***Ralstonia solanacearum* Encounters an Oxidative Environment During Tomato Infection**

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***Ralstonia solanacearum* genes that are induced during tomato infection suggested that this pathogen encounters reactive oxygen species (ROS) during bacterial wilt pathogenesis. The genomes of *R. solanacearum* contain multiple redundant ROS-scavenging enzymes, indirect evidence that this pathogen experiences intense oxidative stress during its life cycle. Over 9% of the bacterium's plant-induced genes were also upregulated by hydrogen peroxide in culture, suggesting that oxidative stress may be linked to life in the plant host. Tomato leaves infected by *R. solanacearum* contained hydrogen peroxide, and concentrations of this ROS increased as pathogen populations increased. Mutagenesis of a plant-induced predicted peroxidase gene, *bcp*, resulted in an *R. solanacearum* strain with reduced ability to detoxify ROS in culture. The *bcp* mutant caused slightly delayed bacterial wilt disease onset in tomato. Moreover, its virulence was significantly reduced on tobacco plants engineered to overproduce hydrogen peroxide, demonstrating that Bcp is necessary for detoxification of plant-derived hydrogen peroxide and providing evidence that host ROS can limit the success of this pathogen. These results reveal that *R. solanacearum* is exposed to ROS during pathogenesis and that it has evolved a redundant and efficient oxidative stress response to adapt to the host environment and cause disease.**

Ralstonia solanacearum is a soil-dwelling gram-negative bacterium that causes bacterial wilt disease in over 200 plant species (Hayward 1991). Due to its large host range and broad geographic distribution, this pathogen causes large crop losses worldwide, especially in developing tropical countries. The bacterium enters the host through wounded roots or sites of secondary root emergence; once inside a host, it quickly spreads and multiplies (Schell 2000). During plant infection, *R. solanacearum* effectively colonizes the xylem vessels, a nutrient-poor and microaerobic environment (Pegg 1985), where the bacterium nonetheless reaches populations of 10^9 CFU/g of plant tissue (Schell 2000). *R. solanacearum* possesses diverse virulence factors that act quantitatively to cause disease; these include extracellular polysaccharide, cell-wall-degrading enzymes, flagellar and twitching motility, chemotaxis, and type III secreted effectors (Arlat et al. 1992; Denny and Baek 1991; Gonzalez and Allen 2003; Kang et al. 2002; Kao et al. 1992; Liu et al. 2005; Tans-Kersten et al. 2001; Yao and Allen 2006). These virulence factors are controlled by a

complex regulatory signal transduction pathway (Schell 2000) that responds to both environmental signals and the quorum-sensing molecule 3-hydroxy palmitic acid methyl ester (Flavier et al. 1997). Although much is understood about these virulence factors and their regulation, little is known about how *R. solanacearum* adapts to effectively colonize, multiply, and spread in the host environment.

Promoter-trapping methods that operate in a specific environment (e.g., a host), commonly known as in vivo expression technology (IVET), have revealed the mechanisms bacteria use to adapt to their environments. IVET identifies genes needed during microbe–host interactions by selecting microbial promoters that are active during the interaction rather than relying on loss-of-function assays (Rediers et al. 2005). A previous IVET screen on *R. solanacearum* during midphase pathogenesis of tomato identified 153 in planta-expressed (*ipx*) genes (Brown and Allen 2004). Approximately 10% of these *ipx* genes are predicted to encode proteins involved in stress response, notably the oxidative stress response, providing indirect evidence that *R. solanacearum* is exposed to an oxidative environment during plant pathogenesis.

When plants recognize potential pathogens, they produce an oxidative burst that consists of superoxide, hydrogen peroxide, and hydroxyl radicals. This oxidative burst, which triggers plant defense signal transduction pathways, occurs in two phases: phase I is short-lived, nonspecific, and occurs within minutes after pathogen recognition and phase II is prolonged and occurs 1.5 to 3 h after pathogen detection (Lamb and Dixon 1997). Phase II of the oxidative burst often leads to disease resistance via the hypersensitive response and the induction of host defense genes (Apel and Hirt 2004). Reactive oxygen species (ROS) generation is mainly attributed to cell-wall-bound peroxidases and NADPH oxidases, similar to those found in macrophages (Torres and Dangel 2005). It is believed that the oxidative burst may have direct antimicrobial effects; however, the amounts of ROS experienced by pathogens during host infection are unknown.

Because ROS cause severe macromolecule damage leading to mutations and cell death, bacteria have evolved complex, specific, and tightly regulated oxidative stress responses to protect themselves (Imlay 2008). The bacterial oxidative stress response includes proteins involved in signal transduction and regulation, ROS-scavenging enzymes, and repair of oxidative damage (Loprasert et al. 1996). Plant-associated bacteria express several oxidative stress response genes during interactions with their hosts (Okinaka et al. 2002; Rediers et al. 2005; Smith et al. 1996; Tamir-Ariel et al. 2007), and some are required for virulence. In *Agrobacterium tumefaciens*, *katA* (catalase) and *sodB* (superoxide dismutase) are necessary for tumorigenesis (Saenkhram et al. 2007; Xu and Pan 2000) and *Erwinia chrysanthemi* *sodA* and *msrA* (methionine sulfoxide reductase)

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mutants have reduced virulence on African violets and chicory leaves, respectively (Hassouni et al. 1999; Santos et al. 2001). However, the effect of host-derived ROS and the role of the oxidative stress response have not been investigated in bacterial wilt disease or in any xylem-dwelling vascular wilt pathogen.

We hypothesized that *R. solanacearum* is exposed to plant-derived ROS during plant pathogenesis and that the oxidative stress response genes of *R. solanacearum* are expressed in planta, where they contribute to survival and adaptation in the host and to disease development. In support of this hypothesis, we found that *R. solanacearum* is exposed to hydrogen peroxide in tomato tissue, and that this pathogen has several redundant oxidative stress response-encoding genes, some of which are expressed during pathogenesis. Our results suggest that the oxidative stress response of *R. solanacearum* is an important adaptation that contributes to its success inside its diverse plant hosts.

RESULTS

R. solanacearum possesses multiple and redundant oxidative stress response genes.

To identify elements of the oxidative stress response in *R. solanacearum*, we searched the complete genome of *R. solanacearum* GMI1000 (Salanoubat et al. 2002) for open reading frames (ORFs) encoding likely orthologs of well-characterized ROS-scavenging enzymes from other bacteria. Amino acid sequences of the predicted scavenging enzymes from GMI1000 were used to search the *R. solanacearum* UW551 8X draft genome (Gabriel et al. 2006) via BLASTP (Altschul et al. 1997). This process identified genes predicted to encode multiple and redundant ROS-scavenging enzymes in both genomes (Table 1). The predicted ROS-scavenging enzymes in the two *R. solanacearum* genomes share high amino acid identities (83 to 99%) and were present in both strains, with the exception of one of the two *katG* genes, which was absent from the UW551 strain genome (Table 1). Predicted proteins include at least five peroxidases, two alkyl hydroperoxide reductase systems, two catalases, and two superoxide dismutases. One of the predicted peroxidases, bacterioferritin co-migratory protein (Bcp), was previously identified as an *ipx* gene in an IVET screen of *R. solanacearum* for promoters specifically upregulated during tomato pathogenesis (Brown and Allen 2004). Bacteria commonly regulate the oxidative stress response with OxyR, for the hydrogen peroxide stress response, and SoxRS, for the superoxide stress response (Imlay 2008). An

OxyR ortholog (closest match outside the genus *Ralstonia*: *Bordetella avium*; E = 2e-128) was found in the available genomes of *R. solanacearum*. However, no likely SoxRS orthologs were found using a cutoff of 35% amino acid identity over the length of the proteins.

Several *R. solanacearum ipx* genes are also upregulated by hydrogen peroxide.

To test whether other *ipx* genes, which included many genes of unknown function, are part of the *R. solanacearum* oxidative stress response, we determined whether hydrogen peroxide could induce expression of the 153 *ipx* fusion strains. The *ipx* strains carry transcriptional fusions to the promoterless *uidA* (*gus*) reporter gene. Expression of the *gus* reporter gene in the presence of 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) generates a visible blue pigment (Jefferson et al. 1986). On unmodified rich medium, the *ipx* colonies were white, indicating lack of fusion gene expression (Brown and Allen 2004). However, 14 *ipx* fusions (9% of all *ipx* genes tested) were blue on rich medium containing hydrogen peroxide (Table 2). Predicted functions of *ipx* genes induced by hydrogen peroxide include hydrogen peroxide detoxification; amino acid, nucleotide, and ascorbate metabolism; sugar transport; signal peptides; transcriptional regulators; and several genes of unknown function.

One of the hydrogen peroxide-inducible *ipx* fusions, *ipx142*, was originally described as backwards; that is, inserted near a promoter in the opposite orientation with respect to the *uidA* gene (Brown and Allen 2004). However, reanalysis of the genomic context around the predicted promoter of *ipx142* in the GMI1000 genome indicates that *trxA* (Rsc1188, encoding a putative thioredoxin, a protein involved in the bacterial oxidative stress response) is in the correct orientation with respect to the *uidA* gene, suggesting that the *trxA* promoter drives *uidA* expression in this strain (Fig. 1A).

R. solanacearum is exposed to hydrogen peroxide in tomato plants.

To determine whether *R. solanacearum* experiences ROS during growth in an important host, we used a stain specific for hydrogen peroxide during *R. solanacearum* infection of tomato leaves. Oxidation of 3,3-diaminobenzidine (DAB) by hydrogen peroxide creates a visible brown precipitate that can be qualitatively assessed (Orozco-Cardenas and Ryan 1999). Leaves infiltrated with concentrations of *R. solanacearum* cells ranging from 5×10^5 to 1×10^9 CFU/ml were cut at the petiole

Table 1. Predicted reactive oxygen species-scavenging enzymes in *Ralstonia solanacearum* genome sequences

Predicted function (gene name)	Gene number ^a		E value, relevant match
	GMI1000	UW551	
AhpC/TSA family peroxidase	RSc0754	RRSL01499	1e-108, <i>Pseudomonas putida</i>
Glutathione peroxidase	RSc2674	RRSL03227	3e-85, <i>Ralstonia pickettii</i>
Glutathione peroxidase	RSc0307	RRSL01638	3e-46, <i>Xanthomonas campestris</i> pv. <i>vesicatoria</i>
Thiol peroxidase (<i>tpx</i>)	RSc2790	RRSL02667	5e-59, <i>P. fluorescens</i> Pf-5
Peroxidase (<i>bcp</i>)	RSc1317	RRSL00135	2e-61, <i>P. fluorescens</i> Pf0-1
Organic hydroperoxide resistance (<i>ohr</i>)	RSp1107	RRSL04748	3e-60, <i>P. atrosepticum</i>
Alkyl hydroperoxide reductase (<i>ahpD</i>)	RSc1645	RRSL04570	4e-55, <i>Bordetella petrii</i>
Alkyl hydroperoxide reductase (<i>ahpC2</i>)	RSc1646	RRSL04571	4e-55, <i>B. petrii</i>
Alkyl hydroperoxide reductase (<i>ahpC1</i>)	RSp0245	RRSL01699	2e-95, <i>X. axonopodis</i> pv. <i>citri</i>
Alkyl hydroperoxide reductase (<i>ahpF</i>)	RSp0246	RRSL01700	0.0, <i>P. fluorescens</i> Pf0-1
Catalase (<i>katGb</i>)	RSc0775	RRSL01521	0.0, <i>Burkholderia pseudomallei</i>
Catalase (<i>katGa</i>)	RSc0776	Absent	9e-133, <i>B. pseudomallei</i>
Catalase (<i>katE</i>)	RSp1581	RRSL02532	0.0, <i>P. aeruginosa</i>
Superoxide dismutase (<i>sodB</i>)	RSc2526	RRSL01451	1e-95, <i>B. petrii</i>
Superoxide dismutase (<i>sodC</i>)	RSc2368	RRSL03640	2e-31, <i>X. campestris</i> pv. <i>vesicatoria</i>

^a Name and numbers are according to GMI1000 and UW551 genomes (Gabriel et al. 2006; Salanoubat et al. 2002). Boldface denotes gene previously identified as plant-induced (Brown and Allen 2004).

after 24 h of incubation and stained with DAB (Fig. 2) or sampled to determine bacterial population size (Table 3). Brown precipitate indicating hydrogen peroxide was visible in leaves inoculated with 5×10^6 CFU/ml and higher, and the stain intensity increased with cell concentration. Leaves inoculated with lower cell densities were indistinguishable from the mock-inoculated control (data not shown). Leaves containing large populations and showing tissue collapse and necrosis produced the most intense color (Fig. 2F).

Leaves of the bacterial wilt-tolerant tomato line Hawaii 7996 were also infused with *R. solanacearum* cells. Although this stain is qualitative and, thus, would not detect small differences in hydrogen peroxide concentrations, the intensity of the DAB stain in this line was similar to that in susceptible leaves inoculated with the same bacterial suspensions (data not shown). Leaves of both plants contained similar numbers of *R. solanacearum* cells at the time of DAB stain (Table 3).

R. solanacearum bcp mutant K1317 has reduced ability to detoxify ROS in culture.

To investigate the virulence contribution of the in planta-expressed and hydrogen peroxide-induced predicted peroxidase Bcp, we created a *bcp* mutant strain, K1317 (Fig. 1B). The *R. solanacearum*-predicted Bcp was 39% identical at the amino acid level to the well-characterized *E. coli* Bcp over 95% of the protein sequence.

R. solanacearum K1317 grew as well as the wild type in minimal media supplemented with glucose and in susceptible tobacco leaves (cv. Bottom Special) (data not shown), suggesting that K1317 is not an auxotroph and does not have any general metabolic defects. However, K1317 was hypersensitive to hydrogen peroxide (Fig. 3A) and did not grow as well as its wild-type parent in media supplemented with the superoxide-generating agent paraquat (Fig. 3B). Introduction of a wild-type copy of *bcp* into K1317 partially restored a wild-type level of hydrogen peroxide resistance (Fig. 3A). Wild-type strain K60 and the K60-bcp mutant were significantly different from each other according to Fischer's least significant difference test ($P < 0.05$). However, the complemented mutant strain gave an intermediate value that was not significantly different from either the wild type or the mutant. The lack of complete complementation might be due to a fitness reduction caused by the complementing vector.

To evaluate the ability of K1317 to cause disease on susceptible tomato cv. Bonny Best, we performed a naturalistic soil soak assay (Tans-Kersten et al. 2001), inoculating unwounded

tomato plants with suspensions of wild-type or K1317 strains. Like the wild-type, K1317 killed all plants by 14 days postinoculation (dpi) (Fig. 4). However, plants inoculated with K1317 showed a slight but significant delay of bacterial wilt symptom onset on days 6 and 7 postinoculation.

R. solanacearum K1317 has reduced ability to kill host plants that overproduce hydrogen peroxide.

To determine whether increased plant-derived ROS can hinder *R. solanacearum* disease development, we infused *R. solanacearum* cells into the leaves of susceptible tobacco plants (cv. Petit Havana SR1) and a transgenic derivative with silenced leaf *catalase 1* gene, which overproduces hydrogen peroxide (Chamnongpol et al. 1996). Leaves of the transgenic plants showed less necrosis and leaf tissue collapse than the leaves of the parental line at 48 h after inoculation (data not shown), and *R. solanacearum* cell numbers were lower in the *catalase 1*-

Fig. 1. Genomic regions of *Ralstonia solanacearum* GMI1000. **A**, Genomic context of in vivo expression technology (IVET) fusion in planta-expressed (*ipx*) 142. Arrows indicate the direction of transcription: white arrows are open reading frames (ORFs) originating from the IVET plasmid and black arrows are ORFs from GMI1000 genome. Dotted lines indicate the location of the IVET fusion in the *R. solanacearum* genome. RSc1189 encodes a protein of unknown function, *trxA* encodes thioredoxin I, *rho* encodes a Rho transcriptional termination factor, *trpEG* encodes anthranilate synthase, and *uidA* encodes the β -glucuronidase reporter gene. **B**, Mutagenesis strategy for *bcp* (RSc1317). Downstream of the *bcp* ORF is RSc1316 (unknown function) and upstream are genes RSc1318 to RSc1322, which are predicted to encode proteins involved in lipopolysaccharide metabolism, and which may form an operon. The *bcp* gene was mutated by introduction of a gentamicin resistance gene cassette (*aacC1*, triangle) into a unique restriction site. These genomic regions are colinear in *R. solanacearum* UW551.

Table 2. *Ralstonia solanacearum* in planta-expressed (*ipx*) genes that were also upregulated by hydrogen peroxide in culture^a

<i>ipx</i> Strain ^b	Gene number		Predicted protein (gene name)
	GMI1000	UW551	
6	RSc0015	RRSL02946	Conserved hypothetical protein
7	RSc0086	RRSL04380	Conserved hypothetical protein; GST superfamily
18	RSc3367	RRSL00854	Hypothetical protein
19	RSc3389	RRSL04168	Hypothetical protein
52	RSp0536	Absent	Transmembrane sugar-proton symporter
65	RSc1317	RRSL00135	Peroxidase (<i>bcp</i>)
71	RSp1530	RRSL04639	L-ascorbate oxidase
74	RSc0076	RRSL02003	Proline-rich signal peptide
81	RSc3030	RRSL00873	Signal peptide protein
88	RSc0280	RRSL01667	Transcription regulator
112	RSc0378	RRSL03283	Anthranilate phosphoribosyl transferase
115	RSc1126	RRSL01951	CTP synthase
119	RSc2095	RRSL01253	Xanthine dehydrogenase
142	RSc1189	RRSL00301	Thioredoxin (<i>trxA</i>) ^c

^a Annotation and gene number according to GMI1000 and UW551 genomes (Gabriel et al. 2006; Salanoubat et al. 2002).

^b From Brown and Allen (2004).

^c Initially described as backwards.

silenced plants compared with those in the parental line (Fig. 5A). However, there was no measurable difference in population size between K60 and K1317 on either plant host (data not shown). Soil-soak virulence assays on these tobacco plants revealed that, by 10 dpi, both strains killed significantly more wild-type tobacco plants than the *catalase 1*-silenced derivative. Moreover, K1317 killed significantly fewer hydrogen peroxide-overproducing plants than K60.

DISCUSSION

As part of the host defense response, plants produce an oxidative burst. This burst occurs in two phases depending on the ability of the microbe to evade or suppress its host's defenses (Espinosa and Alfano 2004). Some successful pathogens only encounter the first, transient phase of the oxidative burst. However, other microbes experience the prolonged phase that

induces the host defense response and resistance (Lamb and Dixon 1997). Some bacterial plant pathogens differentially express oxidative stress response genes during midphase pathogenicity (Okinaka et al. 2002; Rediers et al. 2005; Tamir-Ariel et al. 2007), suggesting that they are exposed to a prolonged phase of the oxidative burst or continuous oxidative stress during host infection. *E. amylovora*, the causal agent of fire blight, has been shown to induce a sustained phase of the oxidative burst during pear leaf infection (Venisse et al. 2001). Thus, successful plant pathogens must possess efficient oxidative stress defense mechanisms in order to infect, spread in their hosts, and cause disease.

An IVET screen of *R. solanacearum* identified 153 genes that are induced during midphase pathogenicity; approximately 10% of these genes have predicted functions in stress defense (Brown and Allen 2004). These results suggest that this pathogen confronts a stressful environment during tomato patho-

Fig. 2. Generation of hydrogen peroxide in susceptible tomato leaves infected with *Ralstonia solanacearum* and visualized by 3,3-diaminobenzidine (DAB) staining after 24 h. Tomato leaves (cv. Bonny Best) were infused as follows: **A**, untreated control; **B**, water-inoculated control; **C**, leaves inoculated with *R. solanacearum* cells at 5×10^6 CFU/ml; **D**, 1×10^7 CFU/ml; **E**, 5×10^7 CFU/ml; **F**, 1×10^9 CFU/ml. This experiment was performed three times independently with similar results; representative results are shown.

genesis. Indeed, mutagenesis of in planta-expressed multidrug efflux pumps in *R. solanacearum* significantly reduced virulence (Brown et al. 2007), providing direct evidence that, during disease, *R. solanacearum* encounters antimicrobial compounds, probably derived from the host defense response. Genes predicted to encode elements of the oxidative stress response were among those identified in the IVET screen (Brown and Allen 2004), suggesting that ROS are among the stresses that *R. solanacearum* must overcome to succeed as a pathogen.

Bioinformatic analyses of two *R. solanacearum* genomes offered further evidence that this pathogen is exposed to ROS during its life cycle. Approximately 15 redundant ROS-scavenging enzymes were identified in two *R. solanacearum* genomes (Table 1), suggesting that *R. solanacearum* is well adapted for oxidative environments. One of the predicted peroxidases, *bcp*, was previously identified in the IVET screen (Brown and Allen 2004).

If *R. solanacearum* encounters oxidative stress during host infection, it seemed likely that some plant-induced genes also respond to oxidative stress. Fourteen of the IVET-identified genes (9%) were induced by hydrogen peroxide in culture (Table 2). This result suggests that these genes are part of an oxidative stress response that is induced while in the host. Predicted functions of several genes induced by hydrogen peroxide sug-

gest a role in the oxidative stress response: pyrimidine synthesis (*ipx 115*) for DNA damage repair; metabolism of the antioxidant ascorbate (*ipx 71*); amino acid biosynthesis (*ipx 112*), which might indicate a role in de novo protein synthesis or protein damage repair; and xanthine dehydrogenase (*ipx 119*), which is involved in purine metabolism resulting in the generation of the antioxidant uric acid (or urate) (Becker 1993). Thioredoxins (*ipx 142*) maintain the reduced state of intracellular disulfide bonds, including those in peroxidases (Imlay

Table 3. *Ralstonia solanacearum* cell populations in tomato leaves infiltrated and stained for hydrogen peroxide^a

Initial inoculum (CFU/ml)	<i>R. solanacearum</i> populations 24 h after inoculation (CFU/cm ² of leaf) ^b	
	Bonny Best	Hawaii 7996
5×10^5	5.2×10^6	1.5×10^7
1×10^6	1.1×10^7	2.6×10^7
5×10^6	4.6×10^7	3.9×10^7
1×10^7	1.2×10^8	1.4×10^8
5×10^7	1.6×10^8	3.6×10^8

^a Results shown are representative of two assays with similar results.

^b Population size at the time of H₂O₂ stain.

Fig. 3. *Ralstonia solanacearum* strain sensitivity to reactive oxygen species. **A**, Disk inhibition assay. A bacterial suspension was added to warm top agar and poured over agar plates. A filter disk containing 10 μ l of hydrogen peroxide was placed in the center of the plate and incubated at 28°C for 24 h. Initial inoculum in top agar: *R. solanacearum* K60/pLAFR3 (wild type), 1.61×10^7 CFU/ml; K1317/pLAFR3, 1.19×10^7 CFU/ml; K1317/pLAFR3::bcp, 6.25×10^6 CFU/ml. Results are the average of two independent experiments with four measurements per experiment; bars represent standard error of the mean. **B**, Growth curve of *R. solanacearum* strains in the presence of 1 μ M paraquat. *R. solanacearum* K60 (wild-type, squares) and K1317 (*bcp* mutant; circles) were grown in minimal medium broth in the presence (filled symbols) or absence (empty symbols) of 1 μ M paraquat (PQ). Results are the average of three independent growth curves, each with three flasks per treatment; bars represent standard error of the mean. Columns with different letters above are significantly different according to the Fisher least significant difference test ($P < 0.05$).

Fig. 4. Disease progress curves of *Ralstonia solanacearum* strains on susceptible tomato plants. Bacterial suspensions of *R. solanacearum* K60 (diamonds) and K1317 (circles) were poured into pots containing unwounded 15-day-old tomato plants. Disease symptoms were evaluated daily for 14 days using a 0-to-4 disease index. Results shown are the average of three independent experiments; bars indicate standard error of the mean. Inoculum ranged from 3.9×10^7 to 5.7×10^7 CFU/g of soil. Asterisks indicate days where wild-type and K1317 strains were significantly different according to analysis of variance ($P < 0.05$).

2008). Genes with predicted functions similar to those in Table 2 have also been identified in IVET screens of other pathogenic and beneficial bacteria (Rediers et al. 2005), suggesting that microbes associated with eukaryotes are exposed to ROS during interaction with their hosts.

The indirect evidence described above implies that *R. solanacearum* encounters oxidative stress during tomato infection. To more directly determine whether this pathogen is exposed to ROS during tomato pathogenesis, we visualized hydrogen peroxide in tomato leaves during infection with a specific stain (Fig. 2). Hydrogen peroxide was present in infected tomato leaves and, although the stain was not quantitative, its concentration appeared to increase as disease progressed and as the pathogen populations grew. This is evidence that *R. solanacearum* is exposed to ROS during mid- to end-stage pathogenesis and suggests that this pathogen might be experiencing the sustained phase of the host-derived oxidative burst that is usually associated with incompatible interactions or a continuous oxidative burst. This finding also suggests that the redundant ROS-scavenging enzymes and the additional IVET-identified genes that are also induced by hydrogen peroxide help *R. solanacearum* overcome this oxidative stress to successfully infect and persist in its hosts, and cause disease.

The similar levels of hydrogen peroxide in wilt-susceptible and wilt-tolerant tomato leaves in response to *R. solanacearum* suggest that the resistance in the tolerant line, Hawaii 7996, is not the result of a heightened oxidative burst. This result is consistent with the finding that *R. solanacearum* readily forms latent infections in Hawaii 7996 (Grimault et al. 1994). However, it is possible that, during natural disease progress, when *R. solanacearum* colonizes the xylem tissue before spreading out into leaves, this bacterium encounters different oxidative conditions (see below). In addition, given the qualitative nature of hydrogen peroxide detection by DAB, it is possible that small differences in hydrogen peroxide concentrations among the different tomato lines would not have been observed.

To test the contribution of *bcp* to the oxidative stress response and virulence of *R. solanacearum*, we created a *bcp* mutant, K1317 (Fig. 1A). In *Escherichia coli*, *bcp* encodes a thioredoxin-dependent peroxidase of the TSA/AhpC family

that is necessary for normal growth in the presence of hydrogen peroxide (Jeong et al. 2000). *Bcp* homologues are necessary for host colonization in the animal pathogens *Helicobacter pylori* and *Brucella melitensis* (Wang et al. 2005; Zygmunt et al. 2006). In addition, *bcp* homologues were identified in IVET screens of other plant-associated bacteria (Hammad et al. 2001; Rediers et al. 2003), suggesting that *bcp* is generally important in host-microbe interactions. The *R. solanacearum bcp* gene (*ipx 65*) is 20-fold induced in planta (Brown and Allen 2004), and was also upregulated in rich media in the presence of hydrogen peroxide (Table 2). *Bcp* was necessary for *R. solanacearum* ROS detoxification in culture, confirming its role in the oxidative stress response of *R. solanacearum*. The combination of bioinformatic analysis and the hypersensitivity of the *bcp* mutant to ROS in culture suggest that *Bcp* encodes a peroxidase in *R. solanacearum* (Fig. 3). However, this protein played a minor role in tomato virulence (Fig. 4), possibly because of compensation from other predicted oxidative stress response genes in *R. solanacearum* (Tables 1 and 2).

R. solanacearum was affected in growth and disease development in tobacco plants that overproduce hydrogen peroxide (Fig. 5), suggesting that host-produced ROS can limit pathogen success. However, because it is a very successful pathogen, *R. solanacearum* can evidently overcome the lower concentrations of ROS present in natural hosts. K1317 grew as well as its wild-type parent when cells were directly infused into tobacco leaves regardless of the tobacco host used (data not shown), suggesting that, under these artificial conditions, *Bcp* does not make a large contribution to ROS detoxification. In contrast, the hydrogen peroxide-overproducing plants were more resistant to K1317 than to the wild-type in a naturalistic soil-soak assay that required bacteria to infect plants through unwounded roots (Fig. 5B). These results indicate that plant-derived ROS can inhibit *R. solanacearum* growth in planta and, further, that *Bcp* is necessary for detoxification of plant-derived hydrogen peroxide. In addition, these results suggest that the oxidative stress encountered during natural disease progress by *R. solanacearum* is different from and possibly greater than that experienced following artificial leaf infusion.

Fig. 5. Behavior of *Ralstonia solanacearum* K60 in bacterial wilt-susceptible tobacco cv. Petit Havana SRI (white bars) and a transgenic derivative that overproduces hydrogen peroxide CatAS1 (gray bars). **A**, *R. solanacearum* cell populations in leaves were quantified via dilution plating. Data represent mean of two independent assays; bars represent standard error of the mean. **B**, Percent dead plants 10 days postinoculation. Inoculum ranged from 3.3×10^7 to 1×10^8 CFU/g of soil. Results shown are the average of three independent experiments; bars represent standard error of the mean. Asterisk indicates treatment that was statistically different according to analysis of variance ($P < 0.05$).

The slight delay in disease onset on susceptible tomato plants inoculated with K1317 on days 6 and 7 might coincide with a delayed host defense response. This late oxidative burst would coincide with maximum *R. solanacearum* populations in the plant and is consistent with the detection of hydrogen peroxide in tomato leaves, where the stain intensity increased with cell populations (Fig. 2). In addition, preliminary results indicate that *R. solanacearum* induces maximum host defense gene expression 7 to 8 days after soil-soak inoculation of susceptible tomato plants (A. Milling and C. Allen, *unpublished results*), significantly later than it occurred during previously studied infections that result in resistance (Lamb and Dixon 1997). Similarly, a *Xanthomonas campestris* pv. *malvacearum* infection leading to disease resulted in a delayed and less intense oxidative burst than an infection leading to resistance (Jalloul et al. 2002). Together, these results suggest that *R. solanacearum* is able to evade or suppress host defenses until it reaches high populations within the host, by which time any host response would be ineffectual.

Together, these findings demonstrate that *R. solanacearum* encounters oxidative stress during plant pathogenesis, and that an effective bacterial oxidative stress response is necessary and important for successful host infection. In addition, these direct and indirect results collectively suggest that the concentrations of ROS experienced by this pathogen during disease may have direct antimicrobial activity. Although Bcp was necessary for ROS detoxification in culture, a *bcp* mutant had only a minor reduction in virulence on tomato, very likely because the pathogen's other peroxidases can partially compensate for the loss of Bcp. This result highlights the limitations of a single-gene mutagenesis approach and reinforces our view that complex molecular interactions give rise to bacterial wilt disease. Given that successful microbial–host interactions result from the interaction of multiple gene products differentially expressed at different stages of the infection, it is not surprising that the *bcp* mutant has a small virulence defect. Consistent with our results, several genes identified in IVET screens of other plant-pathogenic bacteria also don't contribute measurably to virulence (Tamir-Ariel et al. 2007), including some genes identified in *R. solanacearum*'s IVET screen (Gonzalez et al. 2007). Because *R. solanacearum* possesses multiple and redundant genes involved in the oxidative stress response, mutagenesis of

oxyR, the only apparent regulator of *R. solanacearum*'s oxidative stress response, would shed light on the role of the oxidative stress response during host colonization and disease development of *R. solanacearum*.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

The bacterial strains and plasmids used in this study are listed in Table 4. *E. coli* strains were grown in Luria-Bertani medium (Miller 1992) at 37°C. All *R. solanacearum* strains in this study were derived from wild-type biovar 1 phylotype II sequevar 7 strain K60 (Kelman 1954). *R. solanacearum* strains were grown in CPG broth (Hendrick and Sequeira 1984) or CPG solid medium with 0.05% (wt/vol) tetrazolium chloride (Kelman 1954). Antibiotics were added to cultures at the following concentrations when necessary: kanamycin at 25 µg/ml, gentamicin at 12.5 µg/ml, and tetracycline at 15 µg/ml. Growth curves were performed in minimal BMM (Boucher et al. 1985) supplemented with 0.2% (wt/vol) glucose as previously described (Gonzalez and Allen 2003). Growth curves to determine the effect of paraquat (Ultra Scientific, N. Kingstown, RI, U.S.A.) on growth of strain K1317 were performed in BMM supplemented with 0.2% malate buffered with 50 mM morpholineethanesulfonic acid.

In planta growth of *R. solanacearum* strains was measured by quantifying bacterial multiplication in tobacco leaves (*Nicotiana tabacum* cv. Bottom Special or *N. tabacum* cv. Petit Havana SRI and its *catalase 1*-silenced derivative Cat1AS) (Chamnongpol et al. 1996) as previously described (Tans-Kersten et al. 2001).

DNA manipulations.

The isolation of plasmid, cosmid, and genomic DNA as well as polymerase chain reaction (PCR), cloning, and Southern hybridization were carried out by standard protocols (Ausubel et al. 1998). Competent cells of *E. coli* and *R. solanacearum* were created as previously described (Allen et al. 1991). Unless otherwise noted, chemical reagents were purchased from Sigma-Aldrich (St. Louis) and molecular biology reagents and kits were purchased from Promega Corp. (Madison, WI, U.S.A.).

Table 4. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Reference or source
<i>Escherichia coli</i> strain		
DH5α	F [−] <i>endA1 relA φ80lacZΔ supE44 thi-1 recA1 gyrA96</i>	Hanahan 1983
HB101	F [−] , <i>thi-1 hsdS20 supE44 recA13 ara-14 leuB6 proA2 lacY1 galK2 rpsL20 xyl-5, mtl-1</i>	Boyer and Roulland-Dussoix 1969
<i>Ralstonia solanacearum</i> strains		
K60	Wild type; phylotype II, biovar 1, sequevar 7	Kelman 1954
<i>ipx</i> strains	K60 <i>ΔtrpEG</i> ; diverse plant-induced promoters:: <i>trpEG</i> ; Km ^r	Brown and Allen 2004
K1317	K60 <i>bcp</i> :: <i>aacC1</i> ; Gm ^r	This study
PpilA-forward	K60 <i>trpEGΔ pilA</i> promoter (forward orientation) :: <i>trpEG</i> ; Km ^r	Brown and Allen 2004
PpilA-reverse	K60 <i>trpEGΔ pilA</i> promoter (reverse orientation) :: <i>trpEG</i> ; Km ^r	Brown and Allen 2004
Plasmids		
pSTblue-1	Km ^r Ap ^r	Novagen
pLAFR3	Tc ^r	Staskawicz et al. 1987
pUCGM	Gm ^r Ap ^r	Schweizer 1993
pCR-Blunt II-Topo	Km ^r Zeo ^r	Invitrogen
pVO155	Promoter probe plasmid with promoterless <i>uidA</i> gene; Km ^r	Oke and Long 1999
pRK600	pRK2013 <i>ntp</i> ::Tn9; Cm ^r	Finan et al. 1986
pSJYot	<i>oriT</i> from pVO155 cloned into pSTblue-1; Km ^r Ap ^r	J. Yao, <i>unpublished results</i>
pSTbcp	900-bp insert containing <i>bcp</i> in pSTblue-1; Km ^r Ap ^r	This study
pTOPObcp	900-bp insert containing <i>bcp</i> in pCR-Blunt II-Topo	This study
pLAFR3bcp	900-bp <i>EcoRI</i> fragment from pTOPObcp cloned into pLAFR3; Tc ^r	This study
pSTbcp:: <i>aacC1</i>	pSTbcp with 850-bp Gm ^r cassette insertion from pUCGM; Km ^r Ap ^r Gm ^r	This study
pSJYotTbcp:: <i>aacC1</i>	1.8-kb <i>EcoRI</i> fragment from pSRbcp:: <i>aacC1</i> cloned into pSJYot; Km ^r Ap ^r Gm ^r	This study

^a Ap^r, Km^r, Gm^r, Tc^r, and Zeo^r indicate resistance to ampicillin, kanamycin, gentamicin, tetracycline, and zeocin, respectively.

Oligonucleotides and sequence analysis.

DNA and protein sequences were analyzed using Biology Workbench, National Center for Biotechnology Information Blast (Altschul et al. 1997), NEB Cutter, and the genomic databases of phylotype II sequevar 1 (race 3, biovar 2) *R. solanacearum* UW551 and phylotype I sequevar 12 strain GMI1000. Oligonucleotides were purchased from Qiagen Operon (Alameda, CA, U.S.A.) or Integrated DNA Technologies (Coralville, IA, U.S.A.). DNA sequencing was performed at the University of Wisconsin Biotechnology Center (Madison).

Construction of *bcp* mutant K1317.

A 954-bp DNA fragment containing the putative *bcp* ORF was amplified by PCR using primers 5'CATCCGTATATCCC GAGCAT3' and 5'CGGATTTCTGACTCATCGGT3' designed from the GMI1000 genomic sequence. The resulting fragment was AT-cloned into pSTBlue-1, creating pSTbcp, and sequenced. The gentamicin resistance-encoding cassette *aacCI* from pUCGM was introduced into the unique *StuI* site of the *bcp* ORF, creating pSTbcp::Gent. The mutant allele was introduced into *R. solanacearum* K60 through triparental mating using the helper plasmid pRK600 in *E. coli* HB101 and the donor plasmid pSJYoT carrying the *bcp::aacCI* *EcoRI* fragment from pSTbcp::*aacCI*. Allelic replacement of the *bcp* gene in the *R. solanacearum* chromosome was confirmed by PCR and Southern blot analysis (data not shown). The confirmed *bcp* mutant was called K1317.

Complementation of K1317.

Primers 5'CATCCGTATATCCCGAGCAT3' and 5'CGGAT TTCGTACTCATCGGT3' were used to amplify the approximately 900-bp fragment containing the *bcp* gene with *Pfu*Ultra DNA polymerase (Stratagene, La Jolla, CA, U.S.A.), and the resulting product was cloned into pCR-Blunt II-TOPO vector (Invitrogen, Carlsbad, CA, U.S.A.) to create pTOPO-*bcp*. Using *EcoRI* sites, the 954-bp fragment was subcloned into the low copy-number vector pLAFR3 (Staskawicz et al. 1987). For *trans* complementation, electroporation was used to introduce pLAFR3 into wild-type *R. solanacearum* and K1317, and pLAFR3-*bcp* into K1317.

β -Glucuronidase activity filter assay.

Each of the 153 *ipx* fusion strains identified in the previous *R. solanacearum* IVET screen contains a transcriptional fusion to the β -glucuronidase (*uidA*) reporter gene (Brown and Allen 2004). These 153 fusion strains were spotted onto unmodified CPG plates and GPG plates containing 276 μ M H₂O₂. After overnight incubation at room temperature, the resulting colonies were lifted using Whatman no. 1 filters. To determine gene expression, we measured β -glucuronidase activity via a colorimetric assay as previously described (Brown and Allen 2004). White colonies were considered to have little or no gene expression; blue colonies were considered to carry fusions induced by hydrogen peroxide. Those that were blue on CPG with H₂O₂ but remained white on plain CPG were selected. Fusions carrying the constitutively expressed *pilA* promoter cloned in the forward and backward orientation to the *uidA* gene were used as positive and negative controls for β -glucuronidase activity detection and relative color intensity, respectively.

Hydrogen peroxide detection in planta.

Hydrogen peroxide production in plant host tissue was detected with DAB stain, which forms a brown precipitate in the presence of hydrogen peroxide. Four tomato leaves of the wilt-susceptible cv. Bonny Best and the wilt-tolerant tomato line Hawaii 7996 (Thurston 1976) were infused with water or with different concentrations of *R. solanacearum* cells. At 24 h

postinoculation, three leaves were cut at the petiole and placed in DAB solution (pH 3.8) at 1 μ g/ml for 8 h under light at room temperature (Giovanini et al. 2006). Leaves were then bleached in boiling 95% ethanol for 10 min and stored in 70% ethanol. The fourth leaf was used to quantify the *R. solanacearum* populations within the leaf. Three samples were taken per leaf, pooled, ground, and dilution plated in triplicate.

Disk growth inhibition assay.

Overnight broth cultures of *R. solanacearum* were collected by centrifugation and resuspended in sterile distilled water. Optical density was adjusted to an optical density at 600 nm of 0.1, roughly 1×10^8 CFU/ml, and 100 μ l of the cell suspension was added to warm BMM top agar and poured over BMM agar plates with 0.2% malate buffered with 50 mM MES as sole carbon source. Once the top agar solidified, a Whatman no. 1 filter disk containing 10 μ l of 0.44 M hydrogen peroxide was placed in the center of the plate and incubated at 28°C for 24 h. The cell suspension (100 μ l) was dilution plated to determine exact initial cell numbers. The zone of growth inhibition around each disk was measured twice at 90° angles.

Virulence assays.

Susceptible tomato plants cv. Bonny Best, tobacco plants cv. Petit Havana SRI, and a transgenic derivative of Petit Havana SRI engineered to overproduce hydrogen peroxide (Chamnongpol et al. 1996) were used in a naturalistic soil-soak assay as previously described (Tans-Kersten et al. 1998). Briefly, a suspension of bacteria was poured over the potting mix of 15-day-old tomato plants. Plants were kept in a growth chamber with 12-h day-and-night cycles at 28°C. Disease symptoms were evaluated daily for 14 days using a 0-to-4 disease index. The assay was performed in triplicate in both hosts, with 16 plants per treatment in the tomato assays and 10 plants per treatment for the tobacco assays.

Statistical analysis.

Analysis of variance analysis was performed with Minitab Statistical Software (Minitab, Inc., State College, PA, U.S.A.).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Institut National de la Recherche Agronomique *Ralstonia solanacearum* webpage: bioinfo.genotoul.fr/annotation/iANT/bacteria/ralstonia

myCollaborative Annotation Portal *Ralstonia solanacearum* UW551 project: vision.biotech.ufl.edu/mycap/jsp/project/description.jsp?projectID=1

New England Biolabs NEB Cutter software: tools.neb.com/NEBcutter2/index.php

San Diego Supercomputer Center Biology Workbench tool: workbench.sdsc.edu