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KatG and KatE Confer *Acinetobacter* Resistance to Hydrogen Peroxide but Sensitize Bacteria to Killing by Phagocytic Respiratory Burst

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Abstract

Aims—Catalase catalyzes the degradation of H_2O_2. *Acinetobacter* species have four predicted catalase genes, *katA*, *katE*, *katG*, and *katX*. The aims of the present study seek to determine which catalase(s) plays a predominant role in determining the resistance to H_2O_2, and to assess the role of catalase in *Acinetobacter* virulence.

Main Methods—Mutants of *A. baumannii* and *A. nosocomialis* with deficiencies in *katA*, *katE*, *katG*, and *katX* were tested for sensitivity to H_2O_2, either by halo assays or by liquid culture assays. Respiratory burst of neutrophils, in response to *A. nosocomialis*, was assessed by chemiluminescence to examine the effects of catalase on the production of reactive oxygen species (ROS)[1] in neutrophils. Bacterial virulence was assessed using a *Galleria mellonella* larva infection model.

Key findings—The capacities of *A. baumannii* and *A. nosocomialis* to degrade H_2O_2 are largely dependent on *katE*. The resistance of both *A. baumannii* and *A. nosocomialis* to H_2O_2 is primarily determined by the *katG* gene, although *katE* also plays a minor role in H_2O_2 resistance. Bacteria

[1]**Abbreviations used are:** ACB, *Acinetobacter calcocaceticus-baumannii*; ICU, intensive care unit; kat, catalase; MOI, multiplicity of infection; OD, optical density; ROS, reactive oxygen species; WT, wildtype.

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lacking both the *katG* and *katE* genes exhibit the highest sensitivity to H_2O_2. While *A. nosocomialis* bacteria with *katE* and/or *katG* were able to decrease ROS production by neutrophils, these cells also induced a more robust respiratory burst in neutrophils than did cells deficient in both *katE* and *katG*. We also found that *A. nosocomialis* deficient in both *katE* and *katG* was more virulent than the wildtype *A. nosocomialis* strain.

Significance—Our findings suggest that inhibition of *Acinetobacter* catalase may help to overcome the resistance of *Acinetobacter* species to microbicidal H_2O_2 and facilitate bacterial disinfection.

Keywords

Acinetobacter; catalase; KatE; KatG; hydrogen peroxide; reactive oxygen species; neutrophils

INTRODUCTION

Acinetobacter is a catalase-positive, Gram-negative, and non-fermentative short rod bacterial genus known to cause nosocomial infections. The *Acinetobacter calcocaceticus-baumannii* (ACB) species complex comprises four species: *A. baumannii, A. pittii, A. nosocomialis*, and clinically unimportant *A. calcoaceticus*. *A. baumannii* is a well-established opportunistic pathogen that is becoming an increasingly important bacterial species for hospital-acquired infections. It has been estimated that *A. baumannii* accounts for more than 10% of all hospital-acquired infections in the United States and has a >50% mortality rate in patients with sepsis and pneumonia [16]. *A. baumannii* is often resistant to antibiotics and primarily affects people with a compromised immune system, particularly patients in the intensive care units (ICUs) after major surgical operations [3, 12]. While less well characterized, *A. pittii* and *A. nosocomialis*, which are also referred to as *Acinetobacter* genomic species 3 and 13TU, respectively, are highly similar to *A. baumannii* and are also frequently the source of human infections. A comprehensive analysis of the *Acinetobacter* isolates collected between 1995 and 2003 from 31 hospitals throughout the United States identified *A. baumannii* as the most prevalent *Acinetobacter* species, accounting for 63% of all isolates, followed by *A. nosocomialis* (21%) and *A. pittii* (8%) [42]. A similar study on the ACB species complex clinical isolates collected from six hospitals in Singapore indicated that *A. baumannii* constitutes 79% while *A. pittii* and *A. nosocomialis* constitute 9% and 12%, respectively, of the clinical isolates [19].

Acinetobacter species can cause life-threatening infections, particularly in immune compromised patients. *A. baumannii* is a common cause of hospital-acquired skin and soft-tissue infections, bacteremia, secondary meningitis, urinary tract infections, and nosocomial pneumonia, particularly late-onset ventilator-associated pneumonia, due to its ability to colonize indwelling medical devices [2, 6, 10, 26, 31]. It is also a common cause of periodontitis, endocarditis, intra-abdominal abscess, wound and surgical site infections [3, 12]. Multi-drug resistant *A. baumannii* strains have also become major bacterial species responsible for battle wound-associated infections in the United States military personnel injured in Iraq and Afghanistan [4, 6, 8]. *Acinetobacter* infections are notoriously difficult to treat because of their abilities to acquire resistance to a wide array of antibiotics. *A.*

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baumannii strains resistant to broad-spectrum cephalosporins, beta-lactam agents, aminoglycosides, and quinolones have been isolated [24, 36]. Resistance to carbapenems is also on the rise, raising serious concerns about the rapid decrease in clinically available antibiotics to treat these infections. It has been reported that hospitalized patients infected with *A. baumannii* have a mortality of about 30% [32]. For these reasons, strict guidelines have been developed to eliminate the transmission of multidrug-resistant *A. baumannii* in healthcare settings [1, 34]. In particular, emphases have been placed on infection prevention measures, including proper hand hygiene by healthcare providers, environmental and equipment cleaning, and disinfection.

Hydrogen peroxide is a powerful disinfectant with strong bactericidal activity. Vaporized H_2O_2 has been used to control outbreaks of multidrug-resistant *A. baumannii* infections in healthcare facilities [7, 33]. Hydrogen peroxide also plays an important role in the containment of bacterial infections by the immune system. Phagocytosis of bacterial particles by phagocytes, including neutrophils and macrophages, triggers a signal transduction pathway, leading to the assembly of NADPH oxidase complexes at the phagosomal membrane and the production of superoxide [17]. Superoxide is then converted by superoxide dismutase to H_2O_2, which is subsequently converted to a highly bactericidal substance hypochlorous acid by myeloperoxidase. This process is often referred to as the respiratory burst. Highlighting the critical role of the respiratory burst in the containment and clearance of infectious bacterial and fungal pathogens, defects in the respiratory burst process are identified as the underlying cause of chronic granulomatous disease [9]. Since *Acinetobacter* species contain multiple genes encoding for catalases, enzymes that degrade H_2O_2, we hypothesize that strains with mutations in the distinct catalases will exhibit differential sensitivity to H_2O_2. We further postulate that catalases of *Acinetobacter* species may attenuate the production of reactive oxygen species (ROS) by phagocytic cells of the innate immune system, thus, conferring resistance to respiratory burst-mediated killing by neutrophils, monocytes, and macrophages. In the present study, we assessed the sensitivities of various *Acinetobacter* strains lacking distinct catalases to H_2O_2 and examined the effects of catalase deficiency on neutrophil respiratory burst. We assessed the virulence of catalase-deficient *A. nosocomialis* strains using a *Galleria mellonella* larva infection model. Our studies demonstrate the pivotal role of the *katG* gene product in *Acinetobacter* resistance to H_2O_2. Our studies also show that although deletion of both *katE* and *katG* in *Acinetobacter* compromises their ability to attenuate ROS production in neutrophils, these catalase-deficient mutant actually exhibit great virulence in *G. mellonella* larvae, likely due to their attenuated induction of respiratory burst in phagocytes.

MATERIALS AND METHODS

Bacterial strains

The bacterial strains used in this study are listed in Table I. The *A. baumannii* strains were obtained from the University of Washington Transposon Mutant Collection. The parental strain, AB5075, a highly virulent strain of multi-drug resistant *A. baumannii* originally isolated from patients in the United States military health care system, has been previously described [11, 16]. The *A. baumannii* mutants lacking different catalase genes were

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generated by single insertion of the T26 transposons into the genome of AB5075 in the Manoil laboratory at the University of Washington [11]. The *A. nosocomialis* M2 strain has been described previously [5, 13]. Although it was initially regarded as an *A. baumannii* strain, the M2 strain has been re-categorized as an *A. nosocomialis* strain after determining the genomic sequence [5]. *A. nosocomialis* M2 mutants with deficiencies in various catalase genes were generated as previously described [5], using the primers listed in Table II. The authenticity of all genetic deletion mutants were confirmed by PCR and sequencing. All bacterial strains were grown at 37°C in tryptic soy broth medium (Becton, Dickinson and Company, Franklin Lakes, NJ).

Chemicals and reagents

Tryptic soy broth powder was purchased from BD (Becton, Dickenson & Co., Sparks, MD, USA). Hydrogen peroxide and luminol were purchased from Sigma-Aldrich (St Louis, MO). FITC-conjugated rat monoclonal antibody against mouse Ly-6G and APC-conjugated rat monoclonal antibody against mouse CD11b were purchased from Biolegend (San Diego, CA).

Assessment of H_2O_2 sensitivity

For halo assays, bacteria were grown overnight at 37°C with shaking at 300 rpm. The bacteria were sub-cultured by diluting in fresh medium at 1:10 and allowing to grow for 5 h. The cultures were then diluted with fresh tryptic soy broth medium to an optical density (OD) of 0.1 measured at 600 nm. Eighty microliters of the diluted culture was added to 4 ml of heat-melted 0.7% soft agar maintained at 42°C, vortexed thoroughly, and overlaid onto solidified tryptic soy broth with 1.5% agar (10 ml) in petri dishes. The top agar was allowed to solidify for 30 min. Sterilized dry Whatman 3MM filter discs of 8-mm diameter were placed on the soft agar (which contained bacteria), and 10 μl of 2% H_2O_2 was spotted onto the discs. The petri dishes were incubated at 37°C in humidified environment overnight. The diameters of the halos were measured with a digital caliper (General Tools, Secaucus, NJ).

To measure the sensitivity of the bacterial strains to H_2O_2 in liquid culture, bacterial cultures were diluted in fresh medium to a final OD_{600} of 0.01. Hydrogen peroxide was then added to the cell suspensions to different concentrations ranging from 0–0.02% (equivalent to 0–8.5 mM). The mixtures were then added to 96 well plates in triplicate and placed in a SpectraMax M2 spectrophotometer (Molecular Devices, Sunnyvale CA). Bacteria were cultured at 37°C with constant shaking. The optical density of the culture in the wells were measured automatically every 30 min for 16 h.

Assessment of catalase gene expression

A. nosocomialis strain M2 was grown in LB, with shaking at 180 rpm, and cell density determined by measuring optical density at 600 nm. When cultures were in exponential phase, and then in early stationary phase, cells were split into two aliquots, one of which was challenged with 30 mM H_2O_2 for 10 min. Cells were then pelleted at 3,220×g for 10 min at 4°C, the medium removed and cells resuspended in TRIzol (Life Technologies, Carlsbad, CA). RNA was isolated and catalase expression determined as previously described [14]. Briefly, expression of catalase was determined by quantitative reverse transcription (RT)-

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PCR (qRT-PCR) using a one-step QuantiTect SYBR green RT-PCR kit (Qiagen, Valencia, CA), with the primers described in Table III. Five biological replicates and three technical replicates were performed for each condition tested. Threshold cycle (C_T) values were normalized to the value for the endogenous control *gyrA*, and relative quantitation was calculated from the median C_T value using $\Delta\Delta C_T$. Statistical significance was determined using a Student's two-tailed *t* test. A fold change in catalase expression greater than 2-fold and with a p value of <0.05 was assessed as significant.

To assess *katE* and *katG* expression in different growth phases in *A. baumannii*, the wildtype (WT) strain, AB5075, was grown in LB broth overnight at 37°C with shaking at 250 rpm. Subsequently, the cultures were diluted 1:100 (v/v) in fresh medium to 3-ml final volumes, and incubated at 37°C with shaking at 250 rpm. At an optical density of ~0.6 at 600 nm (exponential growth phase) and again at an A_{600}~1.4 (early stationary phase) 1.5 ml of cells were removed for RNA extraction. RNA extractions were carried out using the RNeasy kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's instructions. RNA samples (1 µg per sample) were then digested with DNase (Qiagen, Mississauga, ON, Canada) to remove genomic DNA. cDNA was synthesized using SuperScript® VILO™ cDNA synthesis kit (Life Technologies) along with a no reverse transcriptase control to determine each sample was free of genomic DNA. Levels of *katG* and *katE* cDNAs were assessed by qPCR using SYBR® Select Master Mix (Life Technologies) using primers presented in Table III. The 16s ribosomal RNA was used as an internal control for normalization. Fold of changes of gene expression was calculated from the median C_T value using $\Delta\Delta C_T$.

Catalase activity testing

To categorize the catalase status of the bacteria, a small amount of *A. baumannii* bacteria was collected from an isolated colony after overnight growth 37°C, using a sterile inoculating loop, and placed on a microscope glass slide. A drop of 2% H_2O_2 was placed onto the bacteria. Bubble formation was examined under a microscope. The formation of bubbles indicative of oxygen generation is an indication of catalase positivity.

To quantify catalase activity in stationary phase, single colonies from strains of interest were inoculated into 2 ml of LB (Becton, Dickenson & Co., Sparks, MD, USA) and grown overnight at 37°C with shaking at 200 rpm. The next day catalase activity was measured by mixing 1.94 ml of 50 mM potassium phosphate (pH 7.4) (Becton, Dickenson & Co.) with 50 µl of H_2O_2 (Fisher Scientific, ON, Canada) and 5–10 µl of the overnight culture. A Clark oxygen electrode (Cole-Parmer, QC, Canada 05520–13) connected to a Gilson oxygraph, was then used to measure oxygen generation, as previously described [35]. The catalase units/mg of dry weight was calculated by using the velocity of oxygen production, OD_{600} and dilution factors of the cultures [15]. One unit of catalase activity was defined as the amount that decomposes 1 µmol H_2O_2 in 1min at 37°C. Measurements for each strain were carried out in biological triplicate.

To quantify catalase activity at differential phase of growth, overnight bacterial cultures of *A. baumannii* strains were diluted 1:100 (v/v) in fresh medium, and incubated at 37°C with shaking at 250 rpm. At an optical density of ~0.6 at 600 nm (exponential growth phase) and

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again at an A_{600}~1.4 (early stationary phase) cells were removed for catalase activity determination.

Respiratory burst assays

Neutrophils were purified from the bone marrow of C3H/HeN mice by antibody-mediated depletion of red cells and other leukocytes using a Neutrophil Isolation Kit (Miltenyl Biotech, San Diego, CA). The purified neutrophils were stained with a FITC-conjugated rat monoclonal antibody against mouse Ly-6G, a neutrophil cell surface marker, and an APC-conjugated rat monoclonal antibody against mouse CD11b, a myeloid lineage marker. The percentage of neutrophils in the purified cells was determined by flow cytometry. To prepare for opsonized bacteria, *A. nosocomialis* strains were grown overnight at 37°C in tryptic soy broth, washed three times with phosphate-buffered saline (PBS), and pelleted by centrifugation. Then bacteria ($8 \times 10^6 - 2.4 \times 10^8$ CFU) were suspended in 80 μl mouse serum, and incubated at 37°C for 1 h. Following opsonization, 10 μl of the bacteria/serum mixture were aliquoted into the wells on a 96 well plate. As a control, the same volume of mouse serum was added into additional wells. Respiratory burst assays were initiated by mixing 10^5 neutrophils with opsonized bacteria in the wells containing 100 μM luminol. Respiratory burst activity, measured using chemiluminescence, were monitored by taking sequential images in an IVIS Spectrum imaging system (Caliper Life Sciences, Hopkinton, MA) over 90 min, essentially as described [43]. Respiratory burst activity was calculated as the cumulative chemiluminescence in a given period.

Galleria mellonella larva infection model

The wild type (WT) *A. nosocomialis* M2 and the M2Δ*katE*Δ*katG* strains were grown overnight at 37°C, with shaking at 300 rpm. The cultures were then diluted 10-fold into fresh medium and grown for 3 h. Cells were collected by centrifugation, washed twice in PBS, and resuspended in PBS to a concentration of 2×10^7 CFU/ml. *G. mellonella* larvae (Vanderhorst Wholesale, Saint Marys, OH) were used within 7 days of shipment from the vendor. Larvae were kept in the dark at room temperature before infection. Larvae weighing 200 to 300 mg were used in the survival assays as described previously [16]. Briefly, 5 μl of the bacteria suspension containing 1×10^5 CFU of bacteria was injected into the last left proleg of the larvae using a 10-μl glass syringe (Hamilton, Reno, NV) fitted with a 30-G needle (Novo Nordisk, Princeton, NJ). Each experiment included control groups of non-injected larvae or larvae injected with 5 μl sterile PBS. Injected larvae were incubated at 37°C in humidified environment, and death was assessed everyday over 8 days. Larvae were considered dead if they did not respond to physical stimuli. Experiments were repeated four times using 10–20 larvae per experimental group. Differences in survival between worms infected with the parental WT M2 strain and the M2 Δ*katE*Δ*katG* double mutant of *A. nosocomialis* were determined by Kaplan-Meier analysis with log-rank test, using SigmaPlot software (Systat Software Inc, San Jose, CA).

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RESULTS AND DISCUSSION

Catalase activity in the catalase gene-deficient *A. baumannii* mutants

A.baumannii has four predicted catalase genes *katA* (Ab locus: ABUW_2504), *katE* (Ab locus: ABUW_2436), *katG* (Ab locus: ABUW_3469), and *katX* (Ab locus: ABUW_2059). The *katA* gene is referred to as "catalase" in the *A. baumannii* mutant library database (http://www.gs.washington.edu/labs/manoil/baumannii.htm), while *katX* is referred to as "catalase domain-containing". *katA* encodes a small-subunit mono-functional catalase; *katE* encodes a large-subunit mono-functional catalase; and *katG* encodes a catalase-peroxidase whereas *katX* encodes a small protein with a catalase-domain. The parental WT strain, AB5075, has been described previously [16], and its genome was recently sequenced [11]. Strains with transposon insertions in each individual catalase gene were obtained from the Manoil Laboratory at The University of Washington, and cultured to test catalase activity. Since catalase degrades H_2O_2 to generate O_2, catalase-positive bacteria produce oxygen bubbles upon incubation with H_2O_2. The two *A. baumannii* strains (AB6423 and AB6425) with transposon insertions in *katE* exhibited a catalase-negative phenotype. In contrast, strains with transposon insertions in *katG* (AB9109 and AB9110), *katA* (AB6618 and AB6621), and *katX* (AB5352, 5354, and 5355) exhibited a catalase-positive phenotype that was indistinguishable from WT strain (AB5075) (Table I). This qualitative visual assay of catalase activity was confirmed by a quantitative determination in cells grown into stationary phase. These analyses indicated that strains with transposon insertions in the *katE* gene had virtually no catalase activity, while strains with transposon insertions in *katA*, *katG*, and *katX*, had catalase activities comparable to that of the parental WT strain (Fig. 1). These results are consistent with the idea that KatE is the primary enzyme responsible for H_2O_2 degradation in stationary phase *A. baumannii*.

katG of *A. baumannii* is a determinant of resistance to H_2O_2

To assess the contributions of the different predicted catalases to the resistance of *A. baumannii* to H_2O_2, we performed halo assays. In these assays sensitivity of bacteria to H_2O_2 is indicated by the diameter of the zone of killing encircling the H_2O_2-impregnated filter disc (Fig. 2A). When all *A. baumannii* catalase mutants were tested, H_2O_2 produced a significantly greater zone of killing on the bacterial lawns of both *katG* mutants tested, as compared to the parental strain (Fig. 2B). This stands in contrast to the observation that loss of *katG* had no effect on total catalase activity in stationary phase (Fig. 1). Further, even though disruption of *katE* almost completely eliminated the total catalase activity in stationary phase *A. baumannii*, disruption of *katE* had no significant effect on the sensitivity of *A. baumannii* to H_2O_2. Similarly, loss of either *katA* or *katX* genes had no effect on the sensitivity of *A. baumannii* to H_2O_2. We also compared the sensitivities of the *A. baumannii* parental strain and the catalase mutants to the most common germicides used in both hospitals and laboratories. When the strains were tested against 30% acetic acid, 70% ethanol, bleach (6.15% sodium hypochlorite), and 1.75% iodine, no significant differences were detected (data not shown).

To assess whether similar H_2O_2 sensitivity profiles would be observed with cells grown in liquid culture, we grew the bacteria until they reached the exponential phase, then continued

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growth in the presence of H_2O_2 that ranged in concentration from 0 to 8.5 mM. A representative strain was chosen from each group for analysis. Consistent with the idea that KatG plays a dominant role in determining the resistance of *A. baumannii*, to H_2O_2, the *katG* mutant strain (AB9110) exhibited the greatest sensitivity to H_2O_2 amongst all *A. baumannii* strains tested (Fig. 3C). Conversely, the *katA* and *katX* mutant strains showed similar sensitivities to H_2O_2 as the parental strain (Fig. 3A, 3D), while the *katE* mutant strain (AB6423) of *A. baumannii* appeared only slightly more sensitive to H_2O_2 (Fig. 3B), despite the almost complete absence of measurable catalase activity in stationary phase cultures (Fig. 1). These results suggest that resistance to H_2O_2 in *A. baumannii* is not solely dependent on their ability to degrade H_2O_2.

KatG plays a predominant role in H_2O_2 resistance while KatE also contributes to H_2O_2 resistance

The *Acinetobacter* species *A. nosocomialis* and *A. baumannii* are closely related. In fact, *A. nosocomialis* was only categorized as a new species in 2011 [30]. Based on the genomic sequence, *A. nosocomialis* strain M2 is predicted to have three catalase genes, *katE*, *katG*, and *katX*. The KatE orthologues of *A. baumannii* and *A. nosocomialis* share 98% identity, while the KatG orthologues of the two species have 96% identity. The identity of the KatX orthologues of these two species are significantly lower (78%). *A. nosocomialis* M2 strains which lacked *katE, katG* or *katX*, as well as strains that lacked two catalase genes were tested for catalase activity using the slide-based oxygen bubble forming assay. Similar to what we observed with the *A. baumannii* strains, only the strains lacking the *katE* gene, either alone or in combination with another catalase gene, exhibited a catalase-negative phenotype (Table I). This result is consistent with *katE* being preferentially and highly expressed in stationary phase cells [22, 38].

The sensitivities of the catalase-deficient *A. nosocomialis* strains to H_2O_2 were assessed using the halo assays (Fig. 4A), with results similar to those observed with *A. baumannii* strains. The zone of killing of the Δ*katG* mutant, indicated by the halo, was significantly larger than that of the parental *A. nosocomialis* strain, while the zones of killing of the Δ*katE* and Δ*katX* strains were similar to that of the parental strain (Fig. 4B). Loss of both *katG* and *katE* resulted in an even greater zone of killing, whereas loss of *katG* and *katX* produced a similar level of killing as loss of *katG* alone. These results suggest that in *A. nosocomialis* there is a hierarchy of importance in the catalases with regard to protection against the killing by H_2O_2: KatG plays the predominant role, KatE plays a minor role, while KatX does not appear to be important.

Previously, it has been shown that in *E. coli katG* expression is rapidly induced during the rapid exponential growth phase [22, 27]. Additionally, *katG* transcription is substantially enhanced in response to H_2O_2 through a transcriptional mechanism mediated by OxyR [27], a transcription factor sensitive to oxidative stress [39]. Conversely, *katE* is transcribed at the transition from exponential growth to stationary phase by RNA polymerase containing the alternative sigma subunit σ^s, the product of the rpoS gene [23, 28]. Thus, it is possible that upon inoculation into fresh medium containing H_2O_2, OxyR induces *katG* expression and KatG gradually becomes the enzyme that contributes to the majority of the catalase activity

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mediating the degradation of H_2O_2. In contrast, in stationary phase KatE contributes to most of the catalase activity (Fig. 1), but during exponential growth phase (likely the growth state of cells in both the halo assays and the culture growth assays), KatE contributes a smaller portion of the total catalase activity. To explore this possibility, either exponentially growing *A. nosocomialis* strain M2 or cells in stationary phase were treated with with H_2O_2 then expression of *katE* and *katG* quantified by qRT-PCR (Fig. 5A). The *katE* gene was preferentially expressed in the stationary phase. Stimulation of *A. nosocomialis* cells with H_2O_2 increased *katE* expression at the stationary phase, but had little effect during exponential growth (Fig. 5A, *left graph*). In contrast, basal *katG* expression levels were similar in both stationary and exponential growth phases. H_2O_2 stimulation enhanced katG expression in both phases (Fig. 5A, *middle graph*). The differential expression of the *katE* and *katG* genes at different phases is shown in the right hand graph of Figure 5A.

Similarly, we also examined the expression patterns of *katE* and *katG* in *A. baumannii* during exponential growth and in stationary phase. As observed in *A. nosocomilis*, *katE* expression levels in the parental *A. baumannii* strain (AB5075) were dramatically increased in stationary phase, as compared to during exponential growth (Fig. 5B). In contrast, *katG* expression appeared to be greater during exponential growth as compared to stationary phase. Measurement of the enzymatic activity in exponential growth and stationary phases clearly indicates that catalase activity was substantially greater in stationary phase than in the exponential growth phase in both the parent and the *katG* mutant strains (Fig. 5C). It should be stressed that total catalase activity in the *katG* mutant strain were comparable to that in the parental *A. baumannii* strain during both exponential growth and in stationary phase, whereas catalase activity in the *katE* mutant strain were almost absent in both exponential growth and stationary phases. Thus, although *katG* expression appeared to be greater during exponential growth phase, the H_2O_2-sensitive phenotype cannot be explained by a decrease in total catalase activity in the exponential growth phase. Our results suggest that the H_2O_2-sensitive phenotype of the *katG* mutant is not likely due to a defect in cells' catalase activity, as catalase activity in these *katG* mutant cells was comparable to those of parental cells. This is also consistent with the observation that the *katE* mutant was almost completely deficient in catalase activity but exhibited nearly normal H_2O_2 resistance (Fig. 5C).

A. nosocomialis deficient in both *katE* and *katG* elicit a weaker ROS production by neutrophils and display enhanced virulence in a *G. mellonella* larvae model

Phagocytes such as neutrophils, monocytes, and macrophages produce H_2O_2 and other ROS for bacterial killing through a process often referred to as respiratory burst [17, 29]. We hypothesized that strains deficient in catalase would exhibit a lower capacity to counteract the respiratory burst and so be more sensitive to killing by phagocytic cells. We tested this hypothesis using purified murine neutrophils isolated from mouse bone marrow. Flow cytometry analysis indicated that the cell preparations contained ~92% Ly-6G$^+$CD11b$^+$, ~6% Ly-6G-CD11b$^+$, and ~2% Ly-6G$^-$CD11b$^-$ cells (Fig. 6A). As Ly-6G is only expressed in neutrophils of the CD11b$^+$ myeloid class, we concluded that neutrophils constituted ~92% of the neutrophil preparations, with ~6% were likely monocytes (CD11b$^+$). To assess the effect of bacterial catalase on ROS production in neutrophils in response to bacteria, we

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opsonized WT *A. nosocomialis* strain M2 and the Δ*katE*, Δ*katG*, and Δ*katE*Δ*katG* mutant strains with normal murine serum. The opsonized bacteria were then incubated with purified murine neutrophils in the presence of luminol. The kinetics of ROS production by the neutrophils were documented by measuring the time course of the photon flux. As shown in Figure 5B, normal murine serum did not stimulate a production of ROS. Exposure of neutrophils to all four *A. nosocomialis* strains (M2, Δ*katE*, Δ*katG*, and Δ*katE*Δ*katG*) induced a time-dependent increase in ROS production that reached its peak levels at 45 to 55 min, indicating an increase in respiratory burst activity in neutrophils after phagocytosis of the bacteria (Fig. 6B). Consistent with the notion that KatE and KatG in *A. nosocomialis* cells counteract production of ROS by neutrophils, at a high multiplicity of infection (MOI) of 300 the parent and the Δ*katE* and Δ*katG* mutant strains caused a reduced level of ROS production, but the Δ*katE*Δ*katG* mutant caused an increase in ROS production. The fact that either KatE or KatG was sufficient to reduce peroxide production suggests overlapping function of the two catalases in the degradation of ROS. Paradoxically, ROS production induced by the Δ*katE*Δ*katG* mutant cells at MOI of 10 or 30 was far lower than that induced by the parental *A. nosocomialis* cells, suggesting that the KatE and/or KatG protein(s) facilitate(s) the recognition of bacteria or activation of the respiratory burst by neutrophils (Fig. 6C). In other words, the catalases exhibit competing roles in destroying peroxide to confer resistance to respiratory burst-mediated bacterial killing while at the same time enhancing the respiratory burst of the innate immune cells.

To assess whether loss of both *katE* and *katG* alters the virulence of *Acinetobacter*, we infected *G. mellonella* larvae with 1×10^5 CFU of either the *A. nosocomialis* parent or the Δ*katE*Δ*katG* mutant strain, and assessed survival of the larvae every day for 8 days (Fig. 7). As expected, the parental *A. nosocomialis* strain induced greater mortality in *G. mellonella* larvae than did PBS. Surprisingly, Δ*katE*Δ*katG* mutant cells induced a significantly higher mortality than did the parental *A. nosocomialis* cells. The decreased survival rate of larvae infected with the Δ*katE*Δ*katG* mutant, relative to the parent, indicates that the lack of KatE and KatG actually made these bacteria more virulent. As increased virulence is often associated with increased bacterial resistance to the bactericidal mechanisms of the immune system, these findings suggest that KatE and/or KatG actually hinder the survival of *A. nosocomialis* during the interaction with the immune system, despite conferring bacterial resistance to H_2O_2 (Fig. 6).

General discussion

In this study, we assessed the contributions of different catalases to H_2O_2 resistance in two *Acinetobacter* species. We found that KatG plays a predominant role in the resistance of *Acinetobacter* to H_2O_2 (Figs. 2 and 4), although KatE is the primary catalase responsible for H_2O_2 degradation in stationary phase bacteria (Fig. 1). Our studies also indicate that *katA* and *katX* do not play a detectable role in H_2O_2 resistance. The lack of a role for *katA* and *katX* may be explained by their expression pattern or protein structure. Although *katA* encodes a small-subunit monofunctional catalase, we were unable to detect a catalase activity corresponding to KatA protein (data not shown), suggesting that either *katA* is not expressed in normal culture conditions or its activity is very low, below the limit of detection. KatX is categorized as a “catalase domain-containing” protein, but does not have

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all the structural characteristics of a functional catalase, thus it is unlikely that KatX is an active catalase.

Acinetobacter species are major bacterial pathogens involved in nosocomial infections [42]. Due to their inherent resistance to multiple antibiotics, *Acinetobacter* infections are very difficult to prevent and treat. During the wars in Iraq and Afghanistan, large numbers of *A. baumannii* infection cases were observed in United States military medical facilities treating soldiers wounded in the Middle East [6, 36, 37], highlighting the challenge of this pathogen to the United States military medical service facilities. *Acinetobacter* is also a serious concern in civilian hospitals. Hospitalized patients, especially very ill patients on a ventilator and those with prolonged hospital stays, are particularly susceptible to *Acinetobacter* infections [2, 19, 26, 34]. Patients with open wounds or with invasive devices like urinary catheters are also at greater risk for *Acinetobacter* infection. *Acinetobacter* can be spread to susceptible patients by person-to-person contact or through contact with contaminated surfaces of medical devices or contaminated environments such as bedding and furniture. As *Acinetobacter* can survive on surfaces for a prolonged time, great emphasis has been placed on disinfection through aggressive and monitored cleaning of environmental reservoirs [1, 34]. H_2O_2 vapor has been found to be an effective decontamination agent [7, 33]. In this study, we found that KatG, and to a lesser extent KatE, confers *Acinetobacter* resistance to H_2O_2. Our studies suggest that compounds with catalase-inhibitory properties, if used in combination with H_2O_2, could enhance bacterial killing, and so increase the efficiency of *Acinetobacter* disinfection. While a variety of chemicals have been shown to inhibit catalase activity, including cyanide, azide, hydroxylamine, aminotriazole, and mercaptoethanol [40], most of the inhibitory compounds are either highly toxic or unpleasant, and so not suitable as disinfectants. However, non-toxic broad-specificity catalase inhibitors, if developed, would be very helpful when used in combination with H_2O_2 as disinfectants for the prevention or elimination of *Acinetobacter* pathogens in hospitals.

One of the unexpected findings is that *Acinetobacter* resistance to H_2O_2 is predominantly determined by KatG (Figs. 2 and 3), rather than KatE, the enzyme primarily responsible for the catalase activity during exponential growth and in stationary phase (Figs. 1 & 5C). One plausible explanation for the significantly increased H_2O_2 sensitivity of the *katG* mutant strains, but not the *katE* mutant strains, of *Acinetobacter* is that KatG could constitute a greater portion of the catalase activity during rapid exponential growth, particularly in the presence of H_2O_2. While we cannot completely rule out this possibility, we think that this is unlikely. Although *katE* expression levels in the exponential growth phase were substantially smaller than in the stationary phase in both *A. baumanni* and *A. nosocomialis* (Figs. 5A & B), at least in *A. baumannii*, KatE still constituted the vast majority of the catalase activity in the exponential growth phases (Fig. 5C). The almost complete absence of catalase activity in the *A. baumannii katE* mutant during exponential growth clearly indicates that even in this growth phase KatG does not constitute a significant portion of the catalase activity (Figs. 1 and 5C). The fact that the *katG* mutant of *A. baumannii* had catalase activity comparable to that of the WT parental strain, and yet exhibited elevated H_2O_2 sensitivity, strongly suggests that KatG confers H_2O_2 resistance through a mechanism

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independent of its catalase activity. We favor the idea that the KatG determines the H_2O_2 resistance due to its other enzymatic activity.

KatG is a bifunctional hydroperoxidase I enzyme, with both catalase and peroxidase activity, while KatE is a monofunctional catalase, ie. hydroperoxidase II [21]. The catalase activity uses one molecule of H_2O_2 as the electron donor and a second molecule of H_2O_2 as the electron acceptor, producing oxygen and water. At lower concentrations of H_2O_2, the peroxidase activity is able to utilize a suitable electron donor other than H_2O_2 [15]. For this reason, KatG can, at least in theory, detoxify other peroxide compounds, for example, peroxy acids and peroxy lipids quickly produced as the result of H_2O_2 exposure. It has been reported that H_2O_2 can directly oxidize a variety of cellular components, including DNA, protein, lipid and various cellular organelles [20, 41] as well as common metabolites such as carboxylic acids [18]. It is plausible that through such a mechanism KatG, but not KatE, is able to detoxify the peroxy compounds generated by H_2O_2 and confer cells resistance to H_2O_2. As KatE is capable of degrading H_2O_2, thus eliminating the root source of oxidative damage, it is not surprising that KatE also contributes to the H_2O_2 resistance of the bacteria. This idea is supported by the increased sensitivity in the halo assays demonstrated by the order of the diameters of the halos Δ*katG*Δ*katE* mutant strain>Δ *katG* mutant strain>WT strain (Fig 4). Thus, we propose that both katE and katG are required for optimal H_2O_2 resistance. KatG is responsible for elimination of peroxy damage caused by H_2O_2, while KatE is primarily responsible for the degradation of H_2O_2.

A novel finding of this study is that KatE and KatG do not contribute to the virulence of *A. nosocomialis* (Fig. 7). This is surprising, given that catalase has been shown to contribute to the virulence of *Staphylococcus aureus* toward mice [25]. Mandell showed that a *S. aureus* strain with greater catalase activity is more resistant to neutrophil-mediated killing than *S. aureus* strain with less catalase activity. While the reason underlying such contradiction in these two bacterial species is unclear, we suggest that differential effects of catalase on phagocytic respiratory burst in the two different bacterial pathogens likely play an important role for the observed differences. Increased *S. aureus* virulence in the strain with greater catalase activity is correlated with decreased iodination of bacterial protein [25], presumably due to degradation of neutrophil-produced H_2O_2 by bacterial catalase. Unlike *S. aureus*, the Δ*katE*Δ*katG A. nosocomialis* strain induced a far less robust production of ROS than did the parental strain (Fig. 6). Unlike the parent, which at an MOI less than 300, triggered a robust ROS production which should be able to overwhelm the bacteria's catalase capacity, the Δ*katE*Δ*katG A. nosocomialis* appeared to be able to thwart the potent respiratory burst of the neutrophils (Fig. 6C). In this sense, while KatE and KatG offer the bacteria protection against H_2O_2, they also make them more prone to killing by the neutrophils. On the other hand, the deletion of *katE* and *katG* makes the neutrophils less active against these bacteria, explaining why the *A. nosocomialis* Δ*katE*Δ*katG* strain was more virulent in *G. mellonella* larvae (Fig. 7). While the mechanism underlying the attenuated ability of the Δ*katE*Δ*katG* cells to induce respiratory burst remains unclear, we speculate that lack of the two catalases may have forced the bacteria to adapt compensatory changes, which may have helped the bacteria to stymie the killing by the respiratory burst of the neutrophils.

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CONCLUSIONS

1. **1.** KatG is a more important determinant of resistance of *Acinetobacter* to H_2O_2 than KatE, despite KatE being the predominant catalase during exponential growth and in stationary phase.
2. **2.** KatA and KatX have no observable effect on peroxide resistance. *katX* encodes only a portion of the catalase protein which very likely lacks activity, while we found no evidence for *katA* expression.
3. **3.** Neither KatE nor KatG contribute to virulence in *Acinetobacter*, unlike in *Staphylococcus aureus* toward mice [25]. The latter situation was attributed to faster H_2O_2 degradation by catalase providing protection and survival advantage. The absence of such protection in *Acinetobacter* can be partially explained by the greater burst of H_2O_2 by neutrophils induced by catalase-containing cells compared to catalase-deficient cells. The mechanisms involved remains to be identified.
4. **4.** The combination of a KatG inhibitor with H_2O_2 might be developed as an effective disinfectant for the prevention or elimination of *Acinetobacter* pathogens in hospitals

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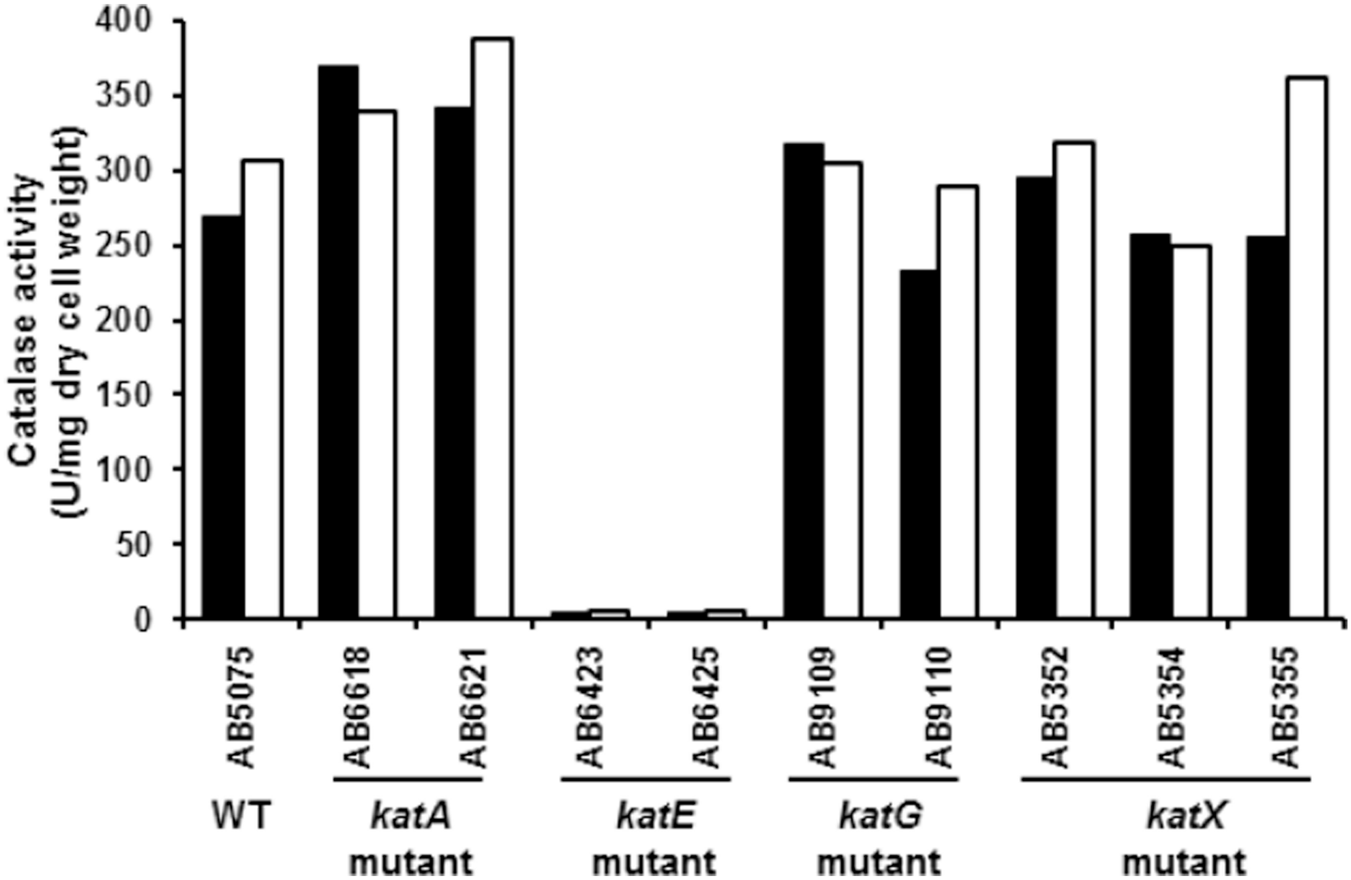

Figure 1.

The catalase activity of the WT and various catalase mutant *A. baumannii* strains. *A. baumannii* cells were cultured overnight, and catalase activity toward H_2O_2 was determined by measuring production using a Clark oxygen electrode connected to a Gilson oxygraph. Values were normalized to the dry weight of bacteria. Results represent data from two independent experiments. Solid and open bars represent results obtained from the two separate experiments.

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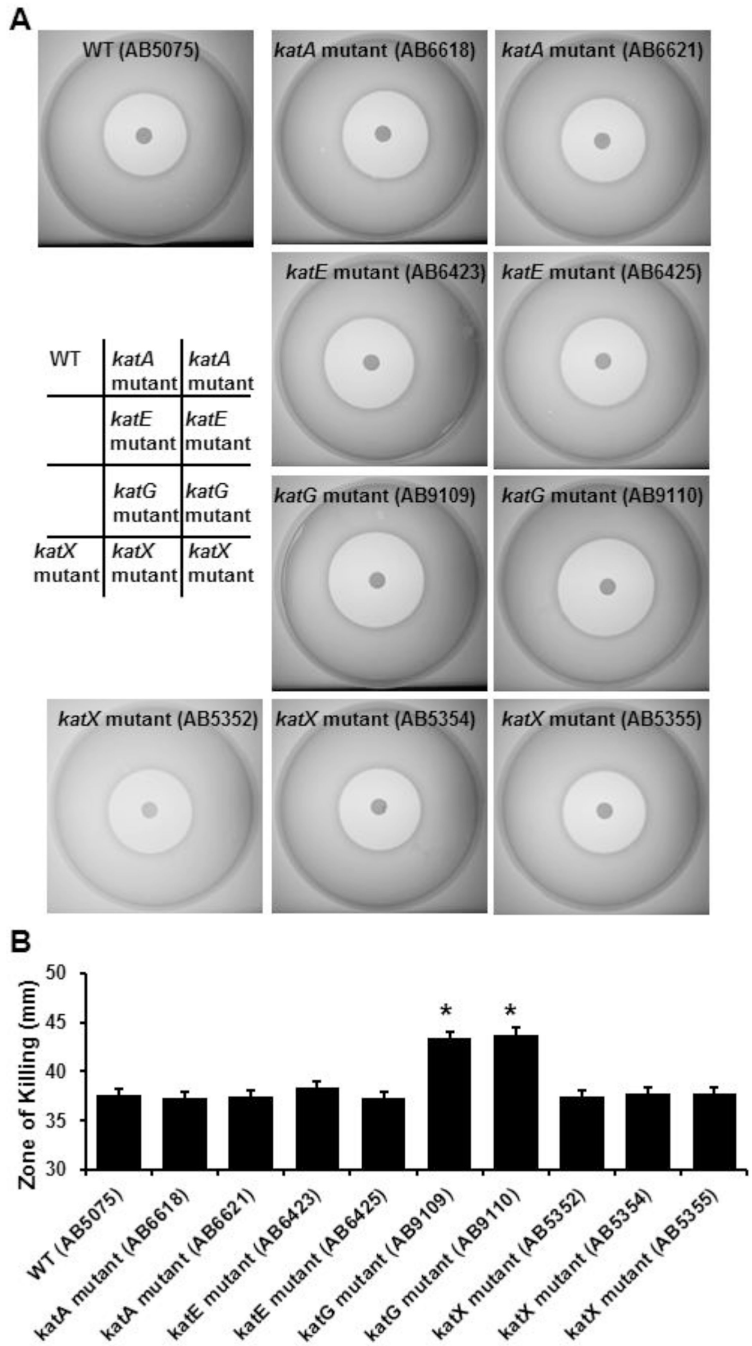

Figure 2.

The sensitivity of the different *A. baumannii* strains to H_2O_2. *A baumannii* strains were cultured overnight, and then grown for 5 hours in fresh medium. Bacteria were adjusted according to their optical density and seeded into soft agar on tryptic soy broth medium containing 1.5% agar. Hydrogen peroxide (2%, 10 μl) was spotted onto sterilized dry filter discs of 8-mm diameter. The plates were incubated at 37°C overnight. The halos were photographed and diameters were measured. **A**. Halo formation caused by H_2O_2 on lawn containing different *A. baumannii* strains. Representative images are shown. **B**. Diameters of the halos caused by H_2O_2 for distinct *A. baumannii* strains. Bars are means ± standard error from 5 independent experiments. *, $p<0.05$, compared to the WT strain (AB5075) (Student's t-test).

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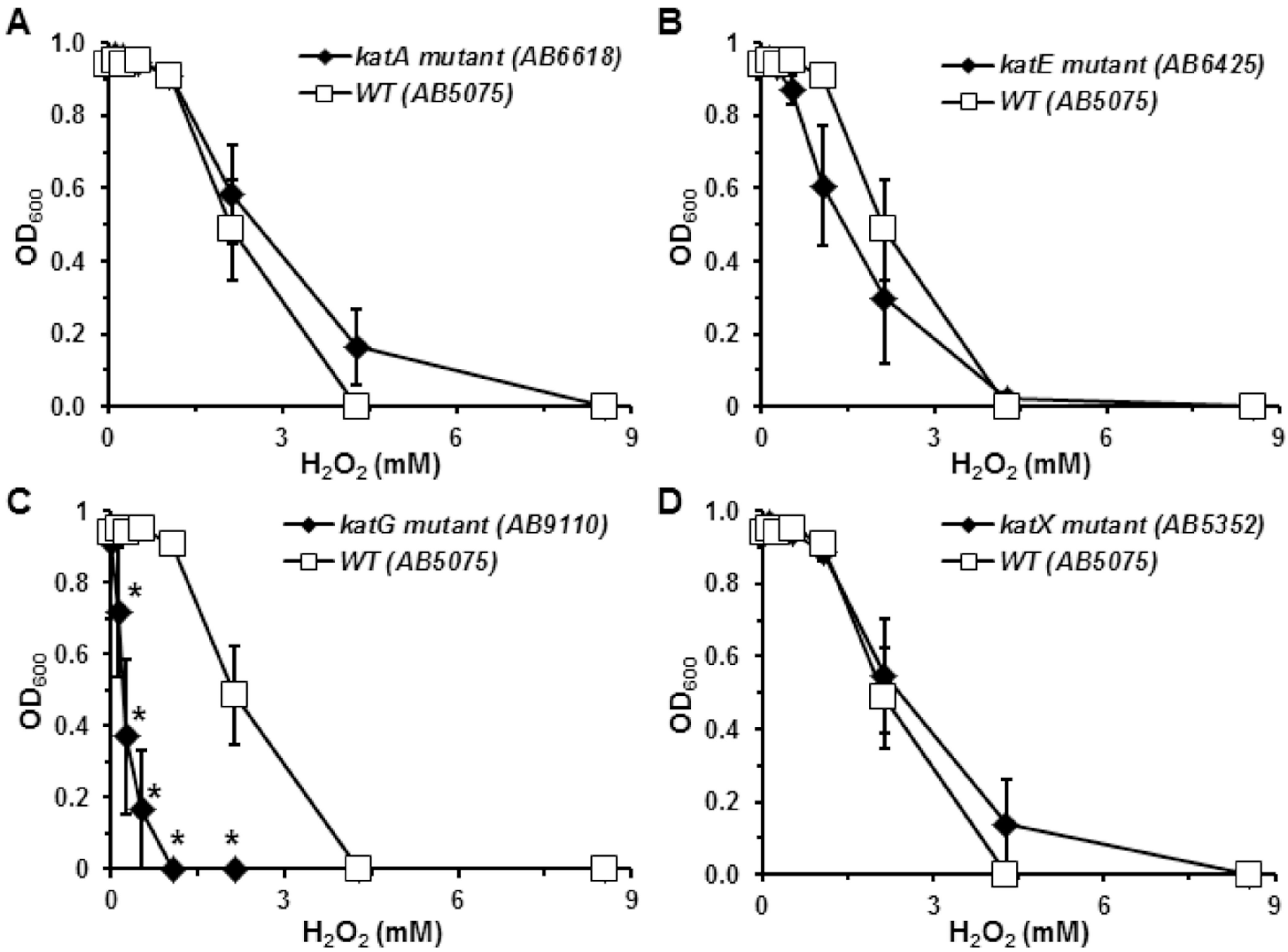

Figure 3.

The inhibitory effects of H_2O_2 on the growth of different *A. baumannii* strains in liquid culture. *A. baumannii* strains were cultured overnight, and then grown for 5 hours in fresh medium. The cultures were diluted to a final optical density of 0.01 into new tryptic soy broth with increasing concentrations of H_2O_2. Bacteria were cultured at 37°C in a spectrophotometer with agitation for 16 h, and OD_{600} were measured every 30 min. Data presented were optical density of different strains reached after 12 h in medium containing the indicated concentrations of H_2O_2. Dose-dependent inhibition of *katA, katE, katG,* and *katX* mutants was presented in **A, B, C**, and **D**. Inhibition of growth of the parental strain by H_2O_2 is presented in comparison with the mutant strains. Note, all assays were done concurrently, so the same parental data is displayed on all four panels. Data were presented as means ± standard error of 5 independent experiments. *, $p<0.05$, compared to the WT strain (AB5075) at the same concentration of H_2O_2 (Student's t-test).

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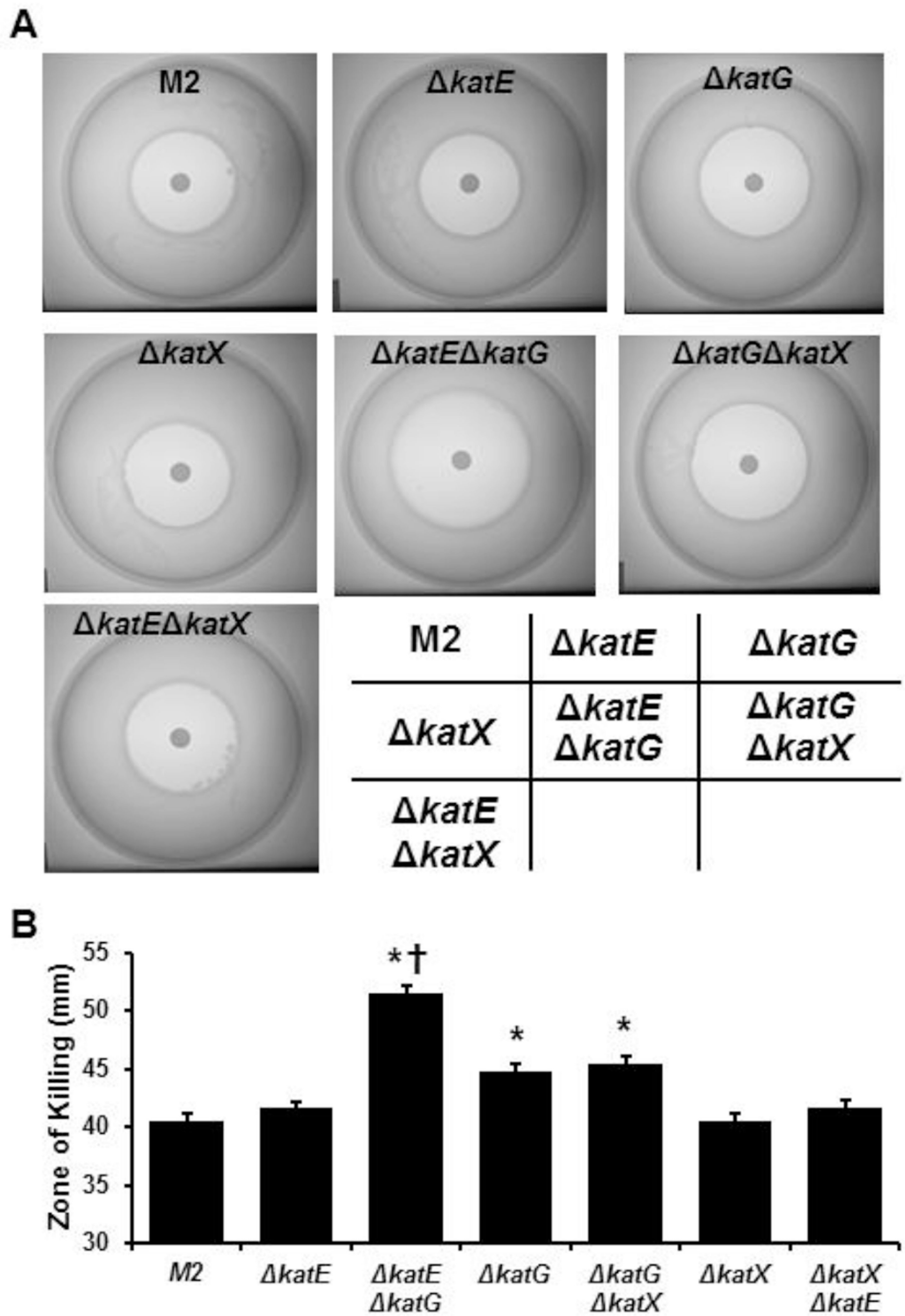

Figure 4.

The sensitivity of WT M2 *A. nosocomialis* strain and its derivatives deficient in *katE*, *katG*, both *katE* and *katG* to H_2O_2. The *A. nosocomialis* strains were cultured overnight, and then grown for 5 hours in fresh medium. Bacteria were adjusted according to their optical densities and seeded into soft agar on tryptic soy broth medium containing 1.5% agar. Hydrogen peroxide (2%, 10 μl) was spotted onto sterilized dry filter discs of 8-mm diameter. The plates were incubated at 37°C overnight. The halos were photographed and diameters of the halos were measured using a digital caliper. **A**. Halo formation caused by H_2O_2 on lawn

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containing different *A. nosocomialis* strains. Representative images are shown. **B**. Diameters of the halos caused by H_2O_2 for distinct *A. nosocomialis* strains. Bars are means ± standard error from 5 independent experiments. *, $p<0.05$, compared to the parental M2 strain. †, $p<0.05$, compared to the Δ*katG* strain. Student's t-test was used for comparison.

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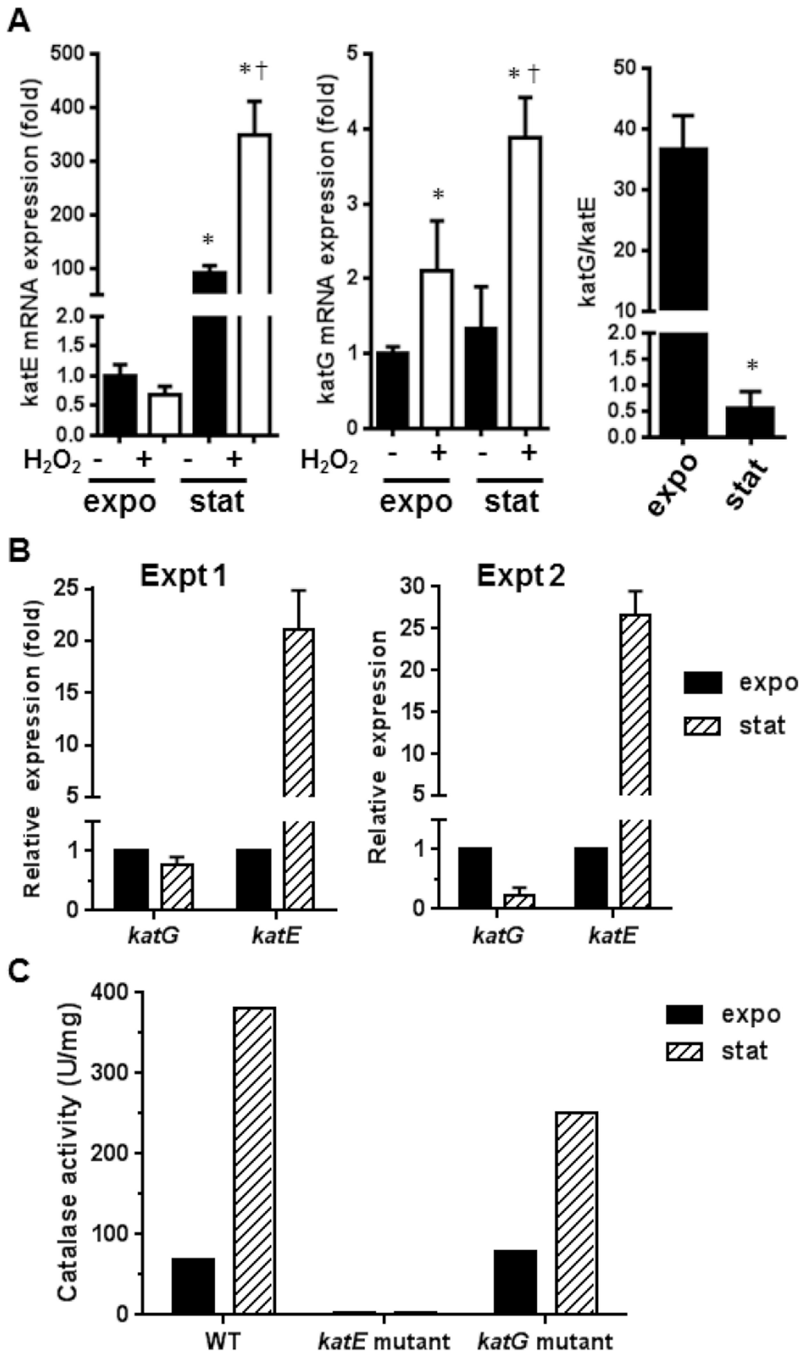

Figure 5.

The effects of growth phase and H_2O_2 on the expression of *katE* and *katG* in *A. nosocomilis* and *A. baumannii* **A**. Expression levels of *katE* and *katG* in exponential growth phase and stationary phase *A. nosocomialis* in the presence and absence of H_2O_2. *A. nosocomialis* M2 strain was grown to exponential growth phase (labeled as expo) or early stationary phase (labeled as stat), and then treated with 30 mM H_2O_2 for 10 min. The mRNA levels of *katE* and *katG* were assessed by qRT-PCR. Five biological replicates and three technical replicates were performed for each condition tested. The expression level at the exponential

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growth phase in the absence of H_2O_2 was set as 1. Comparison between treatment groups were made using Student's two-tailed *t* test. *, p<0.05, comparing to exponential growth phase. †, p<0.05, comparing to cells that were not stimulated with H_2O_2. **B**. Expression levels of *katE* and *katG* in exponential growth phase and stationary phase *A. baumannii*. WT *A. baumannii* strain (AB5075) was grown to exponential growth phase (OD_{600}=0.6) or early stationary phase (OD_{600}=1.4). Cells were harvested to purify total RNA. The mRNA levels of *katE* and *katG* were assessed by qRT-PCR. The expression level at the exponential growth phase was set as 1. Data presented are from two independent experiments. **C**. Catalase activity of WT, the *katE* mutant, and the *katG* mutant at exponential growth phase and stationary phase *A. baumannii*. *A. baumannii* cells were grown to exponential growth phase or early stationary phase as in B, catalase activity in the cell lysate were determined as in Figure 1. Results from a representative experiment were shown.

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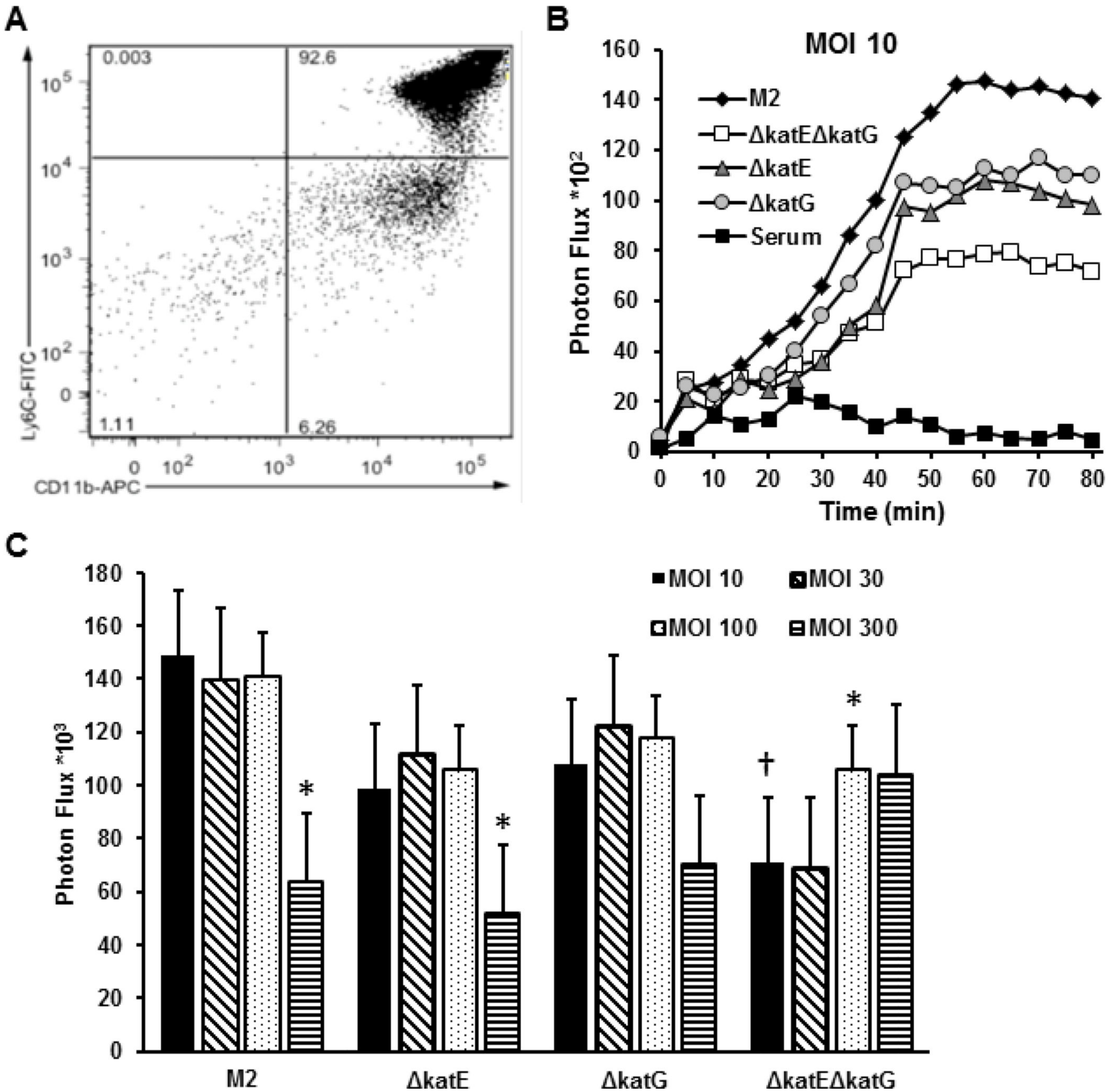

Figure 6.

Neutrophil respiratory burst induced by the *A. nosocomialis* parent and mutant strains deficient in *katE, katG*, or both *katE* and *katG*. **A**. Scot plot of the flow cytometry data on a neutrophil preparation after labeling with FITC-Ly-6G and APC-CD11b. Murine neutrophils were purified from bone marrow of C3H/HeN mice using a neutrophil isolation kit. The purity of neutrophils was assessed by flow cytometry after staining with FITC-Ly-6G and APC-CD11b. **B**. Kinetics of the respiratory burst of neutrophils exposed to parent, Δ*katE*, Δ*katG*, and Δ*katE*Δ*katG A. nosocomialis* at a MOI of 10, or exposed to the same volume of serum. Respiratory burst activity of neutrophils was measured by luminol chemiluminescence after incubating neutrophils with opsonized bacteria and luminol in an IVIS Spectrum system. **C**. Cumulative respiratory burst activity of neutrophils in 90 min after incubation with escalating amounts of parent, Δ*katE*, Δ*katG*, and Δ*katE*Δ*katG A. nosocomialis*. Data are presented as means ± standard errors of the values from 3 independent experiments. *, p<0.05 compared to value at MOI of 10 of the same strain. †, p<0.05, compared to M2 strain at the MOI of 10 (Student's t-test).

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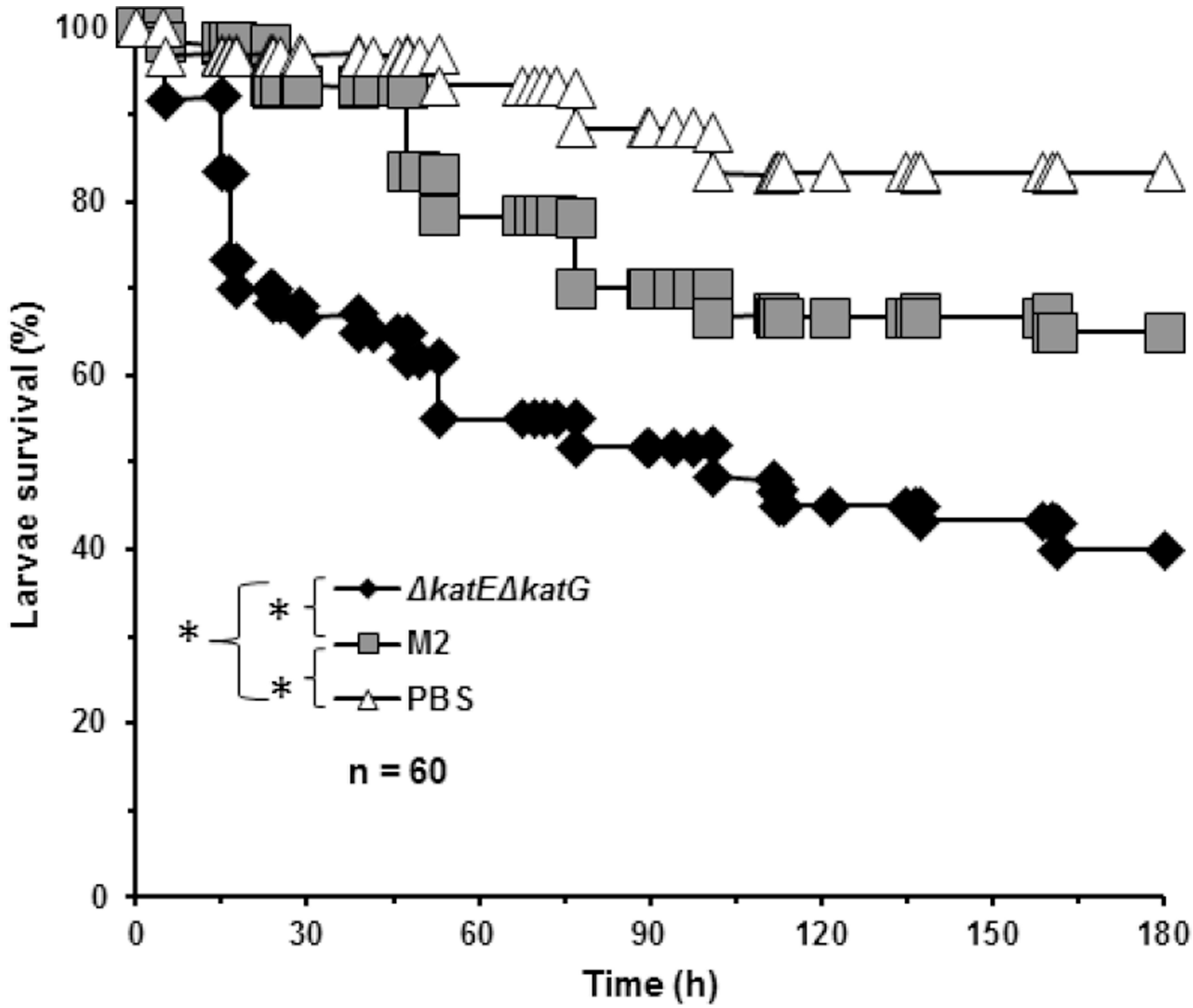

Figure 7.

Survival curves of *G. mellonella* larvae infected with the *A. nosocomialis* parent or the Δ*katE*Δ*katG* mutant strain. Overnight bacterial culture were inoculated into fresh medium and allow to grow for 3 h. Cells were collected by centrifugation, washed and resuspended in PBS to a concentration of 2× 10^7 CFU/ml. Each *G. mellonella* larva was injected with 1× 10^5 CFU of the *A. nosocomialis* parent or the Δ*katE*Δ*katG* mutant strain (in 5 μl PBS), or injected with 5 μl PBS through the last left proleg. Injected larvae were incubated at 37°C in humidified environment. Survival was monitored for 8 days. Survival curves represent data compiled from 4 independent experiments. The survival curves were compared by log-rank test. *, $p<0.05$.

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Table I

Bacterial strains used in this study

Strain	Species	Strain nomenclature	Genotype	Means of disruption	Catalase test
AB5075-UW	*A. baumannii*		WT		+
AB06621	*A. baumannii*	tnab1_kr130916p04q128	*katA* mutant::*T26*	T26	+
AB06618	*A. baumannii*	tnab1_kr121204p05q190	*katA* mutant::*T26*	T26	+
AB06423	*A. baumannii*	tnab1_kr130917p12q154	*katE* mutant::*T26*	T26	−
AB06425	*A. baumannii*	tnab1_kr121213p04q158	*katE* mutant::*T26*	T26	−
AB09109	*A. baumannii*	tnab1_kr121212p02q109	*katG* mutant::*T26*	T26	+
AB09110	*A. baumannii*	tnab1_kr121127p08q183	*katG* mutant::*T26*	T26	+
AB5352	*A. baumannii*	tnab1_kr121204p05q181	*katX* mutant::*T26*	T26	+
AB5354	*A. baumannii*	tnab1_kr121203p08q171	*katX* mutant::*T26*	T26	+
AB5355	*A. baumannii*	tnab1_kr130913p10q131	*katX* mutant::*T26*	T26	+
M2	*A. nosocomialis*		WT		+
M2Δ*katE*	*A. nosocomialis*		Δ*katE*::FRT	Flp-*FRT*	−
M2Δ*katG*	*A. nosocomialis*		Δ*katG*::FRT	Flp-*FRT*	+
M2Δ*katX*	*A. nosocomialis*		Δ*katX*::FRT	Flp-*FRT*	+
M2Δ*katEkatG*	*A. nosocomialis*		Δ*katE*::FRT Δ*katG*::FRT	Flp-*FRT*	−
M2Δ*katEkatX*	*A. nosocomialis*		Δ*katE*::FRT Δ*katX*::FRT	Flp-*FRT*	−
M2Δ*katGkatX*	*A. nosocomialis*		Δ*katG*::FRT Δ*katX*::FRT	Flp-*FRT*	+

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Table II

Primers used for the disruption of the *A. nosocomialis* catalase genes

Primer	Sequence	Function
M2katE_Fwd	ATGATGATCTTTTACGCAGTCT	To clone M2 *katE* and 1-kb flanking DNA into pGEM-T Easy
M2katE_Rev	GCATATTAATCACTATAAAGGGAC	
M2katErecomb_Fwd	TTTTCAACTTCAACCCAAGTTTAACTTTCAAAAC ATAGGTAATGGACATGATTCCGGGGATCCGTCG ACC	Recombineering primers to delete M2 *katE* and replace with Tn903-*sacB*
M2katErecomb_Rev	GAAATCTTCAACTTTCAGGGCTTTTTTATTTAAG CCGGTACGTGTGCGGCTGTAGGCTGGAGCTGCT TCG	
M2katG_Fwd	CGGGATTATGGTAGAGGTA	To clone M2 *katG* and 1-kb flanking DNA into pGEM-T Easy
M2katG_Rev	GCATATTAATCACTATAAAGGGAC	
M2katGrecomb_Fwd	GGTTCCATTAAGAGCTCAAATAAATATTACCCA GAGAGAATATAATCATGATTCCGGGGATCCGTC GACC	Recombineering primers to delete M2 *katG* and replace with Tn903-*sacB*
M2katGrecomb_Rev	GAACTGGCTTTTTATTATTTCGACTTAAATTAAG CTAAGTCAAAACGGTCTGTAGGCTGGAGCTGCT TCG	
M2katX_Fwd	ATCTATTAATATCACTTCTCCAG	To clone M2 *katX* and 1-kb flanking DNA into pGEM-T easy
M2katX_Rev	ATCAATGTTAGCTTTTTATTATCT	
M2katXrecomb_Fwd	ACATATTATTTTAATTCTAAAGTGGACTTTGCA ATAAGGAGCAATGAATGATTCCGGGGATCCGTC GACC	Recombineering primers to delete M2 *katX* and replace with Tn903-*sacB*
M2katXrecomb_Rev	TTTATATGTAAAGTATGACGAACTCACTTTTAA GGCTCTTGTCGAGGGGCTGTAGGCTGGAGCTGC TTCG	

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Table III

Primers used for the assessment of *katE* and *katG* expression

Primer	Sequence	Function
M2 katE_qRTPCR F1	AGATGGCGATAATTTAAATGCAGTGATG	Quantitative PCR for *katE* gene expression in *A. nosocomialis*
M2 katE_qRTPCR R1	CGGTACGTGTGCGGCTATTTGTT	
M2 katG_qRTPCR F1	TTGGTGGTTTACGTGTACTGGGCA	Quantitative PCR for *katG* gene expression in *A. nosocomialis*
M2 katG_qRTPCR R1	CCGTCGGCTTGAGCATAAACCTC	
M2 gyrA_qRTPCR F1	TGATTTCTGATGGTGGTACGCTTGTT	Quantitative PCR for *gyrA* gene expression in *A. nosocomialis*
M2 gyrA_qRTPCR R1	ATACAACTTCTTCGCTATCAGTTTCAGTCGT	
16S_RT_F	CTTCGGACCTTGCGCTAATA	Quantitative PCR for 16S rRNA expression *in A. baumannii*
16S_RT_R	ATCCTCTCAGACCCGCTACA	
katG_RT_F	GGCGATGAAAAAGAATGGTTA	Quantitative PCR for *katG* expression in *A. baumannii*
katG_RT_R	ATTTCTTCATCATCCATTGCC	
katE_RT_F	AACTTTGACTTCGATTTGCTGGA	Quantitative PCR for *katE* expression in *A. baumannii*
katE_RT_R	TGTATGAAAATAGTCGGGCTTGT	