The Monofunctional Catalase KatE of *Xanthomonas axonopodis* pv. *citri* Is Required for Full Virulence in Citrus Plants

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Abstract

**Background:** *Xanthomonas axonopodis* pv. *citri* (Xac) is an obligate aerobic phytopathogen constantly exposed to hydrogen peroxide produced by normal aerobic respiration and by the plant defense response during plant-pathogen interactions. Four putative catalase genes have been identified in silico in the Xac genome, designated as *katE*, *catB*, *srpA* (monofunctional catalases) and *katG* (bifunctional catalase).

**Methodology/Principal Findings:** Xac catalase activity was analyzed using native gel electrophoresis and semi-quantitative RT-PCR. We demonstrated that the catalase activity pattern was regulated in different growth stages displaying the highest levels during the stationary phase. KatE was the most active catalase in this phase of growth. At this stage cells were more resistant to hydrogen peroxide as was determined by the analysis of CFU after the exposition to different H$_2$O$_2$ concentrations. In addition, Xac exhibited an adaptive response to hydrogen peroxide, displaying higher levels of catalase activity and H$_2$O$_2$ resistance after treatment with sub-lethal concentrations of the oxidant. In the plant-like medium XVM2 the expression of KatE was strongly induced and in this medium Xac was more resistant to H$_2$O$_2$. A Xac *katE* mutant strain was constructed by insertional mutagenesis. We observed that catalase induction in stationary phase was lost meanwhile the adaptive response to peroxide was maintained in this mutant. Finally, the Xac *katE* strain was assayed in planta during host plant interaction rendering a less aggressive phenotype with a minor canker formation.

**Conclusions:** Our results confirmed that in contrast to other *Xanthomonas* species, Xac catalase-specific activity is induced during the stationary phase of growth in parallel with the bacterial resistance to peroxide challenge. Moreover, Xac catalases expression pattern is modified in response to any stimuli associated with the plant or the microenvironment it provides. The catalase KatE has been shown to have an important function for the colonization and survival of the bacterium in the citrus plant during the pathogenic process. Our work provides the first genetic evidence to support a monofunctional catalase as a virulence factor in Xac.

Introduction

Aerobic organisms are usually exposed to a variety of reactive oxygen species (ROS) such as the superoxide radical (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and the hydroxyl radical (OH), which are produced by the stepwise one-electron reduction of molecular oxygen [1,2]. Univalent reduction of O$_2$ leads to the production of superoxide, which may be rapidly converted to hydrogen peroxide through spontaneous dismutation or via disproportionation by the action of superoxide dismutases (SODs) [1,3]. The highly reactive hydroxyl radical is generated when hydrogen peroxide reacts with Fe$^{2+}$ in the Fenton reaction, thereby linking cellular iron status to oxidative stress. Thus, exposure to ROS is an unavoidable consequence of aerobic metabolism.

However, ROS are also important components of the host immune response, and many pathogens need to prevent and overcome oxidative stress in order to establish and maintain infections [4]. The use of ROS as antimicrobial agents by the immune system is based on the high reactivity of this species with various cellular components, leading to lesions in DNA, damage to iron-sulphur clusters of key enzymes, oxidation of protein thiolis and peroxidation of lipids in the invading bacteria [5,6].

To cope with the harmful effects of ROS, most aerobic organisms have evolved an arsenal of enzymes involved in either direct detoxification of ROS or repair processes of oxidatively damaged cellular components [2]. Among antioxidant enzymes, catalases (E.E. 1.11.1.6; H$_2$O$_2$:H$_2$O$_2$ oxidoreductase) are central components of the detoxification pathways that prevent formation of the highly reactive hydroxyl radical by catalyzing the dismutation of H$_2$O$_2$ to water and oxygen. Based on their enzymological properties, bacterial catalases have been classified into three types: (i) monofunctional heme-containing catalases,
further subdivided into the small- and large-subunit categories; (ii) bifunctional heme-containing catalase-peroxidases, closely related by sequence and structure to plant peroxidases; and (iii) nonheme or Mn-containing catalases [7,8].

Bacterial catalase levels are largely determined by two factors: the content of H2O2 in the medium and the entry of cells into the stationary phase of growth [7]. Most bacterial species possess multiple catalase isozymes encoded by different genes; which are regulated differently in terms of growth phase and response to oxidative stress, suggesting that they may have different physiological functions [7,9].

*Xanthomonas axonopodis pv. citri* (Xac) is a Gram negative obligate aerobic bacterium. The organism is also the phytopathogen responsible for citrus canker, a severe disease that affects most commercial citrus cultivars [10,11].

One of the earliest responses to pathogen recognition in plant defense is the so-called oxidative burst, which consists of the rapid generation of ROS, primarily H2O2, at the site of attempted invasion [12]. It has been reported that in plant-pathogen incompatible interactions, accumulation of H2O2 occurs in a biphasic manner, being the second phase of the oxidative response responsible for the establishment of disease resistance. During compatible interactions, in which the pathogen is capable of colonizing a susceptible plant and causing disease, only the first peak of lower magnitude is observed, which appears to be a non-specific plant response to a variety of stress stimuli [13].

The capacity of phytopathogenic bacteria to multiply in host plant tissues may be due, in part, to the ability of these organisms to detoxify H2O2. In contrast to other active oxygen species, H2O2 can penetrate through membranes to affect a variety of cellular processes. In this way, catalase is likely very important for Xac in detoxifying H2O2 generated (i) endogenously through normal aerobic respiration, and (ii) by the oxidative burst of plant cells during plant-pathogen interactions.

Four genes encoding putative catalase enzymes have been identified in the Xac genome (http://cancer.lbi.ic.unicamp.br/)

| Table 1. Bacterial strains, plasmids and primers used in this work. |
|---------------------------------------------------|
| **Strain/plasmid** | **Relevant genotype and description** | **Source/reference** |
| **Strains** | | |
| *Xanthomonas axonopodis pv. citri* | | |
| Xcc99-1330 | Wild type, Ap′ | B. I. Canteros |
| XackatE | katE mutant of Xcc99-1330, Km′, Ap′ | This work |
| cXackatE | XackatE complemented, carries pBBR1/katE, Km′, Gm′, Ap′ | This work |
| **Escherichia coli** | | |
| JM109 | HsdR17 endA1 RecA thiA1 endA1 glyA96 relA1 recA1 supE44 lacIqZ D M15 | [16] |
| S17-1 | thi, pro, hsdR, recA with RP4-2(Tc-Mu-Km-Tn7), Sm′ | [21] |
| **Plasmids** | | |
| pGEM-T Easy | PCR cloning and sequencing vector, Ap′ | Promega |
| pGEM/katE | pGEM-T Easy containing 440-bp fragment of katE | This work |
| pK18mobGII | pUC18 derivative, lacZa, gusA, mob site, Km′ | [24] |
| pKmob/katE | pK18mobGII containing 440-bp fragment of katE | This work |
| pBBR1MCS-5 | Broad host-range vector, Gm′ | [25] |
| pBBR1/katE | pBBR1MCS-5 containing katE gene | This work |
| **Primer name** | **Sequence** | **Amplified fragment** |
| **srpA-F** | 5′ attggatccGGGCCAATACGCAGTACAAC 3′ | 432 bp of the XAC3990 gene |
| **srpA-R** | 5′ aattaagcttGATTGAACGATTGCGAATACAC 3′ | |
| **katE-F1** | 5′ attggatccTCGGTATTCATAGCTTCCGTTT 3′ | 440 bp of the XAC1211 gene |
| **katE-R1** | 5′ aattaagcttCTTGTCGCCATGCAGAACAAC 3′ | |
| **katE-F2** | 5′ TGATCATGCGGTGAAGATGG 3′ | 887 bp of the XAC1211 gene |
| **katE-R2** | 5′ GGTCTGGCTACGGAAGAACAG 3′ | |
| **ckatE-F** | 5′ aattaagcttATGACATGGAGGAGCTCTGG 3′ | 2729 bp including XAC1211 |
| **ckatE-R** | 5′ attggatccCTTCCCTAGGGCGGCTAC 3′ | |
| **katG-F** | 5′ CTGGTGAAAGGACAGACAGC 3′ | 405 bp of the XAC1301 gene |
| **katG-R** | 5′ aattaagcttCAGATGGTGAAATCGTTT 3′ | |
| **catB-F** | 5′ attggatccTGACGAAGAACGTGACAACATC 3′ | |
| **catB-R** | 5′ aattaagcttCTTGACAGAGACAGACAGACATC 3′ | |
| **R16S-F** | 5′ TGGTAGTCCAGGCCCTAAGG 3′ | 217 bp of the XAC2491 gene |
| **R16S-R** | 5′ TGGAAAGTTCCTGGTGTGC 3′ | |

Ap, ampicillin; Km, kanamycin; Gm, gentamycin; Sm, streptomycin.
a. Capital letters correspond to nucleotides of the Xac genome sequence and small letters to nucleotides added to facilitate cloning.
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xanthomonas/) [14,15]. Comparative sequence analysis of their deduced amino acid sequences indicate that *spa* (XAC3990), *katE* (XAC1211) and *catB* (XAC4029 and XAC4030) genes encode putative monofunctional catalases while *katG* (XAC1301) encodes a bifunctional catalase-peroxidase. In the present study we investigated the Xac response to hydrogen peroxide and studied the expression patterns of the catalase genes during the bacterial growth cycle and in a plant-like medium. A mutant strain lacking a functional *katE* gene was constructed and used to demonstrate that KatE is the major catalase induced in Xac during the stationary phase of growth. Furthermore, the virulence of the mutant strain was assessed during plant-pathogen interactions with host plants revealing the importance of this catalase in the plant colonization process.

**Materials and Methods**

**Bacterial strains, plasmids and growth conditions**

Bacterial strains and plasmids used in this study are described in Table 1. Xac strains were routinely grown aerobically in Silva Buddenhagen (SB) medium (5 g l⁻¹ sucrose, 5 g l⁻¹ yeast extract, 5 g l⁻¹ peptone, and 1 g l⁻¹ glutamic acid, pH 7.0) at 28°C with shaking at 200 rpm, or on 1.5% Bacto agar-SB plates. For the *in vitro* studies of pathogen responses to plant-like media, cells were grown in nutrient broth (NB, 3 g l⁻¹ beef extract and 5 g l⁻¹ beef peptone) and in the *hup*-inducing minimal medium XV2 (20 mM NaCl, 10 mM (NH₄)₂SO₄, 1 mM CaCl₂, 10 µM FeSO₄, 5 mM MgSO₄, 0.16 mM KH₂PO₄, 0.32 mM K₂HPO₄, 10 mM fructose, 10 mM sucrose and 0.03% (w/v) casein acid hydrolysate (casaminoacid), pH 6.7). *Escherichia coli* strain S17-1 was kindly provided by Blanca I. Canteros (INTA Bella Vista, Argentina).

**Preparation of soluble cell extracts**

Cell extracts were prepared from 10 ml cultures harvested by centrifugation at 10000 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 12000 g for 20 min at 4°C. Protein concentrations in soluble cell extracts were determined by the method of Svedmark and Grossberg [17] with bovine serum albumin as a standard.

**Enzyme activity assay and staining**

Catalase activity in cell extracts was monitored through the decomposition of hydrogen peroxide by following the decrease in absorbance at 240 nm [18]. The assays were performed at 25°C in 50 mM potassium phosphate buffer (pH 7.0), containing 10 mM H₂O₂. An extinction coefficient of 43.6 M⁻¹ cm⁻¹ at 240 nm was used to calculate the specific activity. 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 catalase activity staining, aliquots of cell extracts containing 25–50 µg of soluble protein were electrophoresed on 10% non-denaturing polyacrylamide gels and stained for catalase activity as described by Scandalios [19]. To eliminate the likelihood of multiple, potentially artifactual catalase bands, which can be detected at higher amperage (20 to 30 mA), non-denaturing gels were electrophoresed at 10 mA to resolve these bands.

**Recombinant DNA and microbiological techniques**

All DNA manipulations including plasmid purification, restriction enzyme digestion, DNA ligation and agarose gel electrophoresis were performed with standard techniques [16]. Total bacterial genomic DNA from Xac was isolated using the cetyltrimethylammonium bromide procedure [20]. Plasmids for bacterial conjugations were transferred to Xac by biparental mating from the broad host-range-mobilizing *E. coli* strain S17-1 [21]. Bacterial mixtures were spotted onto Hybond-C membranes, placed on SB-agar and incubated for 48 h at 28°C. Membranes were then washed and bacteria transferred to selective medium as previously described [22].

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**Figure 1. Catalase activity in Xac as influenced by the growth phase.** (A) Xac cultures were grown aerobically in SB medium to early exponential (EE, 4 h), mid-exponential (ME, 8 h), stationary (S, 24 h) and late stationary (LS, 48 h) phases, and soluble extracts were prepared as described in Materials and Methods. Total catalase activity was assayed as described by Beers and Sizer [18] with 10 mM H₂O₂ at 25°C. (B) Equal amounts of protein (25 µg) were separated by 8% non-denaturing PAGE and stained for catalase activity by the method of Scandalios [19]. A simultaneously run Coomassie-stained gel (not shown) indicated equal protein loadings between samples. The positions of the electrophoretically discernible catalase species Kat1, Kat2, and Kat3 are indicated. doi:10.1371/journal.pone.0010803.g001
Survival in the presence of hydrogen peroxide

Survival experiments were performed by subculturing Xac overnight cultures into fresh SB medium at 2% inoculum. After 4 or 24 h of growth (early exponential and stationary phase, respectively) aliquots of the cultures were diluted and plated on SB-agar plates. Hydrogen peroxide was then added to the cultures at final concentrations of 0.25 to 30 mM. After 15 min of exposure to the oxidant, samples were removed, washed once with fresh medium, serially diluted and plated on SB-agar plates.

To assess the \( \text{H}_2\text{O}_2 \) resistance of Xac in a plant-like medium, Xac overnight cultures were subcultured into fresh NB or XVM2 media [23] at 2% inoculum and grown for 7 or 16 h to early exponential and stationary phase respectively. Survival experiments were then performed as previously described using final concentrations of 1 and 30 mM \( \text{H}_2\text{O}_2 \).

For the induction experiments, Xac cultures were grown to early exponential phase and incubated with sub-lethal concentrations of hydrogen peroxide (10, 30 and 100 \( \mu \text{M} \)) 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 SB-agar plates. Cultures were then treated with a range of lethal concentrations of \( \text{H}_2\text{O}_2 \) (0.25 to 5 mM) for 15 min, after which samples were removed, washed once with fresh medium, serially diluted and plated on SB-agar plates.

In all cases, growth of liquid cultures was monitored spectrophotometrically by optical density at 600 nm (OD\(_{600}\)). Colonies were counted after 48 h incubation at 28°C. 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 \( \times 100 \).

RNA extraction and semi-quantitative reverse transcription PCR (RT-PCR)

Total RNA of Xac cells was isolated using TRIzol® reagent (Invitrogen), according to the manufacturer’s instructions. After extraction, the RNA was treated with RNase-free DNase (Promega) and its integrity was checked by agarose gel electrophoresis. Semi-quantitative analyses of transcript levels of \( \text{katE} \), \( \text{srpA} \), \( \text{catB} \) and \( \text{katG} \) were carried out using a two-step RT-PCR approach employing the primers listed in Table 1. For cDNA synthesis, total RNA (1 \( \mu \text{g} \)) was added to a 20 \( \mu \text{l} \) reverse transcription reaction medium containing 4 \( \mu \text{L} \) 5 \( \times \) M-MLV buffer (Promega), 0.5 mM dNTP mixture, 0.5 \( \mu \text{g} \) gene-specific primer, 200 U M-MLV reverse transcriptase (Promega) and incubated for 60 min at 42°C. Reverse transcription was terminated by incubating for 5 min at 94°C. Control reactions, where RT was omitted, were done in parallel for all the samples to rule out the possibility of amplification from contaminating DNA. PCR reactions were carried out with 2 \( \mu \text{l} \) cDNA template under the following conditions: 25 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 1 min, and extension at 72°C for 1 min; with a final extension step at 72°C for 5 min. The number of cycles to be used, avoiding reaching the plateau of the PCRs, was previously determined by taking samples at different number of cycles during the PCR amplification step and analyzing the products obtained by agarose gel electrophoresis. As a constitutive control, a 217-bp fragment of 16S rRNA was amplified using the same PCR conditions but with only 1% of the cDNA synthesis reaction as template due to the high abundance of 16S rRNA in total RNA extracts. RT-PCR products were resolved on 1.5% (w/v) agarose gels, and densitometrically quantified using Gel-Pro Analyzer Software 3.1 (Media Cybernetics).

Construction of the Xac\( \text{katE} \) mutant strain

The Xac\( \text{katE} \) mutant was constructed by insertional inactivation of the \( \text{katE} \) gene on the chromosome by a single homologous recombination. Primers \( \text{katE-F1} \) and \( \text{katE-R1} \) (Table 1), were used to amplify a 440-bp internal fragment of the \( \text{katE} \) coding region using Xac genomic DNA as template. The PCR product was cloned into pGEM-T Easy vector (Promega), and the nucleotide sequence of the insert was confirmed by automated DNA sequencing. Subsequently, a HindIII-BamHI fragment of the PCR product was subcloned into pK11mobGII [24], rendering pKmob/\( \text{katE} \) (Table 1). The recombinant plasmid pKmob/\( \text{katE} \) was transferred from \( \text{E. coli} \) strain S17-1 [21] to the Xac wild-type strain by conjugation. Recombination of the cloned \( \text{katE} \) fragment in the suicide plasmid with the homologous counterpart on the Xac chromosome resulted in the disruption of the \( \text{katE} \) gene. The \( \text{katE} \) mutant was selected on SB-agar plates containing 40 \( \mu \text{g} \) ml\(^{-1} \) Km.

![Figure 2. Hydrogen peroxide resistance of Xac cultures in different growth stages.](https://www.plosone.org/fig10803g002)

**Figure 2. Hydrogen peroxide resistance of Xac cultures in different growth stages.** Cells in early exponential (A) or stationary (B) phase of growth were exposed to the indicated concentrations of \( \text{H}_2\text{O}_2 \) for 15 min. The number of CFU was determined for each culture before and after the peroxide treatment by plating of appropriate dilutions. The percentage of survival is defined as the number of CFU after treatment divided by the number of CFU prior to treatment \( \times 100 \). Data are expressed as the mean ± standard deviation of three independent experiments.

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Inactivation of katE was confirmed by PCR using specific primers katE-F2 and katE-R2, located upstream and downstream of the gene fragment used for the homologous recombination (Table 1).

For mutant complementation, a 2729-bp DNA fragment containing the katE coding region and extending from 603 bp upstream of the 5’ end to 14 bp downstream of the 3’ end of the ORF was amplified using the primer pair katE-F and katE-R (Table 1). The amplified sequence included the putative promoter sequence of the katE gene, previously predicted with SoftBerry (www.softberry.com). The amplified DNA fragment was then cloned into the broad-host-range vector pBBR1MCS-5 [25] to generate the recombinant plasmid pBBR1/katE. This plasmid was transferred into the XackatE mutant strain by conjugation, rendering strain cXackatE (Table 1).

Plant material and plant inoculations

Orange (Citrus sinensis cv. Valencia) was used as the host plant for Xac. All plants were grown in a growth chamber in incandescent light at 25°C with a photoperiod of 16 h. Overnight cultures of Xac WT, XackatE and cXackatE were diluted in 10 mM MgCl₂ to a final concentration of 10⁵ CFU ml⁻¹. For disease symptoms assays, bacterial suspensions were infiltrated into leaves with needleless syringes. In planta growth assays were performed by grinding 0.5 cm diameter leaf discs from infiltrated leaves in 100 μl of 10 mM MgCl₂, followed by serial dilutions and plating onto SB-agar plates. Colonies were counted after 48 h incubation at 28°C.

Results

Catalase activity pattern is regulated in different growth stages

We investigated the growth phase-dependent pattern of catalase activity in Xac by conducting activity assays on soluble extracts from cultures at different growth stages. A typical growth curve of Xac in SB medium is depicted in Figure S1. As shown in Figure 1A, the highest levels of catalase activity were observed in the stationary and late stationary phases (with similar values of ~7 μmol min⁻¹ mg⁻¹), being approximately 2.5-fold higher than those determined for the cultures in exponential growth.

On the other hand, equal amounts of the bacterial extracts were separated by 8% non-denaturing PAGE and subsequently stained for catalase activity (Figure 1B). Three distinct catalase bands were detected throughout all stages of growth: a slow-migrating catalase denoted Kat1, and two bands with similar electrophoretic mobilities that were named Kat2 and Kat3. The activity level of Kat1 increases significantly in the stationary phase of growth, whereas the levels of Kat2 and Kat3 decline in the mid-exponential phase and increase again as cells enter and remain in the stationary phase.

The intensities of the bands detected in the activity gel were measured using Gel-Pro Analyzer Software 3.1 (Media Cybernetics) and the total optical density for each growth stage was calculated. The pattern obtained was consistent with the activity measurements depicted in Figure 1A (data not shown).

Xac in the stationary phase of growth is more resistant to hydrogen peroxide

In order to investigate whether the elevated catalase activity observed in the stationary phase provides Xac cultures with enhanced resistance to oxidative stress, studies of bacterial survival in the presence of H₂O₂ were performed (Figure 2). In early exponential phase, Xac was very sensitive to hydrogen peroxide treatment, with only 0.28% survival following addition of 1 mM H₂O₂ and almost no detectable CFU following treatment with 5 mM H₂O₂. On the other hand, Xac in stationary phase of growth was significantly more resistant to the oxidative stress treatment, with 95% survival following the addition of 5 mM H₂O₂ and 40% survival following treatment with 30 mM H₂O₂.

Identification of the catalase isoforms expressed in the different growth stages

Analysis of the Xac genome sequence revealed the presence of four putative catalase genes designated as katE, srpA, catB and katG [14,15]. Comparative sequence analysis of the encoded proteins were performed by using ClustalX [26] and described in Supporting Information S1 and Figures S2, S3, S4 and S5.

In order to investigate the expression profiles of the Xac catalase genes during growth, we performed semi-quantitative RT-PCR reactions using specific primers designed from the reported gene

Figure 3. Expression analysis of Xac catalase genes as a function of the growth phase. (A) Amplified products of the katE, srpA and katG genes by semi-quantitative RT-PCR using RNA preparations from Xac cultures grown in SB medium to early exponential (EE, 4 h), mid-exponential (ME, 8 h), stationary (S, 24 h) and late stationary (LS, 48 h) phases. 16S rRNA was used as a loading control and to quantitate the amount of RNA in RT-PCRs. (B) Expression profiles obtained by densitometric quantification of band intensities. Experiments were performed in triplicate with similar results; error bars indicate ±1 standard deviation of the mean. IOD, integrated optical density; A.U., arbitrary units.

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sequences (Table 1, Figure 3). As a control for constitutive bacterial expression a fragment of 16S rRNA was simultaneously amplified. Expression of katE was hardly detectable at the early and mid-exponential phases of growth, subsequently increasing to reach 5-fold higher levels during the stationary phase. On the other hand, expression of oppA and katG genes was detected throughout all stages of growth, reaching maximal levels in the mid-exponential phase and decreasing gradually towards the stationary phase. The mRNA levels of katG were almost undetectable in the late stationary phase. The catB gene was not included in the figure since no product was observed in the RT-PCR reactions under the conditions tested. To ascertain the absence of contaminating DNA in bacterial RNA samples control PCR reactions where RT was omitted were carried out in parallel for all samples (data not shown).

**Xac adaptive response to hydrogen peroxide**

The adaptive response to oxidative stress agents is a well-characterized phenomenon observed in many bacteria, in which the exposure to sub-lethal levels of an oxidant leads to the induction of genes involved in the bacterial stress response, ultimately conferring resistance to lethal levels of the same agent or even unrelated compounds (cross protection) [27]. The ability to develop an adaptive response to hydrogen peroxide was investigated in Xac by determining the catalase activity in early exponential cultures incubated with sub-lethal concentrations of H$_2$O$_2$ (10, 30 and 100 μM) for 60 min. As shown in Table 2, a 2-fold induction of catalase activity was observed in cultures treated with 100 μM H$_2$O$_2$ with respect to the untreated control cells.

Based on this observation, the resistance of bacterial cells pre-adapted with sub-lethal levels of H$_2$O$_2$ to a lethal dose of the same agent was assessed. Cultures pre-treated with 10, 30 and 100 μM H$_2$O$_2$ were subsequently challenged with a killing concentration of H$_2$O$_2$ (1 mM, see Figure 2A) and the percentages of survival were determined (Figure 4A). Interestingly, a dose dependent response was observed with these H$_2$O$_2$ concentrations, with a 10-fold increase in resistance after pre-adaptation with 100 μM H$_2$O$_2$. Moreover, Xac cultures pre-treated with 100 μM H$_2$O$_2$ for 1 hour were then incubated with 0.25, 0.5, 1 and 5 mM H$_2$O$_2$ for 15 min (Figure 4B). We found that pre-adapted cells were more resistant than the control cells to all H$_2$O$_2$ concentrations tested, the difference of survival being more pronounced as the H$_2$O$_2$ levels increases. After challenge with 5 mM H$_2$O$_2$ survival of the pre-adapted culture was 100-fold higher than that of the untreated control.

**Table 2. Induction of catalase activity in response to sub-lethal levels of hydrogen peroxide**.

| Culture | Catalase activity (μmol min$^{-1}$ mg$^{-1}$ protein) | Induction (fold) |
|---------|---------------------------------|----------------|
| Uninduced | 3.7 ± 0.3 | - |
| Induced by H$_2$O$_2$ | | |
| 10 μM | 4.5 ± 0.2 | 1.2 |
| 30 μM | 5.7 ± 0.2 | 1.5 |
| 100 μM | 7.6 ± 0.4 | 2.0 |

a. Xac cells were grown in SB medium to early exponential phase and exposed to the indicated concentrations of H$_2$O$_2$ for 1 hour. Catalase activities in soluble cell extracts were measured as described in Materials and Methods.

A medium that mimics the environment of plant intercellular spaces modifies Xac catalase expression pattern

As an initial approach to evaluate the involvement of catalases during plant-pathogen interactions we determined the levels of these enzymes in early exponential and stationary phase cultures grown in NB, a rich standard medium, and in XVM2, a nutrient poor medium that simulates conditions in the apoplastic space of plants, which induces the bacterial hsp (for hypersensitive response
and pathogenicity gene cluster [23]. Typical growth curves of Xac in these media are depicted in Figure S6. As shown in Table 3, cells grown in XVM2 exhibited ~2-fold higher catalase activity levels than cells grown in the standard medium, suggesting a possible induction of these enzymes in the environment found in the intercellular spaces of plant tissues. Moreover, Xac cultures grown in XVM2 were considerably more resistant to killing when exposed to H2O2 than those grown in NB (Table 3).

To address the question if there is a transcriptional induction of any of the catalase genes in the XVM2 medium we performed semiquantitative RT-PCR analysis with early exponential or stationary phase cultures exposed to 1 mM and 30 mM H2O2, respectively, for 15 min. The percentage of survival was defined as the number of CFU after treatment divided by the number of CFU prior to treatment × 100.

Data represent mean ± standard deviation of three independent experiments.

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Table 3. Increase in catalase activity and hydrogen peroxide resistance of Xac cells in a plant-like medium.

| Medium        | Catalase activitya (μmol min⁻¹ mg⁻¹ protein) | H₂O₂ | % Survivalb         |
|---------------|---------------------------------------------|------|---------------------|
| Early exponential phase |                                     |      |                     |
| NB            | 3.2±0.2                                     | 0.25±0.07 | 38±2               |
| XVM2          | 7.5±0.3                                     | 3.4±0.1   | 98±5               |
| Stationary phase |                                         |      |                     |
| NB            | 7.2±0.4                                     | 38±2   |                     |
| XVM2          | 16.3±0.6                                    | 98±5   |                     |

a. Xac cells were grown in the indicated media to early exponential or stationary phase and then harvested. Catalase activities in soluble cell extracts were measured as described in Materials and Methods.
b. Early exponential and stationary phase cultures were exposed to 1 mM and 30 mM H₂O₂, respectively, for 15 min. The percentage of survival is defined as the number of CFU after treatment divided by the number of CFU prior to treatment ×100.

Characterization of a XackatE mutant strain

Having established that katE is transcriptionally induced in Xac during the stationary phase of growth and in the apoplastic space mimicking XVM2 medium, a XackatE mutant strain was then generated by insertional mutagenesis (see Materials and Methods) and genetically verified by PCR analysis (data not shown).

In order to assess the effect of katE disruption on the catalase pattern, soluble extracts from the parental (WT) and mutant (katE) strains in early exponential and stationary phases of growth were analyzed by native gel electrophoresis and catalase staining. As shown in Figure 7A, the upper band observed in the wild-type strain was completely absent in the katE mutant, indicating that this band corresponds to KatE. A complementation assay was also carried out to validate the katE phenotype. This was done by cloning the katE gene under the control of its own promoter sequence in a pBBR1MCS-5 vector [25], which was then conjugated into the katE mutant. The upper catalase band was recovered in the resulting cXackatE strain, corroborating the identity of this catalase (Figure 7A). The intensity of this band was higher than the observed in the wild-type cells, probably due to the low but still multiple copy number of the pBBR1/katE vector in Xac cells.

The growth phase-dependent pattern of catalase activity in the XackatE strain was then investigated by conducting assays on soluble extracts from cultures in different stages of growth. In contrast to wild-type bacteria, no induction was observed in stationary phase cultures of the XackatE mutant, with a constant average value of ~1.6 μmol min⁻¹ mg⁻¹ throughout all the bacterial growth cycle. On the other hand, the cXackatE strain exhibited the same pattern of wild-type cells but with higher activity values.

Furthermore, the mutant strain was more sensitive to hydrogen peroxide treatment in both early exponential and stationary growth phases (Figure 7B).

The XackatE adaptive response to hydrogen peroxide was also analyzed by determining the catalase activity in early exponential cultures incubated with sub-lethal concentrations of the oxidant. As was previously demonstrated for wild-type cells, a ~2-fold

Figure 5. Expression of Xac catalase genes in the plant-mimicking XVM2 medium. (A) Amplified products of the catalase genes by semi-quantitative RT-PCR using RNA preparations from early exponential Xac cultures grown in NB and in XVM2. As a control for constitutive bacterial expression a fragment of 16S RNA was simultaneously amplified. (B) Expression profiles obtained by densitometric quantification of band intensities. Experiments were performed in triplicate with similar results; error bars indicate ±1 standard deviation of the mean. IOD, integrated optical density; A.U., arbitrary units.
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induction of catalase activity was also observed in XackatE cells treated with 100 μM H₂O₂ (Table 4), suggesting that KatE is not responsible for this response.

Interaction of the XackatE mutant with host plants

In order to assess the physiological role of KatE during the infection process, the mutant strain was tested for its ability to trigger disease in citrus leaves. Both wild-type bacteria and XackatE produced typical canker lesions upon infiltration at a concentration of 10⁷ CFU ml⁻¹, with no differences in the time of appearance of the first symptoms (water soaking). However, the magnitude of the lesions and the number of cankers were significantly diminished in the mutant strain compared to wild-type bacteria, even though the infiltration areas and the bacterial densities were equivalent for both strains. On the other hand, infiltration with the cXackatE strain caused the same symptoms and a similar percentage of necrotic area than wild-type cells (Figure 8A).

The degree of virulence of the different strains was also evaluated by conducting bacterial growth curves in planta. As shown in Figure 8B, the magnitudes of leaf injuries correlated with the bacterial growths inside the host. The bacterial number of XackatE recovered from the infected leaves was fewer than that of the wild-type strain at each time analyzed. On the other hand, although complementation restored the bacteria to full virulence on citrus leaves, cXackatE growth on leaves did not reach the values of the wild-type.

Furthermore, in order to rule out the possibility that the lower infectivity of XackatE arise because mutant cells had already been injured during the culture period, infection experiments were conducted with exponentially growing cultures, in which KatE would not be even induced. The results obtained in these inoculations were in agreement with those previously described, indicating that the deficiency suffered by the mutant strain arise during the plant infection (data not shown).

Discussion

X. axonopodis pv. citri holds a strictly aerobic life style and this physiology can lead to the intracellular generation of oxidative stress during normal respiration on molecular oxygen. As a pathogenic microorganism, it encounters a great deal of oxidative stress during the infection process as well. To prevent the accumulation of ROS generated during aerobic respiration or plant interactions, Xac should employ versatile antioxidant defense enzymes, including catalases. The genome of Xac has been completely sequenced, revealing the presence of four putative catalases, four SODs and the OxyR and SoxR sensors [14,15]. The elevated number of genes encoding for antioxidant enzymes in this bacterium provides an indication of the overall relevance of the antioxidant systems for its survival. In this study we focused on the analysis of Xac catalases and their expression patterns in order to elucidate the physiological roles that catalases play in this microorganism.

The pattern of catalase activity was found to be growth phase-regulated in Xac, with the highest levels detected during the stationary phase (Figure 1). This result was unexpected because previous reports in other Xanthomonas species showed that maximum levels of enzyme activity were attained as the cultures were emerging from the lag phase and subsequently declined as growth proceeded [28–30]. On the other hand, growth into stationary phase has been largely documented as one of the main factors influencing catalase levels in a majority of bacteria, as these enzymes would serve a protective role against peroxide during periods of low metabolic activity [7,31,32]. According to our results this may be the case for Xac, possibly suggesting different mechanisms of catalase regulation between species of the Xanthomonas genus. In addition, we showed that resistance levels to H₂O₂ treatment also varies significantly in Xac depending on the growth stage, with stationary phase cells being capable of tolerating up to 30-fold higher concentrations of the oxidant than exponentially growing cells (Figure 2).

The decrease in the activities of oxidant-scavenging enzymes, such as catalase and SOD, observed in other Xanthomonas species during the stationary phase of growth has lead to the proposal that the mechanisms responsible for stationary-phase resistance to oxidants would be independent of the levels of scavenging enzymes [29]. In contrast, our results revealed that Xac resistance to H₂O₂ during the bacterial growth cycle increases in parallel with the expression of catalase-specific activity (Figures 1 and 2), suggesting that the mechanism of resistance to oxidants in this bacterium differs at least partially from those reported for other species of the Xanthomonas genus.
We also demonstrated that Xac catalase activity is regulated at isozymes level. At all stages of Xac growth we were able to detect three bands with catalase activity in non-denaturing gels (Figure 1B), which exhibited differential patterns of expression along the bacterial growth cycle, being the upper band significantly induced during the stationary phase.

Additionally, we analyzed the expression of the complete set of Xac catalase genes (katE, katG, catB and srpA) along the bacterial growth cycle by RT-PCR, showing that the katE gene was strongly induced during the stationary phase, while katG and srpA exhibited a peak level of expression during the mid-exponential phase (Figure 3). On the other hand, transcription of the catB gene was not detected under the conditions tested, suggesting that the gene may be cryptic, that is, present but not expressed in Xac. This may be attributed to the fact that there are two overlapping gene fragments annotated as catB in the Xac genome sequence (XAC4029 and XAC4030), which encode for this putative monofunctional catalase in different open reading frames [14,15]. However, the possibility that catB is expressed in Xac under specific growth conditions not assayed for in this study can not be ruled out.

**Table 4.** Induction of catalase activity in the XackatE mutant in response to sub-lethal levels of hydrogen peroxide.

| Culture | Catalase activity (μmol min⁻¹ mg⁻¹ protein) | Induction (fold) |
|---------|--------------------------------------------|------------------|
| Uninduced | 1.7 ± 0.2 | - |
| Induced by H₂O₂ | | |
| 30 μM | 2.7 ± 0.2 | 1.6 |
| 100 μM | 3.2 ± 0.3 | 1.9 |

* a. XackatE cells were grown in SB medium to early exponential phase and exposed to the indicated concentrations of H₂O₂ for 1 hour. Catalase activities in soluble cell extracts were measured as described in Materials and Methods. Data represent mean ± standard deviation of three independent experiments.

Interestingly, transcript levels of katG and srpA in the mid-exponential phase were equal to or even higher than the total transcript levels observed during the stationary or late-stationary phases (Figure 3). However, total catalase activity detected in the stationary phase was significantly higher than that of the mid-exponential phase (Figure 1A). We speculate that the apparent discrepancy between RNA levels and catalase activities may be a consequence of the rapid rate of bacterial duplication during the exponential phase of the growth cycle, which may cause the limitation of some component (e.g., heme and/or iron) essential for the proper assembly/activity of the enzyme. A potential mechanism of post-transcriptional regulation could also be involved in the control of the catalase expression. Further investigation would be necessary to probe this contention.

We have also demonstrated that interruption of the katE gene has a marked effect on catalase activity in growth-arrested cells. The XackatE mutant strain exhibited a constant low level of activity throughout all the bacterial growth cycle and lower resistance to H₂O₂ than wild-type cells, the difference of survival being more pronounced during the stationary phase (Figure 7B). These findings support the notion that KatE is the isozyme responsible for the increase of catalase activity previously observed for the wild-type strain during the stationary phase. Furthermore, this catalase accounts for a considerable part of the overall hydrogen peroxide resistance in Xac. On the other hand, the catalase content of the XackatE mutant on non-denaturing gels revealed the absence of the upper activity band of the wild-type strain (Kat1 in Figure 1B), which allowed us to conclude that this band corresponds to the KatE isozyme (Figure 7A). Moreover, the absence of this band in both phases of growth supports the notion that the apparently different mobility observed between lanes in Figure 1 was only an artifactual effect of the electrophoretic run. In addition, a decrease in the intensities of the bands with higher electrophoretic mobilities (Kat2 and Kat3) was also observed in these gels, suggesting that the loss of KatE influences the expression of the other catalase isofoms.

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 [27]. We then became interested in the adaptive
response of Xac to H$_2$O$_2$, the major component of the plant oxidative burst [13]. Our results demonstrate that Xac also develops an adaptive response to H$_2$O$_2$, and the level of induced protection correlates with the bacterial ability to induce catalase activity during the pre-adaptation treatment (Table 2). Since the XackatE mutant exhibited the same catalase activity induction than wild-type cells after the oxidative treatment, we suggest that KatE would not be involved in the adaptive response of Xac. Adequacy of the antioxidant system may be critical for Xac interaction with citrus plants, in order to minimize oxidative stress and establish infection. We observed that the total catalase activity and the resistance to H$_2$O$_2$ were significantly higher in the apoplastic space mimicking XVM2 medium than in a rich medium (NB) (Table 3). The expression analysis of the different catalase genes indicated that the monofunctional catalase katE was strongly induced in XVM2 (Figure 5). Consistent with this, analysis of the upstream sequence of the katE gene revealed the presence of an imperfect PIP box (TTCGCN$_{14}$TTCGT) located 1 bp downstream of the predicted −10 promoter sequence. This conserved plant-inducible promoter sequence motif has been suggested to be associated with the regulation of genes induced in planta and also in the XVM2 medium [33]. Our results indicate that Xac catalase expression pattern is modified in response to any stimuli associated with the plant or the microenvironment it provides. It is still not clear if the increase in catalase activity observed in XVM2 was only a result of higher expression of the katE gene (due to possible differences in the catalytic properties with respect to KatG), or enhanced enzyme activity due to post-transcriptional regulation, or both. Nevertheless, the induction of catalase activity in response to the plant environment may serve a protective role against exposure to H$_2$O$_2$ in the early stages of plant infection.

Furthermore, the virulence of the XackatE mutant was considerably attenuated during the compatible interaction with citrus plants, given that the magnitude of the damaged tissue and the number of canker lesions were noticeably reduced compared to the wild-type strain. The phenotypic differences observed between both strains were consistent with the bacterial growth curves in planta (Figure 8). The wild-type virulence was recovered in a complemented strain (cXackatE) allowing us to conclude that the phenotypes observed are indeed caused by the loss of KatE function. Our results indicate that catalase KatE has an important function in the colonization and survival of Xac in the host tissue. Accordingly, in X. campestris pv. campesiris was recently shown that a mutant in KatG, the bifunctional catalase-peroxidase of this bacterium, was unable to infect radish (Raphanus sativus) leaves and cause disease [34]. The impaired ability of the XackatE mutant to infect citrus leaves provides the first genetic evidence to support a monofunctional catalase as a virulence factor in Xac, further indicating that the oxidative burst may play a significant role in pathogen growth restriction during the infection process. Our results collectively suggest that in the apoplast environment Xac may be more resistant to H$_2$O$_2$ as a consequence of KatE induction. Our future aims are to elucidate the regulatory pathways that orchestrate the Xac oxidative stress response during the first stages of plant infection.

Supporting Information

**Supporting Information S1** The monofunctional catalase KatE of *Xanthomonas axonopodis* pv. *citri* is required for full virulence in citrus plants.

Found at: doi:10.1371/journal.pone.0010803.s001 (0.06 MB DOC)

**Figure S1** Growth curve of Xac in SB medium. Xac culture was cultivated aerobically in SB medium at 28°C with shaking at 200 rpm. Aliquots were taken at the indicated times and measured for both optical density at 600 nm (OD$_{600}$, open circles) and colony-forming capacity on SB-agar medium (closed circles).

Found at: doi:10.1371/journal.pone.0010803.s002 (2.04 MB TIF)

**Figure S2** Multiple alignment of the deduced amino acid sequence of Xac KatE with catalases KatE of *X. campestris* pv. *phaseoli* (XcpE) and HPII of *E. coli* (EcollO), performed by using ClustalX [26]. An asterisk indicates complete residue conservation, a colon indicates strong group conservation, a period indicates weak group conservation, and a blank space indicates no conservation of residues.

Found at: doi:10.1371/journal.pone.0010803.s003 (0.47 MB TIF)

**Figure S3** Multiple alignment of the deduced amino acid sequence of Xac SrpA (XacA) with catalases from *X. campestris* pv. *vesicatoria* (Xcv), *X. oryzae* pv. *oryzae* (Xoo), *P. syringae* (Psy) and *P. aeruginosa* (Pae), performed by using ClustalX [26]. An asterisk indicates complete residue conservation, a colon indicates strong...
group conservation, a period indicates weak group conservation, and a blank space indicates no conservation of residues. Found at: doi:10.1371/journal.pone.0010803.s004 (0.45 MB TIF)

**Figure S4** Alignment of the deduced amino acid sequences of Xac CatB precursor (XacBp) (A) and Xac CatB (XacB) (B) with KatA of *X. campesstris pv. phasolii* (XcpA), performed by using ClustalX [26]. An asterisk indicates complete residue conservation, a colon indicates strong group conservation, a period indicates weak group conservation, and a blank space indicates no conservation of residues. Found at: doi:10.1371/journal.pone.0010803.s005 (0.47 MB TIF)

**Figure S5** Multiple alignment of the deduced amino acid sequence of Xac KatG (XacG) with catalases from *X. campesstris pv. veicatoria* (Xev) and *X. campesstris pv. campesstris* (Xcc), and the bifunctional HPI of *E. coli* (Ecol), performed by using ClustalX [26]. An asterisk indicates complete residue conservation, a colon indicates strong group conservation, a period indicates weak group conservation, and a blank space indicates no conservation of residues. Found at: doi:10.1371/journal.pone.0010803.s006 (0.60 MB TIF)

**Figure S6** Growth curves of Xac in NB and XVM2 media. Xac cultures were cultivated aerobically in these media at 28°C with shaking at 200 rpm. Aliquots were taken at the indicated times and measured for optical density at 600 nm (OD_{600}). Found at: doi:10.1371/journal.pone.0010803.s007 (1.88 MB TIF)

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**Author Contributions**

Conceived and designed the experiments: MLT JO EGO. Performed the experiments: MLT SP. Analyzed the data: MLT JO EGO. Contributed reagents/materials/analysis tools: JO EGO. Wrote the paper: MLT EGO.

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