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A *Helicobacter hepaticus* catalase mutant is hypersensitive to oxidative stress and suffers increased DNA damage

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Abstract

Catalase (KatA) is known to play an important role in oxidative stress resistance in many bacterial species and a homologue exists in *Helicobacter hepaticus*, a member of the enterohepatic *Helicobacter* species. Here, a *kataA* mutant was constructed by insertional mutagenesis and its oxidative stress phenotype was investigated. Catalase activity was readily detected [196 units (mg protein crude cell extract)⁻¹] in the wild-type, whereas the mutant strain was deficient in, but not devoid of, activity. In contrast, *Helicobacter pylori kataA* strains lack detectable catalase activity and wild-type *H. pylori* generally contains higher specific activity than *H. hepaticus*. Wild-type *H. hepaticus* cells tolerated 6 % O₂ for growth, whilst the *kataA* mutant could not survive at this oxygen level. Even at the optimal O₂ level, the growth of the *H. hepaticus kataA* strain was severely inhibited, which is also in contrast to *H. pylori kataA* strains. Wild-type *H. hepaticus* cells withstood exposure to 100 mM H₂O₂ but the *kataA* mutant cells were killed by the same treatment. Wild-type cells suffered no significant DNA damage by H₂O₂ treatment (100 mM for 6 min), whilst the same treatment resulted in severe DNA fragmentation in the *kataA* mutant. Thus *H. hepaticus* KatA plays an important role as an antioxidant protein.

INTRODUCTION

As an oxidative stress reagent, H₂O₂ attacks key sites in proteins. Particularly vulnerable are 4Fe–4S clusters associated with cysteinyl sulfurs and the sulfur of methionine residues. Moreover, H₂O₂ reacts with intracellular free iron via the Fenton reaction to produce a more damaging reactive oxygen species, the hydroxyl radical; this reactive species will damage many biomolecules, including DNA (Valko *et al.*, 2005). Organisms express the ubiquitous enzyme catalase, which decomposes H₂O₂ into water and oxygen, thus protecting organisms from the damaging effects of accumulating H₂O₂.

Helicobacter hepaticus is a Gram-negative, microaerophilic bacterium that occurs naturally in many strains of inbred mice (Fox *et al.*, 1994). Although *H. hepaticus* was first isolated from the mouse liver, it is consistently recovered from the intestinal tract and the primary site of colonization was shown to be the lower bowel of mice (Fox *et al.*, 1994, 1996). Chronic infection with *H. hepaticus* causes hepatic lesions and leads to the development of hepatocellular carcinoma (Solnick & Schauer, 2001). *H. hepaticus*-infected mice currently serve as an ideal animal model to study the mechanisms involved in the pathogenesis of human diseases such as hepatocellular carcinoma and inflammatory bowel diseases. This bacterium has become a topic of increasing research effort and was the second *Helicobacter* species (after *Helicobacter pylori*) to have its genome sequenced (Suerbaum *et al.*, 2003). Recent studies have revealed that *H. pylori* combats oxidative stress via a battery of diverse antioxidant systems that contribute to its long-term persistent infection in the host (Harris *et al.*, 2003; Olczak *et al.*, 2002; Seyler *et al.*, 2001; Wang & Maier, 2004). Some of these systems are

mechanistically novel, whereas others (such as superoxide dismutase and catalase) have been described in a number of pathogens. However, little is known about the antioxidant systems in *H. hepaticus*, and only a few targeted mutant strains of *H. hepaticus* have been reported so far (Hong *et al.*, 2006; Mehta *et al.*, 2005; Young *et al.*, 2004); none of these are in predicted enzymic oxidative stress-combating factors. As a first step towards understanding the antioxidant abilities and in particular the role of catalase in *H. hepaticus*, we constructed a gene-targeted *H. hepaticus* catalase mutant.

METHODS

Bacterial strains, culture and growth conditions

H. hepaticus type strain ATCC 51449 was used in this study. *H. hepaticus* was cultured on Brucella agar (BA; Difco) plates supplemented with 10 % defibrinated sheep blood at 37 °C in an incubator. The optimal atmosphere used for growth was 1 % O₂, 5 % CO₂, with the balance of the atmosphere composed of N₂. Different oxygen levels (1, 3 and 6 % O₂) were also applied in oxidative stress resistance assays as indicated. Chloramphenicol (20 µg ml⁻¹) was added to the medium for culture of the *H. hepaticus* mutant. *Escherichia coli* was grown aerobically on Luria–Bertani plates supplemented with ampicillin (100 µg ml⁻¹) or chloramphenicol (50 µg ml⁻¹). The plates were incubated at 37 °C in air.

katA mutant construction

Based on the genome sequence of *H. hepaticus*, primers *katAF* (5'-GTTTTCCGCCTCTTTTTCGC-3') and *katAR* (5'-AACATCGCCTGTGAGACATTCAG-3') were used to amplify a 1200 bp fragment; the amplicon was ligated directly into pGEM-T vector (Promega; linear DNA with a 3' T overhang) and the construct was used to transform *E. coli* DH5α by electrotransformation. The cloned plasmid was then extracted from the culture using a Qiagen extraction kit, and a chloramphenicol-resistance cassette (Cm^R) was inserted into the unique *Hind*III restriction site within the *katA* gene. The recombinant plasmid pGEMT : *katA* : Cm was then introduced into *H. hepaticus* by electrotransformation. Allelic exchange occurred, leading to the formation of the *katA* mutant strain. The mutant was selected on blood agar plates supplemented with chloramphenicol (20 µg ml⁻¹). The mutant was verified by demonstrating the correct-sized insertion of the Cm^R cassette (by PCR) within the *katA* gene.

Oxygen sensitivity assay

A suspension of *katA* mutant or wild-type cells (0.1 ml) with an OD₆₀₀ of 0.8 was evenly spread onto BA plates and incubated for 72 h under different O₂ partial pressures (1, 3 and 6 %) previously set for the experiment. Cells from a whole plate were harvested by suspension in 1 ml PBS and the OD₆₀₀ was measured (or calculated from dilutions) as an indication of relative growth yield.

Sensitivity to H₂O₂

Wild-type and *katA*:: Cm cells grown at 1 % O₂ for 48 h were suspended in PBS and the OD₆₀₀ was adjusted to 1.5. To test sensitivity towards H₂O₂, 200 µl cell suspension was mixed with 1.8 ml 100 mM H₂O₂, vortexed briefly and incubated at room temperature. Every 3 min, 0.1 ml of the cell/H₂O₂ mixture was removed and spread onto blood agar plates. The plates were then incubated under 1 % O₂ at 37 °C. As a control, 0.1 ml of the cell suspension (without exposure to H₂O₂) was taken and processed in the same way. After 48 h incubation, cells from each plate were harvested and suspended in 2 ml PBS buffer and the OD₆₀₀ was recorded.

DNA fragmentation assay

Wild-type or *katA* mutant cells were harvested at the late exponential phase from BA plates and suspended in PBS at an OD₆₀₀ of 0.5. Samples were treated with 100 mM H₂O₂ for 0, 3 or 6 min as described above, and then processed for gel electrophoresis. For the preparation of samples, we followed a procedure described previously (Zirkle & Krieg, 1996) with minor modifications. Briefly, H₂O₂-treated or untreated cells were washed, suspended in TE buffer (50 mM Tris/HCl, 5 mM EDTA, pH 8.0) and mixed with 1.0 % low-melting-point agarose. After solidification, the gel plugs were placed in a lysis solution containing 0.5 % (w/v) Sarkosyl and 0.5 mg proteinase K ml⁻¹ and incubated overnight at room temperature. The gel plugs were then washed with cold TE buffer and submerged in a 0.8 % regular agarose gel. Samples were then subjected to electrophoresis and the gel was stained with ethidium bromide (0.5 µg ml⁻¹) and visualized under UV light.

Catalase activity assay

The catalase activity of cell extracts was determined quantitatively following a method described previously (Wang *et al.*, 2004). Briefly, catalase activity was measured spectrophotometrically at 25 °C by following the decrease in A₂₄₀ ($\epsilon_{240}=43.48 \text{ M}^{-1} \text{ cm}^{-1}$) of 13 mM H₂O₂ in PBS. All assays were repeated to give 12 rate determinations for the first minute of reaction. One unit was defined as the amount of enzyme that catalysed the oxidation of 1 µmol H₂O₂ min⁻¹ under the assay conditions.

RESULTS

Construction of a *H. hepaticus* catalase mutant

In the published genome sequence of *H. hepaticus* (Suerbaum *et al.*, 2003), HH0043 was annotated as the gene encoding catalase. The *katA* gene in the *H. hepaticus* genome was disrupted by insertion of a Cm^R cassette (Wang & Taylor, 1990) via allelic exchange. Transformant colonies were obtained by screening for colonies in a 1 % O₂ environment. To confirm the correct insertion of the antibiotic-resistance cassette within the *katA* gene, genomic DNA prepared from the mutant clone was used as template for PCR with the primer pair katAF/katAR (Fig. 1a). As shown in Fig. 1(b), PCR using wild-type DNA as template produced a product of 1.2 kb, whereas the mutant genomic DNA gave rise to a PCR product of 2.0 kb, indicating insertion of the 0.8 kb Cm^R cassette.

Protein profile and catalase activity in cell extracts

Protein profiles were examined after SDS-PAGE of the wild-type and *katA* mutant cell extracts; the results are shown in Fig. 2. A 55 kDa protein band was present in the wild-type cells but lacking in the mutant cells. This protein was identified as catalase by direct N-terminal sequencing of the protein band, confirming that the strain *H. hepaticus* ATCC 51449 *katA* :: Cm was a true catalase mutant. Based on densitometric measurement of the protein bands on the gel, KatA was estimated to constitute ~1 % of the total protein of the cell.

Catalase activity was determined for the wild-type and *katA* mutant cell extracts. The wild-type cells exhibited a catalase activity of 196±14 units [µmol H₂O₂ min⁻¹ (mg total protein)⁻¹, mean±SD from five replicates]. In contrast, the *katA* mutant cells lost most of their catalase activity. Nevertheless, the residual amount of activity (23±3 units, for five replicates) was significantly higher than the background, which comprised heat-treated cell extract.

Sensitivity to oxidative stress

We examined the sensitivity of the *katA* mutant to O₂ by measuring growth under different O₂ concentration conditions in comparison with the wild-type strain. As *H. hepaticus* does not

usually form discrete individual colonies on plates, we measured the OD₆₀₀ of the cell suspensions as an indication of relative growth yield (Fig. 3). Even at 1 % partial O₂ pressure, the mutant strain grew much slower than the wild-type. Whilst the wild-type could still grow at 6 % O₂ (albeit at a slower rate than at 1 % O₂), the *katA* mutant could not survive under this O₂ condition. This result clearly demonstrated that the *H. hepaticus katA* mutant was hypersensitive to O₂.

To examine sensitivity to H₂O₂, the wild-type and *katA* mutant cells were treated with 100 mM H₂O₂ and samples were removed at 3 min intervals followed by transfer to normal growth conditions at 1 % O₂. After incubating for 48 h, the cells were collected in the same volume of PBS and OD₆₀₀ was measured to represent the relative number of viable cells (Fig. 4). About half of the wild-type cells survived the initial 3 min treatment. Longer exposures to peroxide (up to 21 min) did not significantly reduce cell viability further. In contrast, the majority of the *katA* mutant cells were killed by exposure to H₂O₂ for 3 min and no cells could be recovered after H₂O₂ treatment for 6 min.

Level of DNA fragmentation

To investigate the role of catalase in protecting DNA from damage, the level of DNA fragmentation was examined for the *katA* mutant strain in comparison with the wild type. Exponential phase growth cells were treated with 100 mM H₂O₂ for 0, 3 or 6 min and the samples were analysed for genomic DNA fragmentation by gel electrophoresis (Fig. 5). Little fragmented DNA was observed for the wild-type cells exposed to H₂O₂ for 3 or 6 min. The *katA* mutant cells exposed to H₂O₂ for 3 min contained a small amount of fragmented DNA, and a large amount of the genomic DNA was damaged in the *katA* mutant cells exposed to H₂O₂ for 6 min.

DISCUSSION

H. hepaticus KatA is highly homologous to catalases in both prokaryotes and eukaryotes, with the highest homology being shown to the catalases of *Bordetella pertussis* (65.9 %) and *H. pylori* (62.5 %). The catalase activity in the cell extract of the wild-type strain ATCC 51449 was determined to be approximately 200 units [$\mu\text{mol H}_2\text{O}_2 \text{ min}^{-1} (\text{mg total protein})^{-1}$]. Using the same method, we examined different strains of *H. pylori* that usually express a high level of KatA protein (>1 % of the total protein) and the catalase activity ranged from approximately 1000 to 4000 units (unpublished data). One exception was *H. pylori* strain 26695, which expressed a very low amount of KatA protein and showed a catalase activity of <400 units. *H. hepaticus* also expressed a high level of KatA (~1 % of the total protein, see Fig. 2a), but the specific activity of *H. hepaticus* KatA appears to be lower than that of *H. pylori*. It is possible that *H. hepaticus* encounters lower H₂O₂ levels than *H. pylori in vivo*.

Wild-type *H. hepaticus* cells were able to survive exposure to 100 mM H₂O₂, which is similar to results observed for *H. pylori* (Harris *et al.*, 2002). For comparison, the H₂O₂ concentration used for sensitivity tests of *Campylobacter* is at a much lower level (1 mM) (Day *et al.*, 2000; Grant & Park, 1995). The ability of *H. hepaticus* cells to survive such high concentrations of H₂O₂ is indicative of the importance of catalase to the survival of the bacterium under oxidative stress conditions. Such stress conditions may result from the production of reactive oxygen species by the host cells following *H. hepaticus* infection. There is evidence that the levels of 8-oxo-guanine and lipid peroxidation are significantly higher in *H. hepaticus*-infected mice and that these oxidized macromolecules increase with duration of infection (Singh *et al.*, 2001; Sipowicz *et al.*, 1997). In addition, endogenous production of peroxides by *H. hepaticus* through auto-oxidation of flavoproteins could be a major source of oxidative stress (Imlay, 2003). Our recent studies on *H. hepaticus* alkyl hydroperoxide reductase (AhpC), the enzyme responsible for cell defence against organic peroxide oxidative stress, revealed that

expression of KatA was greatly increased (Fig. 2a, lane 3) in *H. hepaticus* *ahpC* mutant cells as a compensatory response. This also highlights the importance of catalase in oxidative stress defence in *H. hepaticus*.

To define further the role of catalase in *H. hepaticus*, we examined the phenotypes of a *katA* mutant. The *katA* mutant cell extract had a minimal but detectable activity of H₂O₂ dissipation (about 1/10 of the wild-type level). It is noteworthy that the *katA* mutants of *H. pylori* exhibit undetectable catalase activity (Harris *et al.*, 2002; Odenbreit *et al.*, 1996; Wang *et al.*, 2004). The *H. hepaticus* genome sequence does not reveal the existence of another gene encoding a catalase or catalase-like protein. It is likely that the residual amount of activity detected in the *katA* mutant cell extract was due to the presence of other peroxidases (such as AhpC) that may have redundant H₂O₂ reducing activity.

Disruption of the *katA* gene rendered *H. hepaticus* cells hypersensitive to oxidative stress. Even under optimal growth conditions (1 % O₂) for the wild-type, the mutant strain grew very poorly and it could not survive exposure to an atmosphere of >6 % O₂ (Fig. 3). Of note, *H. pylori* *katA* mutants are hypersensitive to H₂O₂, but grow similarly to the wild-type under optimal O₂ conditions (~5 % O₂) (Harris *et al.*, 2002; Odenbreit *et al.*, 1996; Wang *et al.*, 2004). The hypersensitivity of the *H. hepaticus* *katA* mutant to H₂O₂ (Fig. 4) provides evidence for the importance of catalase in the oxidative stress defence repertoire of *H. hepaticus*.

As a powerful oxidant, H₂O₂ and its derivative hydroxyl radical can have a damaging effect on DNA. We determined the relative levels of DNA damage in *H. hepaticus* cells by use of a DNA fragmentation assay. Exposure to H₂O₂ for 6 min completely killed the *katA* mutant cells (Fig. 4), and the result in Fig. 5 showed that these cells contained a large amount of fragmented DNA. These results indicate the importance of KatA in protecting *H. hepaticus* from H₂O₂-mediated DNA damage.

Downstream of *H. hepaticus* *katA* is a gene (*yahD*) encoding an ankyrin-repeat protein of unknown function (Fig. 1a). Homologues of this gene are present in many pathogenic bacteria such as *Campylobacter jejuni*, *Streptomyces avermitilis* and *Pseudomonas putida*, but none of these have been studied. Downstream of *katA* in *H. pylori* is a gene named *kapA*, which is not a homologue of *yahD*. Disruption of *kapA* in *H. pylori* does not affect catalase activity, but nevertheless results in an increased sensitivity to H₂O₂ (Harris *et al.*, 2002). *H. pylori* catalase has been shown to localize in both the cytoplasm and periplasm, and the catalase activity within the periplasm is significantly affected by the loss of KapA (Harris & Hazell, 2003). Through interaction with KatA, KapA may be involved in the translocation of KatA into the periplasm. The effects of *H. hepaticus* *yahD* on catalase activity and H₂O₂ sensitivity need to be investigated, as one of the known roles of ankyrin-repeat proteins is mediating protein-protein inter-actions (Sedgwick & Smerdon, 1999). Future studies including complementation of KatA function and the effect of KatA disruption on host colonization efficiency are required for a further understanding of the importance of catalase in *H. hepaticus* pathogenesis.

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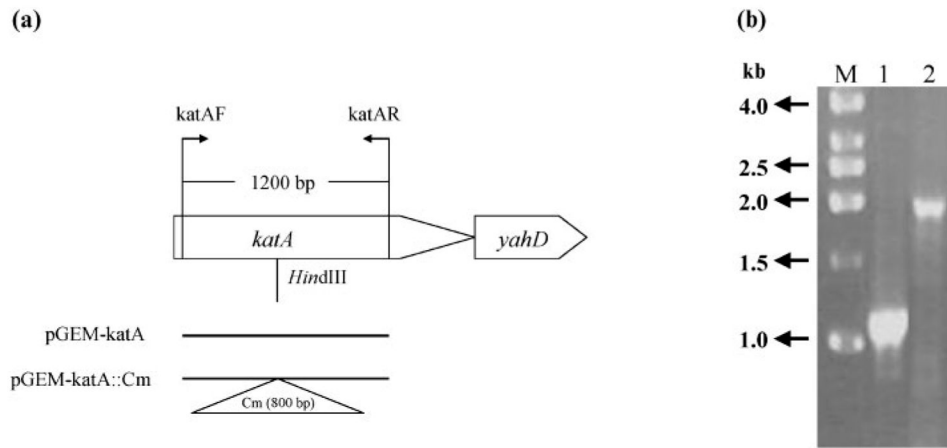


Fig. 1. (a) Organization of the *katA* gene in the *H. hepaticus* genome and DNA constructs for generating the *katA* mutant. A 1200 bp fragment of the *katA* gene was amplified using the *katAF*/*katAR* primer pair and cloned into pGEM-T. A Cm^{R} cassette was then inserted within the *katA* fragment at the *Hind*III restriction site and the plasmid pGEM-*katA* :: Cm was used for transformation of wild-type *H. hepaticus* to create the *katA* mutant. (b) Agarose gel analysis of PCR products using primers *katAF*/*katAR*. Lanes: 1, wild-type *H. hepaticus*; 2, *katA* mutant; M, DNA size standard.

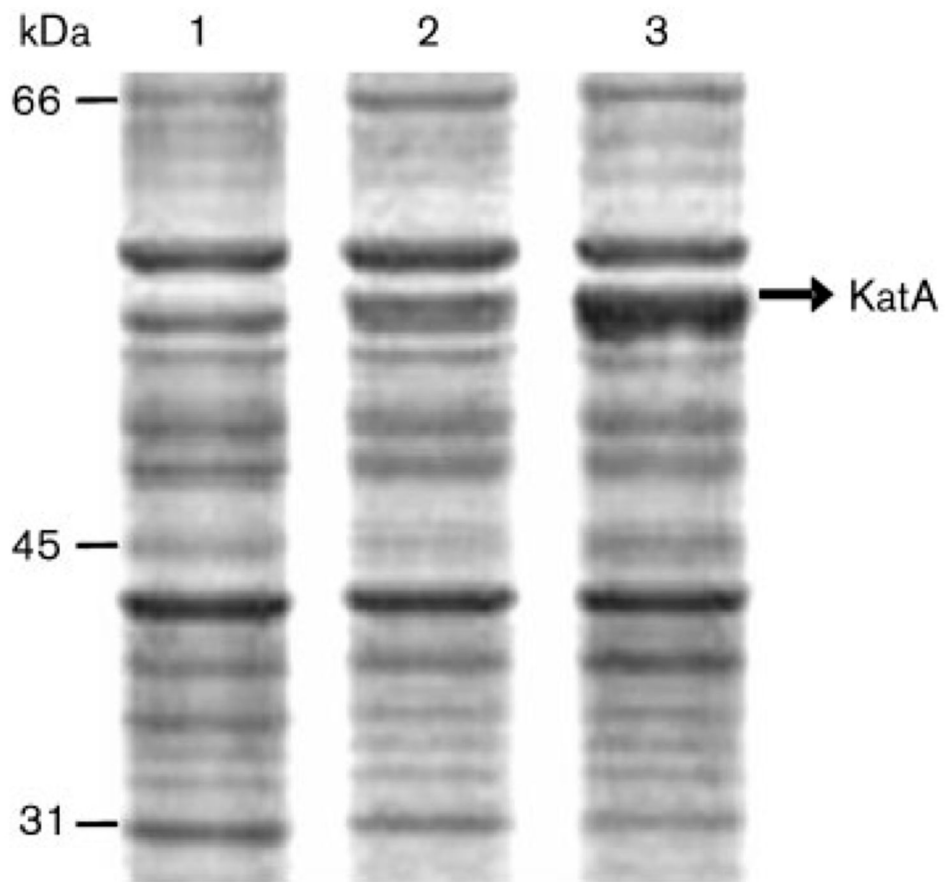


Fig. 2. Protein profiles of *H. hepaticus* cell crude extracts, analysed by SDS-PAGE. Seven micrograms of protein was loaded onto each lane. Lanes: 1, *kata* :: Cm mutant; 2, wild-type cells; 3, *ahpC* mutant (see Discussion). The sizes of protein markers are labelled on the left and the 55 kDa KatA band is marked on the right.

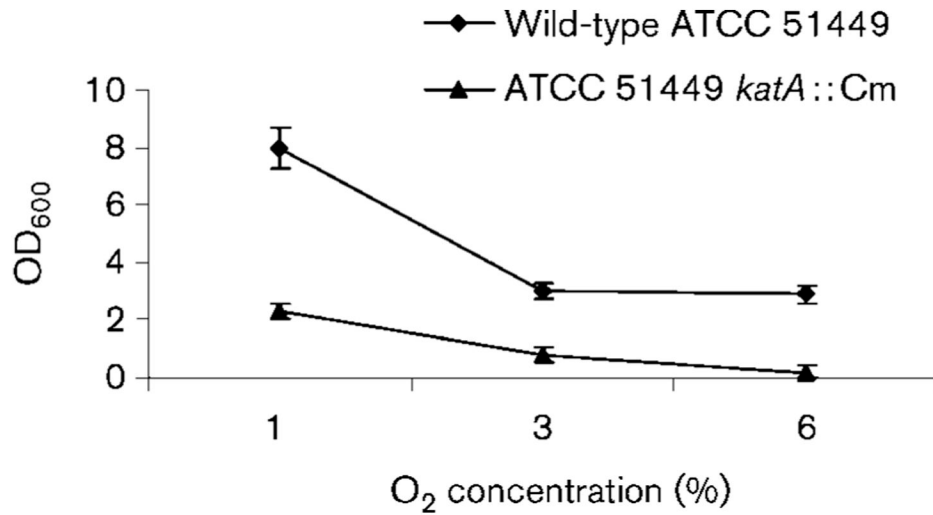


Fig. 3. Oxygen sensitivity of *H. hepaticus* strains. Cells were grown for 72 h under different O₂ partial pressures (1, 3 and 6 %), harvested, suspended in PBS (1 ml per plate) and the OD₆₀₀ was measured. The results are the means±SD of three experiments. According to the Student's *t*-test, the mutant grew significantly slower than the wild-type at the 99 % level of confidence ($P < 0.01$) at all of the O₂ levels tested.

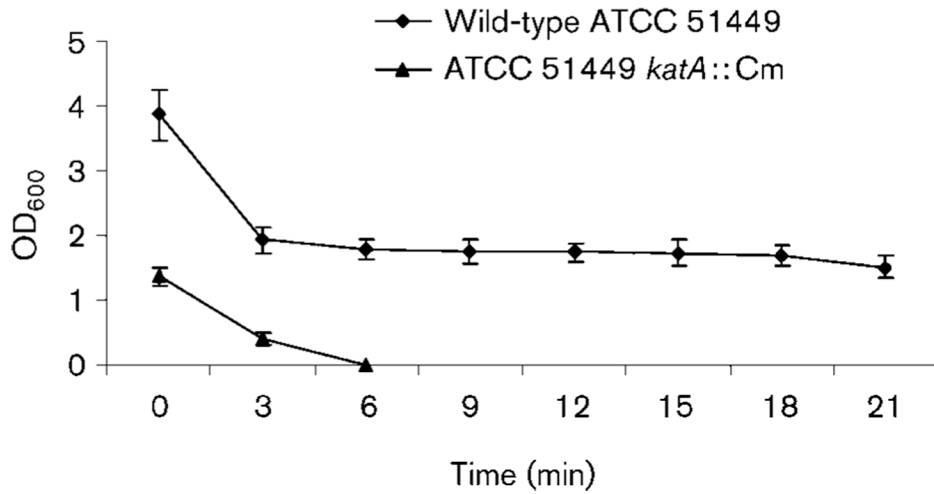


Fig. 4. Sensitivity of *H. hepaticus* wild-type and *katA* :: Cm strains to 100 mM H₂O₂ over a 21 min time period. The y-axis represents the relative number of cells surviving after exposure to H₂O₂. The results are the means±SD of three experiments and survival of the *katA* strain was significantly less than that of the wild-type at all points tested ($P < 0.01$).

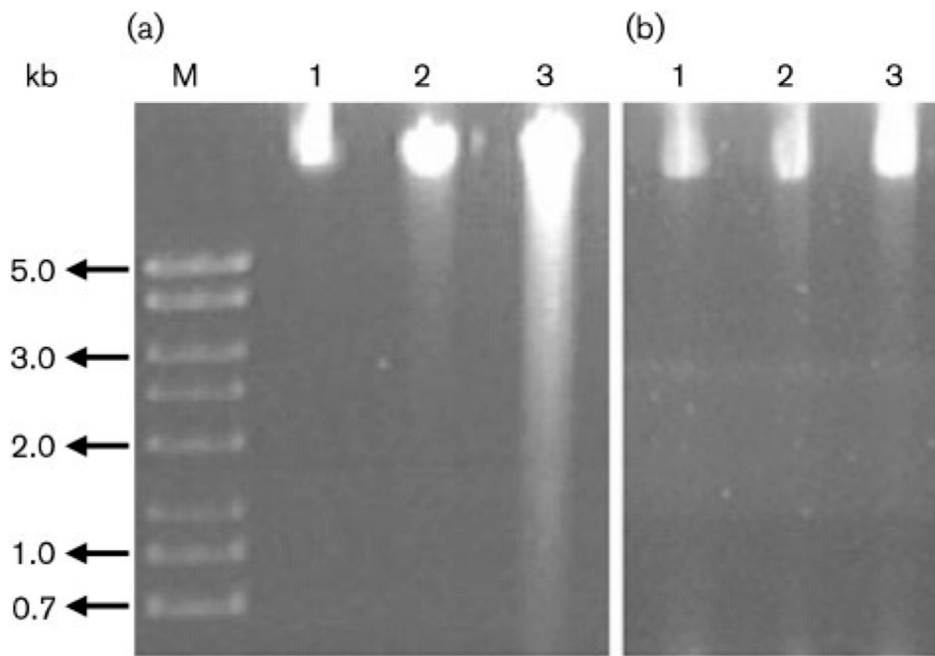


Fig. 5. DNA fragmentation assay. The *katA* mutant (a) or wild-type (b) cells were grown to late exponential phase, harvested in PBS buffer and exposed to 100 mM H_2O_2 for 0, 3 or 6 min (lanes 1–3, respectively). The samples were processed for agarose gel electrophoresis to visualize the relative sizes of DNA fragments. A DNA size marker (kb) is labelled on the left.