**KatE From the Bacterial Plant Pathogen *Ralstonia solanacearum* Is a Monofunctional Catalase Controlled by HrpG That Plays a Major Role in Bacterial Survival to Hydrogen Peroxide**

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*Ralstonia solanacearum* is the causative agent of bacterial wilt disease on a wide range of plant species. Besides the numerous bacterial activities required for host invasion, those involved in the adaptation to the plant environment are key for the success of infection. *R. solanacearum* ability to cope with the oxidative burst produced by the plant is likely one of the activities required to grow parasitically. Among the multiple reactive oxygen species (ROS)-scavenging enzymes predicted in the *R. solanacearum* GMI1000 genome, a single monofunctional catalase (KatE) and two KatG bifunctional catalases were identified. In this work, we show that these catalase activities are active in bacterial protein extracts and demonstrate by gene disruption and mutant complementation that the monofunctional catalase activity is encoded by *katE*. Different strategies were used to evaluate the role of KatE in bacterial physiology and during the infection process that causes bacterial wilt. We show that the activity of the enzyme is maximal during exponential growth in vitro and this growth-phase regulation occurs at the transcriptional level. Our studies also demonstrate that *katE* expression is transcriptionally activated by HrpG, a central regulator of *R. solanacearum* induced upon contact with the plant cells. In addition, we reveal that even though both KatE and KatG catalase activities are induced upon hydrogen peroxide treatment, KatE has a major effect on bacterial survival under oxidative stress conditions and especially in the adaptive response of *R. solanacearum* to this oxidant. The *katE* mutant strain also exhibited differences in the structural characteristics of the biofilms developed on an abiotic surface in comparison to wild-type cells, but not in the overall amount of biofilm production. The role of catalase KatE during the interaction with its host...
INTRODUCTION

*Ralstonia solanacearum* is a gram-negative, soil-borne β-proteobacterium that causes the bacterial wilt disease in more than 200 plant species, including economically important food crops such as potato, tomato, peanut, and eggplant (Allen et al., 2004). In addition to its extremely wide host range, *R. solanacearum* exhibits an increasingly broad geographic distribution and is able to survive for long periods in waterways, soil and in symptomless or latently infected plants (Denny, 2006; Genin and Denny, 2012).

Upon interaction with a susceptible host, the pathogen initiates the infection by entering the roots. After colonisation of the intercellular spaces of the root cortex, the bacterium enters the xylem vessels, spreading rapidly, and systemically through the vascular system. Intensive bacterial multiplication and production of large amounts of exopolysaccharides (EPSs) blocks water traffic in vascular bundles, ultimately resulting in complete wilting, plant death, and the release of the pathogen back to the soil (Genin and Denny, 2012). *R. solanacearum* requires multiple virulence factors that act additively to facilitate infection of the host plant. Bacterial motility mediated by flagella and type IV pili, plant cell wall-degrading enzymes, and type II-secreted proteins enable bacterial penetration into root tissues. Secretion of type III effectors inside plant cells evades plant immune responses and allows disease development (Peeters et al., 2013). In the plant environment, *R. solanacearum* must overcome different types of metabolic stresses in order to survive and proliferate. One of these challenges is the exposure to plant-generated reactive oxygen species (ROS) that accumulate in the apoplast as part of the primary defence response to pathogen invasion (Lamb and Dixon, 1997).

ROS are unavoidable by-products of plant metabolic pathways generated as a result of successive one-electron reductions of molecular oxygen (O2). Under physiological steady state conditions, ROS accumulation is prevented by the action of protective antioxidant systems often confined to specific compartments. However, adverse environmental factors including pathogen infection disturb this fine balance between production and scavenging of ROS leading to a rapid increase in intracellular ROS levels or “oxidative burst” (Apel and Hirt, 2004). In plants challenged with pathogenic microorganisms, including fungi, bacteria, and viruses, the oxidative burst proved to be one of the earliest events after elicitation (Wojtaszek, 1997). In the interaction of *R. solanacearum* with tomato plants, a single-phase ROS increase was detected at 24 h post-inoculation (hpi) of a susceptible cultivar, while a bi-phasic ROS generation with peak levels at 12 and 36 hpi was observed after infection of a resistant tomato variety (Mandal et al., 2011). The second phase of ROS accumulation, usually more prolonged and higher in magnitude, has been correlated with disease resistance via the hypersensitive response during incompatible and non-host interactions (Lamb and Dixon, 1997).

The oxidative burst fulfils multiple functions to plant cells undergoing pathogen attack. ROS promote the oxidative cross-linking of plant cell walls to slow pathogen entry and spread, and act as key signal molecules that mediate the activation of plant defence responses and systemic resistance (Lamb and Dixon, 1997). In addition, the high reactivity of ROS with cellular macromolecules, including DNA and proteins, makes ROS effective antimicrobial agents capable of either killing the pathogen or slowing down its growth (Peng and Kuc, 1992). To counter-attack ROS, oxidative stress response genes were shown to be expressed in plant-associated bacteria during the interaction with their hosts (Smith et al., 1996; Santos et al., 2001; Okinaka et al., 2002; Saenkham et al., 2007; Tamir-Ariel et al., 2007). Particularly, an *in vivo* expression technology (IVET) screen performed in *R. solanacearum* during pathogenesis of tomato plants revealed that at least 15 out of 153 in planta-expressed genes encoded proteins involved in the oxidative stress response, further supporting the notion that an oxidative challenge is associated with plant infection (Brown and Allen, 2004; Flores-Cruz and Allen, 2009).

Hydrogen peroxide (H2O2), the major ROS of the oxidative burst, is an electrically neutral and relatively stable species that can penetrate through cell membranes and diffuse to reach distant cellular components (Wojtaszek, 1997). H2O2 concentrations must be kept at low levels inside bacterial cells due to its ability to oxidize ferrous ions to generate highly reactive hydroxyl radicals (·OH; Fenton reaction), and to react with iron-sulphur clusters of key metabolic enzymes (Mishra and Imlay, 2012). Among the bacterial enzymes evolved to remove ROS and avoid toxicity, catalases (EC 1.11.1.6; H2O2:H2O2 oxidoreductase) constitute the primary scavengers of H2O2 by catalyzing its dismutation to water and oxygen. Based on phylogenetic analyses, three distinct catalase families can be distinguished: typical (monofunctional) heme catalases (KatEs), bifunctional heme catalase-peroxidases (KatGs), and (non-heme) manganese catalases (MnCats) (Zamocky et al., 2012). Most sequenced bacterial genomes encode multiple catalase isozymes that operate in different physiological or environmental conditions (Mishra and Imlay, 2012). Induction of specific catalases has been observed when bacteria detect environmental ROS and...
up upon entry into the stationary phase (Loewen, 1997; Mishra and Imlay, 2012). In addition, recent reports have demonstrated the role of particular catalases during pathogenesis, enhancing the bacterial ability to overcome host-induced oxidative burst (Jittawuttipoka et al., 2009; Tondo et al., 2010; Mishra and Imlay, 2012). The available R. solanacearum GMI1000 genome encodes numerous predicted ROS-scavenging enzymes, including three putative catalases. The RSc0775 (KatGb) and RSc0776 (KatGa) open reading frames (ORFs) encode predicted bifunctional catalase-peroxidases in the bacterial chromosome; whereas RSp1581 (KatE) codes for a predicted typical monofunctional catalase and is located in the megaplasmid, which harbors most R. solanacearum pathogenicity functions (Salanoubat et al., 2002; Genin and Denny, 2012).

Our previous transcriptomic studies in R. solanacearum extracted from roots of early infected potato plants indicated that the transcription of katE and, to a lesser extent, katGb is induced during plant colonisation compared to growth in rich medium (Puigvert et al., 2017). We also identified katE among the genes specifically induced by HrpG, a key R. solanacearum pathogenicity regulator that responds to direct bacterial contact with plant cells (Valls et al., 2006). In addition, R. solanacearum catalases are up-regulated by the transcriptional regulator OxyR, whose deletion impaired bacterial virulence (Flores-Cruz and Allen, 2011). These observations collectively suggest a role for catalases during the infection process, but the contribution of these enzymes to bacterial wilt disease has not been investigated.

Here we present a thorough study of the R. solanacearum KatE. We prove that this gene encodes a bona fide catalase enzyme responsible for one of the two catalase activities detected in this pathogen, describe its expression pattern and study its role during bacterial life in planta.

### MATERIALS AND METHODS

#### Bacterial Strains, Plasmids, and Growth Conditions

Relevant characteristics of the plasmids and bacterial strains used in this work are described in Table 1. The wild-type strain GMI1000 of R. solanacearum and its hrpG-derivative have been previously described (Boucher et al., 1985; Valls et al., 2006). The complemented (ΔhrpG + hrpG) strain was obtained by electroporation of the ΔhrpG mutant with pLT-HrpG, a vector

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant genotype and description</th>
<th>Source/reference</th>
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<tr>
<td><strong>Ralstonia solanacearum</strong></td>
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<tr>
<td>GMI1000</td>
<td>Wild-type strain</td>
<td>Boucher et al., 1985</td>
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<td>ΔkatE</td>
<td>katE deletion mutant in the GMI1000 background, Gm'</td>
<td>This study</td>
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<td>HrpG deletion mutant in the GMI1000 background</td>
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<td>JM109</td>
<td>HsdR17 endA1 recA1 gyrA96 relA1 supE44 &amp;lac-proAB, lacIqZ, lacYZ, F' traD36, proA'B', lacZΔM15</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<tr>
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<td>PCR cloning and sequencing vector, Ap'</td>
<td>Promega Corp.</td>
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<tr>
<td>pGEM-UkatE</td>
<td>PCR-amplified (845-bp) katE upstream fragment, cloned in pGEM-T easy, Ap'</td>
<td>This study</td>
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<tr>
<td>pCM351</td>
<td>Allelic exchange vector, Ap', Tc', Gm'</td>
<td>This study</td>
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<tr>
<td>pCM-UDkatE</td>
<td>Upstream (845-bp) fragment containing katE cloned into EcoRI/NodI and Hpal/SacI sites of pCM351, Ap', Tc', Gm'</td>
<td>This study</td>
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<td>pRCT</td>
<td>pRC containing tetracycline resistance and cloning sites, Ap', Cm', Tc'</td>
<td>Montiero et al., 2012b</td>
</tr>
<tr>
<td>pRCT-katE</td>
<td>PCR-amplified (1960-bp) fragment containing katE ORF and promoter sequence, cloned into Hpal/BgIII sites of pRCT, Ap', Cm', Tc'</td>
<td>This study</td>
</tr>
<tr>
<td>pLT-HrpG</td>
<td>pLAFR3 derivative including the HrpG coding sequence under the control of the Ptac promoter, Tc'</td>
<td>Valls et al., 2006</td>
</tr>
<tr>
<td>pJBA128</td>
<td>Vector containing gptmut3 under a constitutive PlacUV5 promoter, Tc'</td>
<td>Lee et al., 2005</td>
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### Primer name | Sequence | Amplified fragment |
<table>
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<tbody>
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<td>katEU-F</td>
<td>5' tagacctGGATACCTCAGCCTGGCAGC3' (EcoRI)</td>
<td>This study</td>
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<tr>
<td>katEL-R</td>
<td>5' taccggccGCTACGCTGAGTCGAGA3' (NotI)</td>
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<td>katED-F</td>
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<tr>
<td>katED-R</td>
<td>5' tagacctGGATACCTCAGCCTGGCAGC3' (SacI)</td>
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<tr>
<td>ckatE-R</td>
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<tr>
<td>kate-pPCR-F</td>
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<tr>
<td>kate-pPCR-R</td>
<td>5' TGTCCGCGAAGAAGATG3'</td>
<td>This study</td>
</tr>
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Ap', Gm', Tc': resistance to ampicillin (Ap), gentamicin (Gm) and tetracycline (Tc), respectively; PCR, polymerase chain reaction.

*Capital letters correspond to nucleotides of the R. solanacearum GMI1000 genome sequence and small letters to nucleotides added to facilitate cloning (restriction sites underlined).*
that overexpress HrpG from the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible Ptac promoter. The plasmid and transformation procedures are described in (Vals et al., 2006). *R. solanacearum* strains were routinely grown at 28°C in tetrazolium chloride (TZC) agar plates (Kelman, 1954), complete BG medium (10 g/L bactopectone, 1 g/L yeast extract, 1 g/L casamino acids, 0.5% glucose), or MP minimal medium supplemented with 20 mM L-glutamate as a carbon source (Plener et al., 2010). To induce HrpG expression in the complemented Δ*hrpG* + *hrpG* strain IPTG was added to the cultures at a final concentration of 100 μM. Gentamicin and tetracycline were used for selection of *R. solanacearum* strains (5 and 10 μg/mL in liquid and solid cultures, respectively). Bacterial growth was monitored by measuring optical density at 600 nm. 

*Escherichia coli* strains were grown at 37°C in Luria-Bertani (LB) broth supplemented with appropriate antibiotics (Sambrook and Russell, 2001).

**Molecular Biology and Microbiological Techniques**

Molecular cloning procedures, including DNA restriction and analysis, DNA ligation, preparation of competent cells, and transformation of *E. coli* by electroporation, were performed according to standard protocols (Ausubel et al., 1994; Sambrook and Russell, 2001). Plasmid DNA was isolated using Wizard Plus SV Minipreps DNA Purification System (Promega Corp., Madison, WI). Restriction enzymes, DNA ligase, and other DNA enzymes were used according to the manufacturers’ recommendations. Total genomic DNA from *R. solanacearum* was isolated from fresh bacterial cultures as described by Chen and Kuo (Chen and Kuo, 1993). For RNA extraction and quantitative real-time PCR analysis, total RNA was extracted using the SV Total RNA Isolation Kit (Promega) following manufacturer’s instructions for Gram-negative bacteria. cDNA was synthesized using the High Capacity cDNA reverse transcriptase kit (Applied Biosystems) following manufacturer’s instructions. The Sybr Green Master Mix (Sigma Aldrich) was used for quantitative real-time PCR with the LightCycler 480 Instrument (Roche Life Science) using the katE_qPCR-F and katE_qPCR-R primers designed in order to amplify 945-bp (primer pair katEU-F/katEU-R) fragments located upstream and downstream of the gene RSp1581, respectively (Table 1). Specific restriction sites were incorporated to each primer to be used in subsequent cloning steps. PCR amplifications were performed with the proofreading Phusion DNA polymerase (New England Biolabs, Inc., Ipswich, MA, U.S.A.) following the manufacturer’s conditions. The resulting fragments were cloned into pGEM-T easy (Promega Corp.) creating pGEM-UkatE and pGEM-DkatE for the upstream and downstream regions of the katE gene, respectively; and the identity of the inserts were confirmed by sequencing with vector primers SP6 and T7. Inserts were then excised by double digestion with EcoRI /NotI (upstream region) and Hpal/SacI (downstream region), and inserted into the multiple cloning sites of pCM351 (Marx and Lidstrom, 2002) on both sides of the gentamicin resistance cassette, creating pCM-UDkatE. This construction was then linearized by EcoRI and introduced into the wild type *R. solanacearum* GM11000 by natural transformation following the protocol described by Boucher and associates (Boucher et al., 1985). Double recombination events were selected by gentamicin resistance on TZC agar plates and the correct insertion in the genome was confirmed by PCR using primers UkatEU-F and Gent-R, which hybridize upstream of the upper region used for the homologous recombination and in the gentamicin resistance cassette, respectively (Table 1). This mutant strain, denoted as ΔkatE, was used for phenotypic characterization.

For ΔkatE complementation, a 1960-bp DNA fragment containing the katE coding region and extending 430 pb upstream of the 5’ end of the ORF was PCR amplified with primers ckatE-F and ckatE-R (Table 1). The amplified sequence included the putative promoter region of the katE gene as predicted with SoftBerry (www.softberry.com). This amplicon was double digested with Hpal/BglII and cloned into the integration element of the suicide vector pRCT (Monteiro et al., 2012b) to generate recombinant plasmid pRCT-katE. This plasmid was then linearized by NcoI and introduced into the mutant strain ΔkatE by natural transformation as described above. Complemented strains were selected by tetracycline resistance on TZC agar plates. The complemented mutant strain selected for further studies was designated ΔkatE + katE.

**Enzyme Activity Assay and Staining**

*R. solanacearum* soluble cell extracts were prepared from 10 mL cultures harvested by centrifugation at 4,000 g for 10 min at 4°C. Bacteria were washed and resuspended in 500 μL of ice-cold 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM PMSF, and then disrupted by intermittent sonication. Suspensions were clarified by centrifugation at 12,000 g for 20 min at 4°C. Protein concentrations in soluble cell extracts were determined by the Sedmak and Grossberg method (Sedmak and Grossberg, 1977) with bovine serum albumin as standard. Catalase activity in cell extracts was monitored through the decomposition of hydrogen peroxide by following the decrease in absorbance at 240 nm (Beers and Sizer, 1952). The assays were performed at 25°C in 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM H2O2. To calculate the catalase specific activity an extinction coefficient of 43.6 M⁻¹ cm⁻¹ at 240 nm was used. One unit of catalase activity was defined as the amount of activity required to decompose 1 μmol of H2O2 per minute under the assay conditions.

For evaluation of catalase activity in gels, soluble protein extracts (15–25 μg) were separated by continuous electrophoresis in 8% (w/v) non-denaturing polyacrylamide gels in glycine buffer (pH 9.5). To eliminate the likelihood of multiple, potentially artifactual catalase bands, non-denaturing gels were electrophoresed.
cultures were washed, diluted and plated on BG-agar plates. Peroxidase activity staining was performed according to Kang and associates (Kang et al., 1999) with some modifications. Briefly, aliquots of cell extracts containing 100 μg of soluble protein were electrophoresed on 8% (w/v) native polyacrylamide gels as previously described. Gels were then incubated in 0.1 M Tris-HCl (pH 7.5) containing 0.1 mg/mL 3,3′-diaminobenzidine, 9 mM H2O2, and 0.4 mg/mL NiCl2 for approximately 30 min in the dark, until appearance of the bands. Coomassie-stained gels were run in parallel to those used for catalase and peroxidase activity measurements to ascertain comparable protein loadings between samples.

**Bacterial Survival in the Presence of Hydrogen Peroxide**

To test bacterial resistance to hydrogen peroxide *R. solanacearum* overnight cultures were inoculated into fresh BG medium and grown to early exponential phase (6.5 h at 28°C and 200 rpm). Aliquots of the cultures were diluted and plated on BG-agar in order to quantify the bacterial population and then hydrogen peroxide was added to the cultures at final concentrations of 1 and 2.5 mM. After 15 min of exposure to the oxidant, samples were removed, washed once with fresh medium, serially diluted and plated on BG-agar plates.

For the induction experiments, *R. solanacearum* cultures were grown to early exponential phase (6.5 h) and incubated with sub-lethal concentrations of hydrogen peroxide (25, 50, and 100 μM) for an additional hour before being used in the killing experiments. After the induction treatment, aliquots of the cultures were washed, diluted and plated on BG-agar plates. Cultures were then treated with a lethal concentration of H2O2 (5 mM) for 15 min, after which samples were taken, washed once with fresh medium, serially diluted and plated on BG-agar plates.

In all cases, growth of liquid cultures was monitored spectrophotometrically by optical density at 600 nm (OD600). The percentage of survival was defined as the number of colony forming units (CFU) after treatment divided by the number of CFU prior to treatment ×100.

**Biofilm Observation and Quantification**

For analyses of biofilm formation *R. solanacearum* strains were modified to express the fluorescent protein (GFP) by electroporation with plasmid pJBA128 (Lee et al., 2005). Saturated cultures of the GFP-labeled bacteria in BG medium were adjusted to an optical density at 600 nm of 0.1 and diluted 1:20 in fresh CPG medium (1 g/L casamino acids, 5 g/L glucose and 10 g/L bacteriological peptone). Then, 300 μL of the bacterial suspensions were placed onto chamber-covered glass slides (nu155411, Lab-Tek, NUNC, Naperville, IL, U.S.A.) that were statically incubated in a humidified PVC box at 28°C. All microscopic observations were performed on a Zeiss LSM880 confocal laser scanning microscope (Carl Zeiss, Jena, Germany) equipped with an argon laser and detector and filter sets for monitoring of GFP expression (excitation, 488 nm; emission, 517 nm). The images obtained were analyzed with ImageJ software (https://imagej.nih.gov).

Biofilm quantification analyses were carried out following crystal violet assay. In short, CPG overnight cultures were adjusted in CPG to an OD600 of 0.1. Next, 95 μL of fresh CPG and 5 μL of the adjusted culture to OD600 of 0.1 were added in each of the 96-well polystyrene microplates (Greiner, Kremsmünster, Austria) and incubated without shaking at 30°C during 24 h. After incubation, biomass growth was measured at OD600. Next, 100 μL of 0.1% crystal violet stain was added to each well and incubated at room temperature for 30 min. Wells were washed three times with MQ-water and the stained biofilm was solubilised with 100 μL of 95% ethanol and measured at OD580. Measurements were performed using SpectraMax multi-plate reader and the results normalised to biomass (OD580/OD600).

**Pathogenicity and Bacterial Multiplication Assays in Tomato**

The susceptible tomato (*Solanum lycopersicum*) cv. Marmande cultivar was grown under long-day light conditions at 25°C and 60% relative humidity. Prior to infection, three- to four-week-old plants were acclimated for 3 days at 27°C with constant light conditions (12 h light/12 h darkness). For the pathogenicity assays, plants not watered for two days were drench inoculated without root wounding with 40 mL of the bacterial suspension adjusted to 10⁷ CFU/mL from an overnight culture. 20–25 plants were inoculated per strain and wilting symptoms were recorded per plant using an established semi-quantitative wilting scale ranging from 0 (no wilting) to 4 (death) (Monteiro et al., 2012b).

For bacterial growth assays *in planta*, tomato leaves were vacuum-infiltrated submerging the aerial plant into water or 10⁵ CFU/mL bacterial suspensions for 20 s. In both cases, the adjuvant Silwet L-77 was added (80 μL/L suspension) to facilitate infiltration. At day 0 and 3 post infiltration, bacterial concentrations in the plant tissues were measured. To this end, three 5-mm diameter disks per biological replicate were taken from infiltrated leaves, homogenised and 10 μL of serial ten-fold dilutions were plated in selective plates. The plates were incubated at 28°C until colonies could be counted. Three biological replicas were used per bacterial strain.

**Statistical Analyses**

Quantitative analyses were performed with at least three independent biological samples. Data were subjected to a multifactorial mixed model ANOVA and Tukey’s multiple comparison tests along with residual analysis and validation using Infostat software (Infostat 2006H, http://www.infostat.com.ar).

**RESULTS**

*R. solanacearum* RSp1581 Encodes an Active Monofunctional Catalase Induced During Exponential Growth

In order to investigate the role of *katE* in the *R. solanacearum* physiology and plant interaction, we generated a *katE* deletion mutant by genetic replacement of the RSp1581 open reading
frame by a gentamicin resistance cassette in the GMI1000 strain background. The resulting mutant, named ΔkatE, exhibited typical colony morphology on tetrazolium chloride (TZC)-containing agar plates and similar growth curves to its wild-type parental strain, demonstrating that disruption of katE does not affect bacterial growth in vitro (Supplementary Figure 1). To analyze the effect of katE deletion on R. solanacearum catalase activity, soluble protein extracts from cultures grown in BG medium to early exponential and stationary phase were separated on non-denaturing polyacrylamide gels and stained for catalase activity. As shown in Figure 1A, we detected two distinct catalase bands at both growth stages in the wild-type strain GMI1000. On the contrary, the upper, slow-migrating band was completely absent in the ΔkatE mutant, suggesting that this band corresponds to the KatE isozyme. As a final proof that the RSp1581 open reading frame is functional and encodes this enzyme, complementation of ΔkatE with a single copy of the open reading frame under its own promoter restored the catalase activity pattern. Soluble protein extracts of the wild type, ΔkatE and complemented (ΔkatE + katE) strains were also run in parallel on a non-denaturing polyacrylamide gel stained for peroxidase activity (Figure 1B). This assay revealed that the fast-migrating catalase band detected in all three strains exhibits peroxidase activity as well, suggesting that it corresponds to one of the KatG isozymes identified in the R. solanacearum genome (Salanoubat et al., 2002). In addition, the upper KatE band did not appear in the peroxidase assay further corroborating its monofunctional enzymatic nature.

The activity levels of KatE observed in native gels (Figure 1A) seemed to indicate that expression of this gene in R. solanacearum is regulated by growth phase, as previously reported for other bacterial species (Loewen, 1997; Vattanaviboon and Mongkolsuk, 2000; Tondo et al., 2010). To test this hypothesis, we measured the katE mRNA levels in early exponential and stationary phase cultures by quantitative real-time RT-PCR. As illustrated in Figure 1C, mRNA levels of katE significantly decreased in stationary wild type cells, being approximately 5-fold lower in the stationary phase with respect to early exponential growth phase. This expression pattern was similar in the complemented ΔkatE strain whereas expression was undetectable in the katE mutant.

**KatE Expression Is Transcriptionally Activated by the HrpG Regulator**

Using genome-wide expression analyses in R. solanacearum, we previously identified katE among a group of virulence and environmental adaptation genes specifically regulated by the HrpG transcriptional regulator (Valls et al., 2006). To better investigate the role of HrpG in the regulation of katE, we measured katE transcript levels in the wild-type GMI1000 strain, a hrpG deletion mutant (ΔhrpG) and the complemented mutant strain overexpressing this regulator (ΔhrpG + hrpG). katE mRNA levels were significantly lower in the ΔhrpG strain with respect to the wild-type or the complemented overexpressing strain (Figure 2A). This effect was more pronounced (significant differences in 95% Tukey HSD test) in minimal medium -known to specifically induce HrpG activity- than in cells grown in rich BG medium (Figure 2A). To evaluate the influence of this regulation at the protein level, we then measured the effect of hrpG on the catalase activity. Measurements of catalase activity in native polyacrylamide gels revealed the same expression pattern obtained for katE transcripts, with markedly lower levels in the ΔhrpG background that could be complemented by overexpression of this regulator (Figure 2B). These results show a clear correlation between hrpG and katE transcript levels and with the catalase activity as well.

**R. solanacearum KatE Activity Is Enhanced Upon H$_2$O$_2$ Treatment and Protects Against Oxidative Stress**

To assess the involvement of catalases in the R. solanacearum oxidative stress response, we exposed early exponential phase...
was observed in the katE mutant after peroxide exposure, suggesting an impaired ability to face the oxidative challenge.

Resistance of bacterial cells to lethal doses of H₂O₂ was then evaluated. As illustrated in Figure 3C, the ΔkatE mutant exhibited increased sensitivity to the oxidant compared to the parental wild-type strain, a phenotype that was more pronounced at higher H₂O₂ doses and maximal at the highest concentration tested (2.5 mM). Moreover, pre-adaptation of the cultures with a sub-lethal concentration of H₂O₂ (100 µM) led to a significant increase in the resistance of wild-type cells to an elevated dose (5 mM) of the agent (Figure 3D). This effect, commonly known as adaptive response, was not observed in the ΔkatE strain, which did not evidence higher tolerance to the oxidant after the adaptation treatment, reinforcing the notion that katE encodes the only catalase activity that contributes to bacterial adaptation to an oxidative environment.

Biofilm Formation Is Affected by the Deletion of katE

Bacterial antioxidant activities have been shown to influence biofilm formation (Kim et al., 2006; Simmons and Dybvig, 2015; Tondo et al., 2016). To analyze the structural characteristics of the *R. solanacearum* biofilm, we generated Green Fluorescent Protein (GFP)-labeled strain derivatives (Table 1) and observed their growth development on chambered cover glass slides by confocal laser scanning microscopy over a 5-day period. At two days post inoculation (dpi), formation of cell aggregates was apparent for the wild-type strain (Figure 4A), and a well-established biofilm with more complex structures was clearly observed at 5 dpi. In contrast, the katE mutant failed to form a structured biofilm after 5 days, exhibiting minor aggregation and reduced interstitial spaces. Besides observing the biofilm structure, we also quantified the amount of biofilm produced by measuring the intensity of crystal violet staining after growth on 96-well plates. As shown in Figure 4B, these experiments resulted in comparable quantities of biofilm in the wild type, the katE mutant and its genetically complemented derivative, demonstrating that KatE influences the development of biofilm structures but does not alter the overall amount of biofilm produced.

Pathogenicity Tests

As mentioned previously, katE transcription is activated by the master regulator of pathogenicity HrpG (Figure 2). In addition, our preliminary data show that katE from *R. solanacearum* strain UY031 is highly expressed when the bacterium grows in the plant apoplast and in the xylem (unpublished data). This information, together with our finding that catalase activity was key to survive oxidative stress led us to test whether it is required for *R. solanacearum* GMI1000 pathogenicity on tomato, its natural host. Plants of the susceptible tomato cultivar Marmande were inoculated with suspensions of the wild type, mutant, and complemented strains by soil drenching and symptom appearance was recorded over time (Figure 5A). No statistical differences in wilting symptoms in plants inoculated with the wild type, the katE disruption mutant or the complemented strain were observed in three biological replicas,
suggesting no major role of the gene in the virulence of \textit{R. solanacearum} GMI1000. The importance of apoplastic ROS led us to quantify whether bacterial fitness was affected during growth in this plant compartment. To this end, we infiltrated susceptible tomato leaves with solutions of the wild type, the \textit{D}kat\textit{E}, and the complemented strain and quantified bacterial concentrations in recovered leaf disk samples immediately after inoculation and at three days post inoculation (dpi). Results from a representative experiment are presented in Figure 5B and show that no differences in bacterial multiplication in the apoplast were observed for any of the three tested strains.

**DISCUSSION**

It has been shown that hydrogen peroxide is a central component of the oxidative burst during plant-pathogen interaction, as it accumulates in plants attacked by pathogenic microorganisms including fungi, bacteria and viruses (Baker and Orlandi, 1995; Wojtaszek, 1997). In this context, the antioxidant system adequacy by the invading microorganism must be fundamental to minimize the oxidative stress generated by the host plant, thus achieving the establishment of the infection. In this work, we demonstrated that monofunctional KatE and bifunctional KatG catalase activities can be detected in \textit{R. solanacearum} soluble protein extracts using non-denaturing polyacrylamide gels (Figure 1A). Furthermore, a single mutant in the \textit{katE} gene was generated and genetically complemented corroborating that the upper band revealed in the native gel corresponds to the KatE catalase.

We evaluated catalase activities during the different growth phases, detecting that the monofunctional catalase was induced during exponential growth (Figures 1A, C). These results collectively suggest that \textit{katE} expression is growth phase regulated at the transcriptional level. Similar results were previously reported for other bacteria such as \textit{E. coli}, \textit{Xanthomonas campestris} pv. \textit{campestris} and \textit{Xanthomonas citri} subsp. \textit{citri}, although the expression pattern of particular catalase isozymes may vary between species (Loewen, 1997; Vattanaviboon and Mongkolsuk, 1997).
In X. citri subsp. citri, katE gene was also regulated by growth phase but contrary to the pattern observed in R. solanacearum, it exhibited an strong induction in stationary phase cells (Tondo et al., 2010). Our results show that katE is transcriptionally activated by HrpG but also responds to other inducing cues besides the growth phase, as shown by the higher transcriptional output observed upon growth in BG rich medium than in minimal medium, a condition known to induce HrpG activity (Figure 2A). This specific induction in rich medium independently of HrpG is corroborated by the high katE mRNA levels in the ΔhrpG mutant strain grown in this medium. Finally, katE expression seems to be controlled mostly at the transcriptional level, as the levels of the KatE enzyme mostly correlate with its mRNA abundance, although protein stability may be increased post-translationally in minimal medium, as indicated by the fact that it can be detected in the ΔhrpG mutant strain grown in this condition, where it shows minimal transcription levels (Figures 2A, B).

On the other hand, we studied the participation of the two Ralstonia catalases in the resistance against the oxidizing compound hydrogen peroxide. R. solanacearum exponential cultures were exposed to sub-lethal doses of peroxide, detecting...
a clear induction of both catalase isozymes under oxidative stress (Figure 3A). These results are in agreement with those obtained by Florez-Cruz and Allen, who observed an OxyR-dependent induction of katE and katG mRNA levels after exposure to H$_2$O$_2$ (Flores-Cruz and Allen, 2009). Here, the contribution of KatE to this response was analyzed (Figure 3B). Quantification of R. solanacearum catalase activity in the ΔkatE mutant showed that it is almost residual and that its induction is undetectable (Figure 3B). The catalase activity was recovered in the complemented strain, where katE was reintroduced into the mutant background, and showing that KatE plays a significant role in the R. solanacearum protection to oxidative stress. To prove this, bacterial cultures were confronted to lethal doses of H$_2$O$_2$ detecting that the ΔkatE mutant was more susceptible to the oxidative compound than the wild type strain (Figure 3C). This is in agreement with the reported observations that disruption of the monofunctional catalase katE in X. citri subsp. citri and katB in Pseudomonas syringae pv. tomato DC3000 rendered these bacteria more susceptible to oxidative stress (Tondo et al., 2010; Guo et al., 2012). The other monofunctional catalase in P. syringae pv. tomato (KatE), which is clearly less induced by exposure to exogenous H$_2$O$_2$, also showed a minor role in resistance to the oxidative compound (Guo et al., 2012).

The adaptive response to oxidative agents has been previously proposed to play a fundamental role in plant-pathogen interactions, allowing bacteria to withstand increased oxidative stress conditions (Ausubel, 2005). Exposure to sub-lethal concentrations of oxidative stress agents usually have a priming effect on bacteria, which then tolerate higher doses of the same oxidant (adaptive response), and even others (cross-protection). These responses are due to the induction of numerous genes involved in oxidant removal and damage repair, including catalases (Demple, 1991; Tartaglia et al., 1991). Evaluation of this response in R. solanacearum showed that katE mutant does not significantly induce catalase activity upon treatment with low doses of H$_2$O$_2$ and its remained activity is not enough to protect bacteria against higher doses of the oxidant (Figures 3B, D). Consequently, even though KatG activity was found induced in peroxide-treated cultures according to in-gel catalase staining, our results suggest a minor role for the additional KatG catalases in the response to H$_2$O$_2$, being KatE the only catalase activity contributing to the bacterial adaptive response to an oxidative environment.

Our finding that KatE catalase activity was essential for survival in oxidative environments and the fact that ROS is a major player in plant defence responses (Wojtaszec, 1997; Flores-Cruz and Allen, 2009) led us to investigate its role in bacterial virulence. Surprisingly, we found no effect of the katE mutation on pathogenicity assays on tomato (Figure 5A). This could be due to the limited sensitivity of soil drench inoculation and disease scoring to detect minor differences in bacterial pathogenicity. An alternative explanation is that ROS accumulate mainly in the apoplast (Lamb and Dixon, 1997) and R. solanacearum grows mostly inside the xylem vessels of host plants. Thus, we measured the capacity of the bacterium to multiply in the tomato apoplast as a more quantitative measurement of its virulence and fitness. Again, disruption of katE did not cause any effect (Figure 5B). Although bacterial multiplication in the host is not always correlated with its aggressiveness (Angot et al., 2006), this result was somehow unexpected due to the important role played by the KatE catalase in in vitro protection to oxidative stress, a condition that is commonly encountered by bacteria inside the plant host (Lamb and Dixon, 1997).

In addition, the katE mutant strain did not show reduced ability to produce biofilms, another important trait for the wilting disease development (Figure 4). Biofilm-growing cells usually experience endogenous oxidative stress and many antioxidant systems were shown to be induced under this growth condition (Resch et al., 2005; Ram et al., 2005; Mikkelsen et al., 2007; Shanks et al., 2007; Chung et al., 2016). In fact, the role of catalase and superoxide dismutase in the development of mature biofilms was previously demonstrated in X. citri subsp. citri and E. coli, respectively (Kim et al., 2006; Tondo et al., 2016). According to our results, disruption of katE in R. solanacearum only alters the structure of the biofilm produced on an abiotic surface, but not the overall quantity of biofilm production. This is in agreement with previous reports indicating that perturbations of the physiological steady-state levels of ROS or the addition of catalase to the medium affects the quality and structural characteristics of the biofilms developed by Azotobacter vinelandii (Villa et al., 2012) and Mycoplasma pneumoniae (Simmons and Dybvig, 2015), with diverse effects on the amounts of biofilm produced.

However, the minor role that KatE seems to play in planta is in agreement with a previous screening for R. solanacearum genes essential for growth in planta, in which katE was not identified (Brown and Allen, 2004). The two possible explanations for the undetectable effect of R. solanacearum katE disruption on plant infection are, that ROS are not key players in the defence against this pathogen in tomato cv Marmande or that functional redundency with other genes with catalase activity exists. The three catalases in P. syringae pv. tomato DC3000 are all plant induced and play non-redundant roles in virulence (Guo et al., 2012). Our results corroborate the hypothesis proposed by Guo et al. that catalases play different roles in each plant pathogen where they independently adapted to overcome the plant defensive production of H$_2$O$_2$. Our ongoing characterisation of the KatG catalases-peroxidases will be essential to shed light into this question.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/Supplementary Material.

AUTHOR CONTRIBUTIONS

MLT, MV, and EO conceived and designed the work. MLT, RP-J, and AV performed the experiments. LP and RP-J contributed to
statistical analyses. EO and MV provided reagents and materials. All authors contributed to analysis and interpretation of results. MLT, RP-J, MV, and EO wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.01156/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | Growth curves of R. solanacearum GM1000 wild-type (WT), katE mutant (katE) and complemented (katE + katE) strains in BG medium. R. solanacearum cultures were grown aerobically at 28°C with shaking at 200 rpm. Aliquots were taken at the indicated times and measured for colony-forming capacity by serial dilution and plating on BG-agar. Colonies were counted after 48 h incubation at 28°C.


Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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