population size; bars indicate the 95% confidence interval of the mean. Mean stem populations of UW551cco were lower than UW551-rif, according to the paired *t*-test ($P = 0.075$). **C**, *Ralstonia solanacearum* UW551cco did not adhere to roots as well as wild type. Sterile seedlings of tomato cultivar 'Bonny Best' were incubated without aeration for 1.5 or 4 h in 1.0×10^7 CFU/ml suspensions of either UW551 or UW551cco. Rinsed roots were ground and dilution plated. At 1.5 h, the average adhering populations of UW551cco (white bars) were lower than those of UW551 (dark bars) ($P = 0.061$). After 4 h of incubation, populations of UW551 and UW551cco were not significantly different ($P = 0.158$), but wild-type cell densities were still double those of the *cco* mutant. Bars represent the standard error of the mean.

and Gansert 2007). Oxygen in xylem is also reported to be low, around 2 ppm (Pegg 1985). *R. solanacearum* lives in both environments and so must adapt to niches in which oxygen may not be readily available.

Cbb₃-Cco is present in several plant-associated soilborne bacteria including *B. japonicum*, *R. etli*, *Azorhizobium caulinodans*, *Azospirillum brasilense*, *Pseudomonas stutzeri*, *P. aeruginosa*, and photosynthetic *Rhodobacter* spp. (Comolli and Donohue 2004; Koch et al 1998; Mandon et al. 1994; Marchal et al. 1998; Presig et al. 1996; Urbani et al. 2001). The *rex4* 4Fe4S, annotated *ccoG*, is in an apparent operon encoding proteins for synthesis and assembly of a subgroup of Cbb₃-Cco found almost exclusively in the proteobacteria. Several proteobacteria that have *cbb₃-cco* can reportedly adapt to microaerobic conditions when they are dependent on aerobic respiration (Oh and Kaplan 2004). *R. solanacearum* is considered an obligate aerobe (Holt 1994). Consistent with this adaptation hypothesis, expression of the UW551 *rex4::gus* fusion increased more than 30-fold when the bacterium grew in 0.5% rather than 21% oxygen. In other bacteria, this operon is also highly upregulated under low-oxygen tensions (Comolli and Donohue 2004; Mandon et al. 1994; Preisig et al. 1996).

The *R. solanacearum cbb₃-cco* locus encodes apparent homologs to other Cbb₃-cco structural and assembly proteins, most notably from *B. japonicum*. The *R. solanacearum* CcoG contains the conserved ferredoxin motif, CcoN contains the conserved histidine residues required for full function in *B. japonicum*, and CcoI contains the conserved ATPase catalytic domains (Zufferey et al. 1998). In the proteobacteria, *ccoGHIS* is always near *ccoNOQP*, and the *Ralstonia* species complex is no exception. Interestingly, a rearrangement of the *cbb₃-cco* gene clusters is conserved among *Ralstonia* spp., and it appears that *ccoIS* was translocated upstream of *ccoNOQP* in *R. solanacearum*.

We hypothesized that *R. solanacearum* needs a functioning *cbb₃-type* Cco to sustain respiration in the rhizosphere and in the xylem, microaerobic environments relevant to bacterial wilt pathogenesis. To test this hypothesis, we constructed a *R. solanacearum ccoN* mutant, UW551cco. CcoN accepts electrons from CcoP and CcoO, which are membrane-bound mono- and dihememes, respectively (Oh and Kaplan 2004); a *ccoN* mutant of *B. japonicum* was unable to form the oxidase (Zufferey et al. 1996). The genomic arrangement of the *cbb₃-cco* region suggested that *ccoN*, *ccoO*, and *ccoP* might be co-transcribed, such that disrupting *ccoN* would have polar effects on *ccoO* and *ccoP*. However, this does not appear to be the case, since the DNA fragment that successfully complemented UW551cco to wild-type behavior contained only a complete copy of the *ccoN* ORF, its immediate upstream region, and part of the *ccoO* ORF. The UW551cco mutant's phenotype offered biological evidence supporting the annotation of the *cbb₃-cco* locus. At the atmospheric oxygen concentration of 21%, UW551cco grew as well as its parent strain, but its growth was significantly impaired at the microaerobic concentration of 0.5% O₂. The decrease in growth was even more pronounced after 5 days of incubation, indicating that this oxidase is important for *R. solanacearum* R3bv2 respiration when oxygen tensions are low.

Collectively, our tomato experiments indicate that *R. solanacearum* uses this high-affinity oxidase to effectively colonize host plants and cause disease. The *cco* mutant caused delayed wilt disease on tomato in both a naturalistic soil-soak assay and after direct inoculation into the vasculature, indicating that *R. solanacearum* encounters low-oxygen environments during growth in host xylem tissue and possibly also during the root colonization and infection process. However, all inoculated plants did eventually wilt. *R. solanacearum* may have an alter-

native process for respiration when oxygen is limiting. Alternatively, the mutant's delayed wilting phenotype could be due to slower multiplication in the low-oxygen environment of the tomato xylem, similar to its poor growth under low-oxygen conditions in culture. This effect may be additive, with reduced fitness during the first stages of infection in the fluctuating oxygen conditions of the rhizosphere. Consistent with this hypothesis, *cco* mutant population sizes in tomato stems were smaller than those of the wild type during the period when disease progress was delayed. By 8 days after inoculation, populations of the two strains inside tomato stems were similar, although tomato plants inoculated with UW551cco still had markedly lower disease severity than those inoculated with wild type. We speculate that, at this point, alternative mechanisms for dealing with reduced oxygen may compensate for lack of a functional Cbb₃-Cco or that UW551cco simply takes longer to multiply but, given time, can eventually attain wild-type levels. Under natural field conditions the *cco* mutant would probably suffer markedly reduced fitness. The ability of the *cco* mutant to invade and colonize host roots would depend on the oxygen tension of the surrounding soil and rhizosphere. The rhizosphere is characterized by high rates of oxygen depletion that depend on water volume, soil temperature, and respiring biomass (roots and microbes) (Drew and Stolzy 1996; Hawkes et al 2007).

To better understand the role of Cbb₃-Cco in *R. solanacearum* rhizosphere fitness, we coinoculated plants with the wild type and *cco* mutant in a competition assay. The wild-type strain generally outcompeted the *cco* mutant strain at the onset of wilting symptoms, although stem populations varied considerably. This could be due to the fluctuating oxygen levels in the rhizosphere, where increased oxygen levels could restore wild-type respiration, or possibly to compensation by other oxidases in *R. solanacearum* that can transport electrons under reduced oxygen availability.

R. solanacearum has other oxidases useful in respiration, including the aa₃-type, which has a relatively low affinity for oxygen (Sakamoto and Sone 2004). In *R. sphaeroides*, biosynthesis of Cbb₃-Cco increases under microaerobic conditions while aa₃ oxidase expression is reduced, so that Cbb₃-Cco becomes the predominant Cco (Oh and Kaplan 2004). *R. solanacearum* UW551 does, however, possess a cytochrome bd-type of oxidase, which is believed to be important in aerobic respiration (Richardson 2000) and functions with Cbb₃-Cco to support hypoxic growth of *A. caulinodans* (Kaminski et al. 1996). Such a terminal oxidase could explain the ability of the pathogen to overcome a low-oxygen environment during colonization and wilting. Some *R. solanacearum* phylotype II strains, such as R3bv2 UW551, cannot use nitrate as an alternative electron acceptor because they lack NosZ (C. Allen and E. T. Gonzalez, unpublished data). *R. solanacearum* UW551 did not grow under strictly anaerobic conditions (data not shown), suggesting the importance of the high oxygen-affinity oxidases in its aerobic respiration. *R. solanacearum* is a microaerophilic organism that tends to move toward moderate rather than high oxygen levels (Yao and Allen 2007). This behavior may reflect adaptation to the microaerobic environments of the plant rhizosphere and xylem (Yao and Allen 2007). Pathogen attachment is generally required for biofilm formation on host roots, but the role of biofilms in bacterial wilt is uncertain. We speculate that when this pathogen adheres to plant roots and forms aggregates, it is forced to respire while embedded in a low-oxygen tension matrix. UW551 did not form biofilms in an artificial PVC well assay when tested at temperatures ranging from 4 to 28°C over a period of 10 days (J. Colburn-Clifford and C. Allen, unpublished results), although *R. solanacearum* strain K60 does form aggregates on tomato roots (Yao 2006).

A CcoO mutant of *P. aeruginosa* was reduced in late-stage biofilm formation, suggesting that Cbb₃-Cco could also be important in *R. solanacearum* aggregation on roots (Southey-Pillig et al. 2005). Significantly more wild-type than *cco* mutant bacteria adhered to tomato roots following incubation in *R. solanacearum* suspensions. The quantifiable differences in population densities along the root could explain some of the reduced virulence of UW551cco. In situ gene expression analyses could determine if Cbb₃-Cco supports aerobic respiration when cells are aggregated on host roots.

The *rex* screen identified *pehR* and *pilP*, twitching motility genes that potentially play a role in biofilm formation. The *cco* mutant also had reduced twitching motility (data not shown), but we suspect this was a consequence of the mutant's reduced fitness in the low-oxygen environment of our twitching assay.

Additional evidence supports the importance of this high-affinity Cco in *R. solanacearum*. The *ccoNOQP* genes are considered 'core' genes (Guidot et al. 2007), meaning that these are found in the genomes of all or nearly all *R. solanacearum* strains studied to date. Our previous IVET screen found the *rex4* ferredoxin (*ipx38*) was up-regulated during mid-phase disease in *R. solanacearum* phylotype II strain K60 (Brown and Allen 2004). Moreover, recent microarray analysis of UW551 showed that *ccoG* was up-regulated 10.3-fold in planta ($P = 0.019$). Similarly, the log₂ (absolute) expression levels of *ccoNOQP* were moderately high in planta, 11.5, 9.7, 9.4, and 11.8, respectively, during active bacterial wilt disease ($P = 0.024, 0.022, 0.013$, and 0.022 , respectively) (F. Meng, J. Jacobs, L. Babujee, and C. Allen unpublished results).

This study identified *R. solanacearum* R3bv2 genes involved in rhizosphere fitness and bacterial wilt virulence. While not at all exhaustive, this gene set provides insight into this pathogen's behavior in the rhizosphere of its tomato host. The screen,

which used TRE in a soft agar medium rather than the natural tomato rhizosphere environment, certainly affected the traits identified. A complementary although technically daunting approach would be to use direct transcriptomic analysis to find *R. solanacearum* genes that are expressed in the rhizosphere before infection but not during pathogenesis in the stem.

Our results also suggest a potential approach to bacterial wilt disease management. Can we identify one or more components of root exudate that induce pathogen genes critical for wilt disease initiation? Such relationships exist in other soil-borne pathogens. For example, sorgolactone and sesquiterpene derivatives exuded from sorghum roots induce germination of seeds of the root parasites belonging to genus *Striga* (Ejeta 2007). Sorghum genotypes that produced very low levels of these stimulating chemicals were resistant to colonization (Ejeta 2007). Can we similarly exploit the tomato-*R. solanacearum* rhizosphere interaction? Potential approaches include inactivating the putative plant signals through breeding for host lines that do not produce signal, the addition of compounds that inactivate plant signals, or selecting for signals that inhibit attachment to roots (Whipps 2001). Breeding has also been used to improve the effectiveness of biocontrol agent colonization in the rhizosphere (Smith et al. 1997). These points should be considered as we strive to translate our findings into application.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions.

All *R. solanacearum* strains used in this study were derived from the wild-type R3bv2 strain UW551 (Williamson et al. 2002) (Table 2). *R. solanacearum* strains were cultured at 28°C in casamino peptone glucose (CPG) broth or CPG solid medium

Table 2. Strains and plasmids used in this study

Strain or plasmid	Description	Source
<i>Escherichia coli</i>		
DH5α	<i>F⁻ endA1 relA Φ80 lacZΔM15 hsdR17 supE44 thi-1 recA1 gyrA96</i>	Invitrogen, Carlsbad, CA, U.S.A.
HB101	<i>F⁻ thi-1 hsd20 supE44 recA13 ara-14 leuB6 proA2 lacY1 galK2 psdL20 xyl-5 mtl-1</i>	Boyer and Rouillan-Dussoix 1969
<i>Ralstonia solanacearum</i>		
UW551	Wild-type race 3, biovar 2, isolated from geranium	Williamson et al. 2002
UW551cheW	UW551cheW:: <i>aacC1</i> , Gm ^r	This study
<i>PPiA</i> -forward	UW551cheW; <i>pilA</i> promoter (forward orientation):: <i>cheW</i> ; Km ^r	This study
<i>PPiA</i> -reverse	UW551cheW; <i>pilA</i> promoter (reverse orientation):: <i>cheW</i> ; Km ^r	This study
<i>PflhDC</i> -forward	UW551cheW; <i>flhDC</i> promoter (forward orientation):: <i>cheW</i>	This study
<i>PflhDC</i> -reverse	UW551cheW; <i>flhDC</i> promoter (reverse orientation):: <i>cheW</i>	This study
<i>PcheW</i> -forward	UW551cheW; <i>cheW</i> promoter (forward orientation):: <i>cheW</i>	This study
<i>PcheW</i> -reverse	UW551cheW; <i>cheW</i> promoter (reverse orientation):: <i>cheW</i>	This study
UW551cheW <i>rex4</i>	UW551cheW:: <i>aacC1</i> , Gmr <i>rex4</i> (4Fe4S::gus), Gm ^r , Km ^r	This study
UW551cco	UW551cco:: <i>aacC1</i> , Gm ^r	This study
UW551cco(pUFcco)	UW551cco complemented with pUFJ10cco, Gm ^r , Km ^r	This study
UW551-rif	UW551 spontaneous mutant, Rif ^r	Swanson et al. 2005
UW551-GFP	UW551 constitutively expressing GFP, Tc ^r	This study
UW551cco-GFP	UW551-2 constitutively expressing GFP, Tc ^r	This study
Plasmids		
pSTBlue-1	Cloning vector, Ap ^r , Km ^r	Novagen, Madison, WI, U.S.A.
pLAFR3	Broad-host range cosmid vector, Tc ^r	Taskawicz et al. 1986
pVO155	Cloning vector, Km ^r , <i>oriT</i> , <i>uidA</i>	Oke and Long 1999
pRK600	pRK2013 ntp::Tn9, Cm ^r , Nm ^s	Finan et al. 1986
pJCcheW	pVO155 with promoterless UW551 <i>cheW</i>	This study
pJYdcheWgm	K60 <i>cheW</i> :: <i>aacC1</i> in pSTJYoT, Ap ^r , Gm ^r , Km ^r	Yao 2006
pLJYcheW2.0	pLAFR3 containing <i>cheW</i> with promoter, Tc ^r	Yao 2006
pUCGm	Plasmid with <i>aacC1</i> , Gm ^r	Schweizer 1993
pSTcco	PCR-amplified 1.6-kb fragment of UW551 RRS _L _01401 in pSTBlue-1, Ap ^r , Km ^r	This study
pSTcco::Gm	0.85-kb Gm ^r cassette in <i>Nco</i> I site of RRS _L _01401 in pSTcco, Ap ^r , Km ^r , Gm ^r	This study
pUFJ10	Cosmid vector, Gm ^r , Km ^r	Gabriel et al. 2006
pUFJ10::cco	PCR-amplified 2.4-kb fragment of UW551 RRS _L _01401 with promoter in pUFJ10, Gm ^r , Km ^r	This study

^a Ap^r, Cm^r, Gm^r, Km^r, Rif^r, Tc^r, and Cm^r = ampicillin, chloramphenicol, gentamicin, kanamycin, rifampicin, tetracycline, and chloramphenicol resistance, respectively, and Nm^s = neomycin sensitivity. GFP = green fluorescent protein, PCR = polymerase chain reaction.

(Hendrick and Sequeira 1984; Kelman 1954). Boucher's minimal medium (BMM) (Boucher et al. 1985) was used when minimal medium was required. Taxis agar (TA) consisted of BMM buffered with 20 mM morpholineethanesulfonic acid to pH 6.5, supplemented with 4 mM aspartic acid as the carbon source. TA was semisolidified with 0.2% noble agar and TRE were added at 1% concentration, when required.

E. coli strains were grown at 37°C, using Luria-Bertani (Ausubel et al. 1995) solid or liquid media supplemented with an appropriate antibiotic. Antibiotics were added to media for final concentrations (milligrams per liter) as follows: kanamycin (Km), 25; Gm, 5; chloramphenicol, 15; naladixic acid, 4; tetracycline, 25; and rifampicin, 25. Unless otherwise noted, media components were obtained from Difco Laboratories (Detroit) and chemicals from Sigma-Aldrich (St. Louis).

DNA manipulation and sequence analysis.

Standard methods were used to isolate chromosomal and plasmid DNA and to perform cloning and PCR (Ausubel et al. 1995). *E. coli* and *R. solanacearum* were transformed by electroporation as previously described (Allen et al. 1991). Triparental mating was used to introduce plasmid constructs with donor plasmid pJC*cheW* and helper plasmid pRK600 in *E. coli* HB101 (Finan et al. 1986). Oligonucleotide synthesis was performed by Integrated DNA Technologies (Coralville, IA, U.S.A.). Sequencing was performed at the University of Wisconsin-Madison Biotechnology Center (Madison, WI, U.S.A.), using automated fluorescent sequencing. Biology Workbench, Softberry, and NEBcutter databases, the UW551 genome database, and National Center for Biotechnology Information and the Integrated Microbial Genomics databases were used to view and analyze DNA sequences.

Construction of UW551Δ*cheW*.

A UW551*cheW* deletion mutant was constructed by double recombination, using plasmid pJYd*cheW*Gm (Yao and Allen 2006) in which the entire *cheW* gene was replaced in the UW551 genome by the Gm-resistance cassette (*aacCI*). Proper deletion of *cheW* and replacement by *aacCI* was confirmed by PCR and Southern blot analysis. Wild-type swimming motility of the mutant was confirmed by microscopy, and lack of taxis was confirmed on taxis agar. To complement this mutant, pLJY*cheW*2.0 (Yao and Allen 2006) was electroporated into the *cheW* mutant strain. Restoration of chemotaxis was confirmed on taxis agar.

Construction of pJC*cheW*.

The promoter-trapping vector pJC*cheW* was derived from pVO155 (Oke and Long 1999). pJC*cheW* carries the promoterless *cheW* ORF sequence cloned directly upstream of the promoterless *uidA* (*gus*) reporter gene as well as the Km- and ampicillin-resistance genes used for selection. The promoterless *cheW* gene was PCR-amplified from *R. solanacearum* UW551 (*cheW* forward and reverse primers, respectively, 5'-GCTCTAGATGATTGATTGAGGGAGCA-3' and 5'-GCTCTAGATTGTGTCCGTCGTTGTTTC-3'). Both primers contained *Xba*I restriction sites to aid in cloning. The sense primer contained stop codons in all three reading frames and a putative ribosome binding site to generate a functional transcriptional fusion. To ensure that pJC*cheW* functioned correctly, we constructed three control plasmids containing known promoters. Plasmids carrying the native *cheW* promoter in both the forward (pP*cheW*-forward) and reverse (pP*cheW*-reverse) orientation with respect to *cheW* were created by inserting a PCR-amplified, 517-bp DNA fragment (forward primer 5'-TCAGC TCATCAATCTGGTCG-3' and reverse primer 5'-AGGTAATG TCGGAAAACGGA-3') containing the putative *cheW* pro-

motor region directly upstream of the promoterless *cheW* ORF. Additional control plasmids carried the highly expressed pilin structural gene promoter *PpilA* as well as the promoter of the flagellar regulator *FlhDC*, *PflhDC* (Brown and Allen 2004; Kang et al. 2002). Promoter fragments were obtained by restriction digestion, were treated with Klenow (NEB), and were ligated into pJC*cheW* at the *Xho*I site. Constructs were analyzed by restriction digestion to confirm the orientation of the promoter regions. All constructs were tested for the ability to restore UW551Δ*cheW* chemotaxis on taxis agar, and gene expression was determined quantitatively by a GUS activity assay (Jefferson 1986).

Construction of the promoter-trapping library.

R. solanacearum UW551 genomic DNA libraries containing potential promoter sequences were created as described (Oke and Long 1999) and were ligated into pJC*cheW*. Two separate libraries were constructed with an average insert size of 800 bp, as assessed by gel analysis. Over 65,000 colonies from the libraries were introduced into UW551Δ*cheW* in eight separate triparental matings. Integration of the plasmid into the *cheW* mutant strain occurs through single recombination at the site of homology between the chromosome and the cloned DNA fragment; the plasmid could not recombine at the *cheW* locus because it was deleted from UW551Δ*cheW*. Strains with integrated pJC*cheW* were selected on CPG agar amended with Km and were used in subsequent *rex* screening.

Preparation of TRE.

Seeds of the wilt-susceptible tomato cultivar 'Bonny Best' were surface-sanitized as previously described, with some modification. Briefly, seeds were agitated for 30 min in sterile water, 5 min in 95% ethanol, 5 min with sterile water, 5 min in 20% bleach solution, and then, rinsed five times for 5 min with sterile water. Seeds were incubated overnight at 4°C for vernalization and were then germinated either on 1% water agar or directly on sterile cellulose filters at 28°C for 48 to 72 h, when rootlets and stems began to emerge. For each extraction, 20 to 30 seedlings were transferred to a sterile 1L filter apparatus (0.2 μm, Corning Glass, Corning, NY, U.S.A.) and were allowed to grow an additional 13 to 16 days, or until secondary roots began to form. Seedlings were maintained on one-tenth strength filter-sterilized Hoagland's solution. For the TRE collection, 5 to 10 ml of sterile Hoagland's solution was added to the filter and allowed to sit overnight, and the solution was collected by vacuum filtration and stored at -80°C in 1-ml aliquots. Root exudates were tested for sterility and the ability to support UW551 growth on TA and were characterized for protein content, using a bicinchoninic acid assay (Pierce, Rockford, IL, U.S.A.), and for reducing sugar content, using a modified Prado assay (Prado et al. 1998).

Screening the library: the *rex* screen.

Strains carrying the integrated pJC*cheW* were pooled and plated onto CPG agar amended with Km and Gm. Cells were washed, were collected by centrifugation, and were adjusted to an optical density at 600 nm (OD₆₀₀) of 0.05 (approximately 5 × 10⁷ CFU/ml). Bacterial suspension (2 μl, 1 × 10⁵ CFU) were placed into the center of a taxis agar plate supplemented with 4 mM aspartic acid and 1% (vol/vol) TRE and were incubated at 28°C until measurable taxis halos formed. For each library, the number of inoculated plates ranged from 30 to 45. Any strain that carried a DNA fragment capable of restoring chemotaxis to the nontactile *cheW* mutant formed a halo by moving up the nutrient gradient created by bacterial growth on the taxis media. Cells that had migrated (*che*+) were recovered by cutting the halo out of the taxis plate. Resulting colonies were

screened simultaneously on TA and TA with 1% TRE, with the wild-type strain as the control. Those strains that formed halos as large or larger than the wild-type control on TA plus TRE were saved for further analysis.

Analysis of the *rex* library.

To characterize active *rex* promoters, we transferred the integrated pJCheW+insert from UW551 Δ cheW using retrotransfer (Rainey et al. 1997), a conjugation procedure that recreates the original *rex* fusion in *E. coli* DH5 α . The inserted DNA fragment in purified pJCheW+insert was sequenced from the 5' end of the transcriptional fusion in the recovered *rex* fusions, using primer 5'-GTTTCCTTGACTTCCATTGC-3'. These sequences were compared with the UW551 genome database to determine the likely promoter driving *cheW* expression. The expression levels of *rex* fusion strains were determined by measuring their relative GUS activity following growth in minimal media with and without 1% TRE.

Quantitative GUS activity assays.

GUS activity was measured as described (Jefferson 1986). To validate promoter activities in pJCheW and to measure expression of *rex* fusions, cells were collected from overnight culture and were resuspended and extracted for GUS activity. Total cell number was determined by dilution plating an aliquot. For samples grown under low-oxygen conditions, 1 ml of suspension was transferred immediately into GUS extraction buffer and an additional 100 μ l was removed to determine cell numbers. GUS activity was thus determined as nanomole 4-methylumbelliferone released per minute per cell.

Selection and mutagenesis of a *cbb₃*-type *cco* in UW551.

Mutagenesis of *cbb₃*-cco. We mutated RRSL_01401, the first ORF in an operon encoding for structural proteins of a *cbb₃*-type Cco (*cbb₃*-cco), using marker-assisted gene replacement. Briefly, an approximately 1.8-kb region of RRSL_01401 was amplified using primers 5'-CAGACCTTCAATTA CCGGT-3' and 5'-CTTGACAGATAGGCCACCAC-3'. The product was A-T cloned into pSTBlue-1 (Km^r) (Novagen, Inc., Madison, WI, U.S.A.) to create pSTcco. pUCGm (Schweizer 1993) was digested with *Bam*H1 to liberate the *aacC1* Gm-resistance gene cassette. The insert of pSTcco was digested with *Nco*I, and *aacC1* was blunt-end cloned into the site using Klenow, creating pSTcco::Gm. pSTcco::Gm was introduced into UW551 by electroporation, and transformants were selected for Gm resistance and Km sensitivity. The mutation in the resulting strain, UW551cco, was confirmed by PCR and Southern blot analysis.

Complementation of UW551cco. A 2.4-kb region of RRSL_01401 including the likely upstream promoter sequence was amplified using primers 5'-CTTTGTGCTGATGGCAG TG-3' and 5'-GAGTAGTGCCGTAGCGTTC-3' and were A-T cloned into pSTBlue-1 (Km^r) (Novagen) to create pSTcco-comp. This insert was liberated by *Eco*RI digestion and cloned into *Eco*RI-digested pUFJ10 (Gm^r, Km^r) (Gabriel et al. 2006) to create the complementation plasmid pUFcco (Km^r). pUFcco was electroporated into competent UW551cco to create the complemented strain UW551cco(pUFcco).

Virulence assays.

We tested the virulence of the *cbb₃*-cco mutant on 'Bonny Best' tomato plants, using either a naturalistic soil-soak or by direct inoculation through a cut petiole as described (Tans-Kersten et al. 1998). Briefly, for soil-soak, a bacterial suspension was poured directly into individual pots containing 21-day-old unwounded tomato plants to a final cell density of 5×10^7 to 1×10^8 CFU/g soil. For petiole inoculations, approxi-

mately 200 cells were applied to the cut petiole of the first true leaf of 21- to 22-day-old tomato plants. Bacterial wilt progress was measured as previously described (Yao and Allen 2007). All plant assays had 16 plants per treatment and were performed three times.

Population studies.

Plant colonization. To quantify tomato-stem colonization by wild-type and mutant strains, we measured bacterial population sizes in plants following petiole inoculation, as described above. At 4, 6, and 8 days after inoculation, approximately 1 cm of tomato stem was harvested from above and just below the inoculation point. The plant tissue was ground in 1 ml of sterile water and the dilution was plated onto CPG and was amended with antibiotic when necessary, and was then incubated for 48 h at 28°C. Colonies were counted to determine number of cells per gram of stem tissue. Each experiment contained at least three plants per treatment and was repeated four times, for a total of 19 to 20 plants per strain.

Competition study. Plants (21 to 22 days old) were soil-soak inoculated with a 1:1 mixture of UW551-rif and the *cco* mutant strain. When each plant developed wilting symptoms, a 1-cm portion of the stem was cut at approximately 1 cm above the soil line to avoid soil contamination. The stem tissue was ground and a dilution was plated as described above, to determine the number of colony-forming units per gram of stem tissue of each strain. Each experiment contained at least five plants per treatment and was repeated four times on a total of 27 plants.

Growth and survival under low-oxygen conditions.

To measure bacterial growth in defined oxygen levels, initial inoculum was prepared as described above. Each strain was adjusted to an OD₆₀₀ = 0.01 (1×10^7 CFU/ml) in 125-ml side-arm flasks and was then subjected to vacuum for 5 min with swirling every 30 s, to release and remove oxygen from the medium. The suspension was immediately dispensed in 25-ml volumes into 50 ml of vented-cap tissue-culture flasks (Falcon; Franklin Lakes, NJ, U.S.A.), and a flask containing each strain was placed in plastic storage bags designed for vacuum (Reynolds, Richmond, VA, U.S.A.). Air was immediately removed from the bags by applying vacuum and then replaced with a mixture of 21, 5, or 0.5% oxygen gas with nitrogen gas balance (Airgas North Central, Carol Stream, IL, U.S.A.). Vacuum purging and oxygen replacement was repeated twice more for each treatment to fully purge the bags. Suspensions were then incubated at 22 to 24°C with gentle rotation at 100 rpm to facilitate the gas exchange within the bag. Resazurin strips (Oxoid, Hants, U.K.) confirmed the expected oxygen levels inside each bag. After 24 or 120 h, each bottle was sampled and dilution was plated as described above.

Visualization and colonization

of *R. solanacearum* strains on tomato roots.

GFP-expressing strains of UW551 and UW551cco were prepared by natural transformation using genomic DNA from K60-GFP, a phylotype II sequevar 7 (race 1 biovar 1) strain of *R. solanacearum* that expresses GFP (Bertolla et al. 1997; Yao and Allen 2007). UW551-GFP was previously shown to have no growth or virulence defect compared with its parent UW551 (J. Yao and C. Allen *unpublished results*). Tetracycline-resistant transformants were visualized under a fluorescent microscope to confirm the expected green fluorescent phenotype.

Inoculation, observation, and quantification of *R. solanacearum* on tomato roots. 'Bonny Best' seeds were surface-sterilized and germinated on 1% water agar amended with strepto-

mycin to avoid seed-borne contaminants. Germinated seeds were transferred to sterile Magenta boxes and were maintained under light on one-tenth strength Hoagland's solution. Seedlings (12 to 16 days old) were incubated without aeration in suspensions of GFP-labeled strains (1×10^7 CFU ml⁻¹) for 1.5 h, to allow bacteria to adhere to roots but not colonize them. Roots were then excised, gently rinsed and blotted on tissue, measured, ground in sterile water, and dilution-plated to determine the populations of adhering cells.

To observe root colonization progress, roots incubated with *R. solanacearum* for 4 h were excised, rinsed, gently blotted on tissue, and visualized under a fluorescent microscope as described (Yao and Allen 2006). To compare *R. solanacearum* population sizes on these root surfaces, a matched set of seedlings from the 4-h timepoint were treated similarly and measured and were then ground in 1 ml of sterile water and dilution-plated.

Statistical analysis.

Strain growth was analyzed by mixed model or general linear model analysis of variance with Tukey's significant difference test. Virulence assay data were analyzed by repeated measures analysis of variance (Tans-Kersten et al. 1998). Log transformation was used to analyze tomato stem populations. The Mann-Whitney median test was used to further analyze population data from single strain inoculations, and the paired *t*-test was used to analyze population data from the competition assay. Data analyses were conducted in JMP Ver. 7.0.2 and Ver. 8 (SAS Institute, Raleigh, NC, U.S.A.) or in Minitab Release 14.13 (State College, PA, U.S.A.).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Integrated Microbial Genomes database:
img.jgi.doe.gov/cgi-bin/pub/main.cgi
 myCAP(my Collaborative Annotation Portal UW551 genome database:
vision.biotech.ufl.edu/mycap
 National Center for Biotechnology Information website:
www.ncbi.nlm.nih.gov
 New England Biolabs's NEBcutter tools.neb.com/NEBcutter2/index.php
 San Diego Supercomputer Center's Biology Workbench:
workbench.sdsc.edu
 Softberry website: linux1.softberry.com/berry.phtml