Catalases and PhoB/PhoR system independently contribute to oxidative stress resistance in Vibrio cholerae O1

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All cells are subjected to oxidative stress, a condition under which reactive oxygen species (ROS) production exceeds elimination. Bacterial defences against ROS include synthesis of antioxidant enzymes like peroxidases and catalases. Vibrio cholerae can produce two distinct catalases, KatB and KatG, which contribute to ROS homeostasis. In this study, we analysed the mechanism behind katG and katB expression in two V. cholerae O1 pandemic strains, O395 and N16961, of classical and El Tor biotypes, respectively. Both strains express these genes, especially at stationary phase. However, El Tor N16961 produces higher KatB and KatG levels and is much more resistant to peroxide challenge than the classical strain, confirming a direct relationship between catalase activity and oxidative stress resistance. Moreover, we showed that katG and katB expression levels depend on inorganic phosphate (Pi) availability, in contrast to other bacterial species. In N16961, katB and katG expression is reduced under Pi limitation relative to Pi abundance. Total catalase activity in N16961 and its phoB mutant cells was similar, independently of growth conditions, indicating that the PhoB/PhoR system is not required for katB and katG expression. However, N16961 cells from Pi-limited cultures were 50–100-fold more resistant to H₂O₂ challenge and accumulated less ROS than phoB mutant cells. Together, these findings suggest that, besides KatB and KatG, the PhoB/PhoR system is an important protective factor against ROS in V. cholerae N16961. They also corroborate previous results from our and other groups, suggesting that the PhoB/PhoR system is fundamental for V. cholerae biology.

INTRODUCTION

Under aerobic conditions, all cells produce reactive oxygen species (ROS) as aerobic metabolism by-products. Bacteria can also be exposed to high levels of ROS generated by the host defence system (Vallet-Gely et al., 2008) or in response to unfavourable environmental conditions (Shimizu, 2013). ROS accumulation can lead to oxidative stress, which can damage macromolecules such as nucleic acids, proteins and lipids, and to avoid such harmful effects, bacteria have evolved mechanisms to sense ROS levels and to counteract their effects (Lushchak, 2011a).

Bacterial ROS-sensing mechanisms involve a number of transcription factors that regulate antioxidant enzyme expression (Dubbs & Mongkolsuk, 2012). These enzymes include superoxide dismutases, which catalyse superoxide anion (O²⁻) dismutation to H₂O₂, and hydroperoxidases (catalases and peroxidases), which cleave the peroxidic bond in H₂O₂ and also in organic peroxides (Lushchak, 2011b). Hydroperoxidase gene expression regulation is well studied in Escherichia coli, a Gram-negative bacterium that produces two catalases. HPI, also known as KatG, is a bifunctional catalase/peroxidase whose expression is H₂O₂ induced in an OxyR-dependent manner (Storz et al. 1990). E. coli OxyR is a well-characterized bacterial redox-sensing transcription factor that activates a regulon of more than 20 antioxidant genes. HPII, also known as KatE, is a typical catalase whose gene is controlled by RpoS, the primary regulator of stationary-phase genes, and is not induced by H₂O₂ [reviewed in Schellhorn (1995)]. Both KatG and KatE are involved in oxidative stress response in E. coli (Jung & Kim, 2003).
**Vibrio cholerae** is a Gram-negative bacterium that can be found in aquatic environments, especially in brackish water, either free-living or associated with biotic or abiotic surfaces (Colwell, 1996). Several strains are facultative pathogens that can colonize human small intestine and cause a watery diarrhoeic disease known as cholera (Finkelstein, 1996). Seven cholera pandemics have been reported, all caused by *V. cholerae* O1 serogroup strains (Salim et al., 2005). Some *V. cholerae* strains can produce two different catalases, KatG (VC1560) and KatB (VC1585), both of which contribute to their oxidative stress resistance (Wang et al., 2012). However, little is known about katG and katB expression regulation in this species. It has been shown that, in *V. cholerae* El Tor bio- type strain C7258, katB is repressed by the histone-like nucleoid structuring protein by transcriptional silencing (Silva et al., 2008). Differently from *E. coli*, in *V. cholerae* El Tor strain C6706, KatB and KatG production is independent of OxyR (Wang et al., 2012).

Under natural conditions, aquatic environments are usually poor in nutrients such as phosphorus, an essential element for all forms of life. *V. cholerae* survival in such a habitat depends on its ability to cope with phosphorus deficiency. Inorganic phosphate (Pi) is the main source of P for bacteria and the maintenance of bacterial Pi homeostasis is controlled by the two-component system PhoB/PhoR (Wanner, 1993). This system regulates expression of genes involved in Pi uptake and metabolism and in many other processes, including pathogenesis (Goulart et al., 2010; Osorio et al., 2004; von Kruger et al., 1999, 2006). More recently, we demonstrated for the first time that the *V. cholerae* O1 PhoB/PhoR system is also required for full growth and RpoS accumulation in stationary-phase culture under high Pi level, indicating that it is a fundamental system for bacterium biology (Lery et al., 2013). In some bacterial species, Pi limitation induces catalase expression in a PhoB-dependent manner, increasing their ability to cope with oxidative stress in such environments (Yuan et al., 2005). In previous work, we identified many *V. cholerae* stress response-related gene products whose expression is dependent on PhoB/PhoR and/or Pi levels (von Kruger et al., 2006). Although catalases were not among the proteins identified in the mentioned study, we cannot discard a relationship between kat gene expression and PhoB/PhoR or Pi availability in *V. cholerae*. This was investigated in the present study using two *V. cholerae* O1 isolates, the sixth pandemic classical biotype O395 and the seventh pandemic El Tor biotype N16961 (Salim et al., 2005). Our results led us to conclude that KatG and KatB seem to be involved in oxidative stress response in both strains, confirming previous results (Wang et al., 2012). However, differences were found between strains O395 and N16961 with respect to catalase gene expression profiles and total catalase activity. Moreover, katB and katG expression in *V. cholerae* N16961, unlike in other bacterial species (Yuan et al., 2005), is repressed under Pi limitation in a PhoB-independent manner. However, under this condition, an active PhoB/PhoR system is required for WT resistance to ROS.

**METHODS**

**Strains and growth conditions.** *V. cholerae* spontaneous streptomycin-resistant O1 strains El Tor N16961, classical O395 and WK10, an N16961 isogenic *phoB*/*phoR* mutant strain (von Kruger et al., 1999, 2006), were routinely grown at 37 °C in LB broth under aeration or on solid medium (1.5% agar in LB). For growth under defined Pi concentrations, MG (165 mM MOPS, pH 7.4; 80 mM NaCl; 20 mM KCl; 20 mM NH₄Cl; 3 mM Na₂SO₄; 1 mM MgCl₂; 200 µM CaCl₂; 3 µM FeCl₃; 0.4% glucose; 10 µM thiamine) was used with KH₂PO₄ at 65 mM (high phosphate medium, MGHP) or 65 µM (low phosphate medium, MGLP) (von Kruger et al., 1999). For genetic manipulations, *E. coli* Top10 (Invitrogen) was used. Antibiotics were added to media when required at the following concentrations: streptomycin, 100 µg/ml; ampicillin, 100 µg/ml and kanamycin, 50 µg/ml. Bacterial growth was spectrophotometrically determined by following OD₆₀₀ for 8 h.

**Genetic procedures.** Fragments of 1.7 and 2.1 kb containing, respectively, genetic *V. cholerae* N16961 katB (vc1585) and katG (vc1560) genes were PCR amplified using N16961 strain chromosomal DNA as template and primers KatB-fw-KatB-drv and KatGB-fw-KatGB-drv listed in Table 1. Fragments were inserted into pBAD TOPO (Invitrogen), downstream *araBAD* promoter, according to supplier’s recommendations, to generate arabinose-inducible overexpression systems pBAD-katB and pBAD-katG. *katB* and *perA* genes from *V. cholerae* N16961 are 99% identical with the same genes in O395 strain.

Fragments 300 bp long of the katB regulatory region in N16961 and O395 were PCR amplified from the chromosomal DNA from each strain using primers KatBpro1 and KatBpro2 (Table 1). The fragments were digested with *NdeI* and *Xhol* and ligated to pCK52 (Macián et al., 1994) digested with the same enzymes, to create plasmids pkB16 and pKB395. These contained the *katB* regulatory region from each strain fused to the lacZ gene sequence. All constructions were confirmed by digestion and sequencing.

**Induction of catalases expression.** To induce catalases expression from pBAD-katB and pBAD-katG, plasmids were transformed into O395-competent cells prepared as previously described (von Kruger et al., 2006). Transformants were grown in LB until mid-exponential phase (OD₆₀₀=0.5–0.7) and arabinose at 0.002, 0.02 or 0.2% was added to three separate cultures. Glucose (0.2%) was added to another culture to repress *katB* gene expression from the *araBAD* promoter (negative control). All cultures were incubated for further 4 h at 37 °C under aeration before catalase activity was assayed.

**Enzyme assays.** Catalase activity was determined in solution and in gel after non-denaturing PAGE. For both procedures, *V. cholerae* cells were cultivated in LB to mid-exponential (OD₆₀₀=0.5–0.7) or stationary (OD₆₀₀=2.5) phase or in MGHP and MGLP for 10 h. Cells in a volume corresponding to OD₆₀₀=6 were collected by centrifugation (16 000 g for 15 min at 4 °C), suspended in 500 µl of 50 mM potassium phosphate buffer (pH 7.0) containing 5 mM EDTA, 10% glycerol and 25 µM PMSF and disrupted by sonication. Unbroken cells and large aggregates were removed by centrifugation as mentioned above. Lyase protein concentration was determined with Bio-Rad Protein Assay Dye Reagent.

To measure catalase activity in solution, a lyase volume containing a minimum of 12.5 µg proteins was diluted in 1 ml water to which 500 µl of 59 mM H₂O₂ in 50 mM potassium phosphate buffer (pH 7.0) was added. H₂O₂ decomposition was monitored spectrophotometrically by adding

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Table 1. Plasmids and primers used in this work

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<tr>
<th>Name</th>
<th>Description</th>
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measuring OD_{260} for 3 min. The exponential decay of OD_{260} per minute \((dOD_{260} / dt)\) was determined and catalase units (U) were defined as \((dOD_{260} / dt)\times 1000\times 43.6^{-1}\) (43.6 represents \(e\) value for H_{2}O_{2} in \(mM^{-1} cm^{-1}\)).

Native PAGE was used to detect protein bands with catalase activity. Briefly, protein lysate (50–100 µg) was resolved by non-denaturing 4–8 % polyacrylamide gradient gel electrophoresis after which the gel was stained specifically for catalase activity using potassium ferricyanide solution (Woodbury et al., 1971).

\(\beta\)-Galactosidase activities were measured in cell lysates, as previously described (Miller 1972), and expressed in U (µg protein)\(^{-1}\) (Protein Assay Dye Reagent; Bio-Rad).

**MS protein identification.** Whole-cell lysates (30 µg of proteins) were analysed by SDS-PAGE (12.5 %). Gel was stained with Coomassie Brilliant Blue and protein bands were cut from the gel, trypsin digested (Shevchenko et al., 1996) and analysed by MS ESI-Q-TOF Micro (Waters), coupled to a nanoUPLC (nano ultraperformance liquid chromatograph) (nanoACQUITY; Waters), as recommended by Taylor et al. (2007). Raw data were processed using the ProteinLynx 2x4 and proteins were identified as previously described (von Kruger et al., 2006).

**Bacterial survival assays after \(H_{2}O_{2}\) treatment.** Aliquots of 500 µl of the cultures used for catalase assay were incubated with \(H_{2}O_{2}\) (15 or 30 mM, depending on strain and growth condition) for up to 20 min, at 37 °C, without aeration. Cell survival was calculated by determining c. f.u. ml\(^{-1}\) after challenge in comparison to non-treated culture (control, 100 %).

**ROS detection.** Intracellular ROS levels were measured using dihydrodichlorofluorescein diacetate. N16961 and WK10 were grown for 10 h in MGHP or MGLP. Aliquots of 10 µl and 100 µl of the cultures in MGHP and MGLP, respectively, were harvested by centrifugation (16 000 g for 4 min at room temperature) and re-suspended in 1 ml of 0.9 % NaCl. The suspensions were diluted and plated onto 1.5 % LB agar containing 100 µg ml\(^{-1}\) streptomycin for viable c.f.u. determination. Dihydrodichlorofluorescein diacetate at a final concentration of 10 µM was then added to the bacterial suspension, followed by 30 min of incubation at 37 °C. Fluorescence was measured at 485/530 nm excitation/emission using a microplate reader (Victor3, PerkinElmer) and the results were expressed as fluorescence intensity×1000/c.f.u. ml\(^{-1}\).

**RESULTS**

Expression of **katG** and **katB** in V. cholerae O1

N16961 and O395 is growth phase dependent and varies between the strains

Genome analysis revealed that classical O395 and El Tor N16961 V. cholerae O1 strains have two distinct catalase genes, **katG** (VC1560) and **katB** (VC1585), which encode, respectively, KatG and KatB. Previous study has shown that both proteins contribute to oxidative stress resistance in El Tor strain C6706 (Wang et al., 2012). However, nothing is known about the expression and functions of these genes in the pandemic strains O395 and N16961. In order to investigate whether O395 and N16961 produce catalases, cells were grown in rich medium (LB) and enzyme total activity was measured in exponential- and stationary-phase cells (Fig. 1a, b). In N16961, total catalase activity was growth stage dependent, being higher in stationary-phase cells. O395 cells, on the other hand, displayed lower and similar catalase activity in both culture phases.

N16961 and O395 exponential- and stationary-phase, whole-cell lysates were also subjected to non-denaturing PAGE followed by in-gel catalase activity staining (Woodbury et al., 1971). Two catalase activity regions (I and II) exhibiting distinct migration patterns were detected in gel lanes corresponding to N16961 and O395 stationary-phase cells. However, both catalase bands from N16961 were stronger than those from O395 (Fig. 1b). Exponential cells, catalase electrophoretic pattern, on the other hand, differed between strains. While N16961 presented only the slow-migrating catalase (band I), no activity was detected in O395 exponential cells, under conditions analysed (Fig. 1c). This can be explained by the fact that the gel staining is less sensitive than the catalase quantitative assay and also because enzyme activity in exponential O395 cells seems to be highly variable (Fig. 1b).
To identify the catalases, bands of high enzymatic activity (I and II from N16961 stationary-phase cells’ electrophoretic profile; Fig. 1c) were excised and treated with trypsin, and the resulting peptides were analysed by MS. While no proteins were identified in band I, several *V. cholerae* proteins were identified in band II (Table S1, available in the online Supplementary Material), including catalase/peroxidase KatG (VC1560).

As strains O395 and N16961 have potential to produce two catalases, KatG and KatB, we hypothesized that KatB, although it was not identified in the former experiment, could be the slow-moving catalase in the band I (Fig. 1c). To test this assumption, two plasmids, pBAD-katB and pBAD-katG, carrying, respectively, KatB- and KatG-encoding genes upstream of an arabinose-inducible promoter, were constructed and introduced into *V. cholerae* O395 since it shows low endogenous catalase activity.

0.02% arabinose was used in the subsequent experiments. Expression of both *katG* and *katB* in transformed O395 cells was induced by arabinose added to mid-exponential-phase cultures. Among arabinose concentrations tested, 0.02% was the lowest required for higher expression of both *katG* and *katB* (Fig. S1). Thus, 0.02% arabinose was used in the subsequent experiments. Expression of *katB* and *katG* in induced O395 cells carrying either pBAD-katB or pBAD-katG was evaluated by SDS-PAGE and catalase activity assay. A protein band of molecular mass between 45 and 66 kDa (Fig. 2a), consistent with the theoretical KatB molecular mass of 64 kDa, was only seen in the electrophoretic profile of O395/pBAD-katB induced cells, which also presented strong catalase activity at band I (Fig. 2b). These results indicate that *katB* was expressed in O395/pBAD-katB cells upon arabinose induction and that KatB is the slow-migrating catalase in band I, also seen in Fig. 1(c).

The protein profile of the induced O395/pBAD-katG cells, on the other hand, presented an extra band of molecular mass between 66 and 97 kDa (Fig. 2a), consistent with the theoretical KatG molecular mass of 86 kDa, which is not present in non-induced cells (Fig. 2a). Induced cells showed high catalase activity at band II (Fig. 2b) corroborating MS KatG identification in this gel region (Fig. 1c).

In summary, *V. cholerae* O1 strains El Tor N16961 and classical O395 can express *katG* and *katB* in a growth phase-dependent manner. However, N16961 produces significantly higher catalase levels than classical strain O395. This is not surprising since it has been demonstrated that El Tor strains have greater ability to survive stressful conditions.

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**Fig. 1.** Catalase activity in exponential- and stationary-phase cells of the *V. cholerae* strains El Tor N16961 and classical O395. Cells were grown to exponential or stationary growth phase in LB at 37 °C, under aeration. (a) N16961 and O395 growth curves showing exponential- (black arrow) and stationary- (white arrow) phase sampling points. (b) Catalase-specific activity was determined in cell lysates. Columns represent the mean of three individual experiments with standard deviation as error bars. (c) Exponential- and stationary-phase, whole-cell lysate proteins (60 µg) were resolved by electrophoresis on a 4–8% gradient non-denaturing polyacrylamide gel followed by in-gel catalase activity staining. The arrows indicate two bands of catalase activity that will be referred as bands I and II in the text.

**Fig. 2.** Protein profiles of O395 cells carrying pBAD-katB or pBAD-katG. O395 cells carrying pBAD-katB or pBAD-katG were grown in LB for 4 h, at 37 °C, under aeration, after arabinose addition to mid-exponential-phase cultures. Cells were then collected and lysates were prepared and gel analysed. (a) Whole-cell lysate electrophoretic profiles (30 µg of proteins per lane) after SDS-PAGE (12.5%). Arrows indicate protein bands only seen on arabinose-treated cells profile. (b) Whole-cell lysate catalase activity (50 µg of proteins per lane) after non-denaturing 4–8% gradient gel electrophoresis and specific staining.
KatB and KatG are involved in oxidative stress response in *V. cholerae*

In order to investigate whether KatB or KatG plays roles in oxidative stress resistance in *V. cholerae*, non-transformed O395 cells and those carrying pBAD-katB or pBAD-katG were cultivated in LB to mid-exponential phase, at 37 °C, under aeration. At this point, arabinose (to induce araBAD promoter) or glucose (to avoid araBAD promoter leaking) was added to cultures, which were incubated for a further 4 h. Catalase-specific activities and cell survival rates were estimated upon 20 min exposure to 30 mM H$_{2}$O$_{2}$ (Fig. 3). O395/pBAD-katB cells treated with arabinose presented the highest catalase-specific activity detected, followed by those carrying pBAD-katG, compared to non-transformed cells or uninduced transformants. The survival rates of cells challenged with H$_{2}$O$_{2}$ were directly related to their catalase activity. Accordingly, O395/pBAD-katB cells were the most resistant (near 100% survival), followed by pBAD-katG cells (around 10% survival). Cells showing very low catalase levels were very sensitive to H$_{2}$O$_{2}$, with survival rates significantly lower (Fig. 3).

Taken together, these findings show that KatG and KatB catalases seem to be important factors for *V. cholerae* O1 N16961 and O395 strains' oxidative stress response, corroborating data from a previous study on El Tor strain C670 (Wang et al., 2012). However, KatB seems to play a critical role in the process (Fig. 3), in agreement with the increased expression of *katB* gene in *V. cholerae* O1 El Tor strain A1552 variants highly resistant to H$_{2}$O$_{2}$ (Yildiz et al., 2004).

**Expression regulation of *katB* gene differs between *V. cholerae* O1 strains O395 and N16961**

Considering that KatB and KatG are differently expressed in O395 and N16961 cells, we compared a 300 bp region upstream of the putative translation initiation site of *katB* and *katG* from both strains. While *katG* regulatory regions in both strains are identical, *katB* regulatory regions share 98% identity (four nucleotide modifications and one gap in O395 sequence; Fig. S2). For that reason, we decided to investigate *katB* expression regulation in O395 and N16961 strains. To this end, 300 bp DNA fragments upstream of the putative translation initiation sites of *katB* from both N16961 and O395 were fused to lacZ in the pIC552 plasmid (Macián et al., 1994) to create pkBN16 and pkBO395 plasmids (Table 1). Plasmids pkBN16 and pkBO395 were then used to transform *V. cholerae* N16961 and O395 and β-galactosidase activity (lacZ gene product) was measured in exponential (OD$_{600}$ ~0.5–0.7) and stationary-phase (OD$_{600}$ >2.5) cells grown in LB, at 37 °C, under aeration (Fig. 4). β-Galactosidase activity level was higher in stationary-phase cells of both strains, relative to exponential-phase ones, corroborating results presented before (Fig. 1b, c). Moreover, similar activity levels were observed in cells of each strain harbouring either pkBN16 or pkBO395, indicating that *katB* regulatory regions from *V. cholerae* N16961 and O395 can activate lacZ transcription similarly (Fig. 4). However, β-galactosidase activity levels in O395-transformed cells, from both culture phases, were about threefold lower than in O395 cells, under aeration, until exponential phase. Cultures were divided into two flasks, where 0.2% glucose (Glc) or 0.02% arabinose (Ara) was added. Flasks were incubated for 4 h at 37 °C, under aeration, before catalase activity was measured. To determine cell survival upon exposure to peroxide, aliquots of these cultures were taken and H$_{2}$O$_{2}$ at final concentration of 30 mM was added to each one. Samples were collected 5, 10 and 20 min after H$_{2}$O$_{2}$ addition, diluted and plated onto LB agar. Cell survival was calculated by determining c.f.u. number after challenge and expressed as a percentage of c.f.u. obtained for the control culture (non-exposed to H$_{2}$O$_{2}$). Columns represent the mean of four independent experiments with standard deviation as error bars. Each value at the top of the figure represents the mean catalase activity in cells from the same cultures used for assessing H$_{2}$O$_{2}$ sensitivity.

![Fig. 3](image-url)
the corresponding ones in N16961 transformants (Fig. 4). These results are in perfect agreement with O395 cells’ low catalase activity (Fig. 1b, c) and are strong evidence that katB expression regulation differs between strains O395 and N16961. Nonetheless, this cannot be attributed to the differences in katB regulatory regions between these strains (Fig. S2) and it may, in fact, depend on strain-specific regulatory elements.

**katB and katG gene expression is repressed under Pi limitation in a PhoB/PhoR-independent manner but this system is required for oxidative stress resistance in V. cholerae O1 N16961**

In previous work, we identified many V. cholerae O1 proteins synthesized in response to Pi limitation and/or PhoB cellular levels with roles in oxidative and other stress conditions (von Kruger et al., 2006). Although catalases were not among those proteins, we cannot discard an interaction between kat gene expression and Pi metabolism. Induction of catalase katA expression by Pi limitation was experimentally demonstrated for three different proteobacteria, Sinorhizobium meliloti, Pseudomonas aeruginosa and Agrobacterium tumefaciens (Yuan et al., 2005). In these species, katA transcription requires PhoB, and Pho-box elements were identified upstream of katA genes (Yuan et al., 2005, 2006). Since nothing is known about a possible interaction between catalase production and Pi metabolism, we used the high catalase producer strain N16961 (Fig. 1b, c) and WK10, a phoB mutant of N16961, to examine the involvement of PhoB/PhoR system in the expression regulation of katB and katG. To this end, specific catalase activity was measured in N16961 and WK10 cells grown in MGHP and MGLP with appropriate antibiotics, at 37 °C, under aeration, for 10 h (stationary phase). Total catalase activity did not differ between the strains, but the cells grown under Pi abundance presented about fourfold the activity detected in cells from Pi-limited cultures (Fig. 5a).

We next investigated whether Pi limitation and PhoB/PhoR system were involved in katB and katG gene expression by electrophoresis of cell lysates on non-denaturing polyacrylamide gel, followed by in-gel catalase activity staining. The electrophoretic patterns of the catalases produced by N16961 and WK10 in MGHP and MGLP were highly similar (Fig. 5b), presenting two bands corresponding to KatB and KatG, as in Figs 1(c) and 2(b). Although KatB- and KatG-specific activity levels did not vary between the strains, they were affected by Pi concentration in culture media, being lower in cells grown under Pi limitation (Fig. 5b), corroborating data presented before (Fig. 5a). These results contrast with those obtained for other species (Yuan et al., 2005, 2006) and suggest that katB and katG gene expression in V. cholerae is repressed under Pi-limiting conditions in a PhoB/PhoR-independent process.

N16961 and WK10 cells grown in MGHP and MGLP were also exposed to H2O2 for 5, 10 and 20 min and their survival rates were determined. The high catalase level in N16961 and WK10 cells from MGHP cultures correlated well with their survival rates upon challenge with H2O2 compared to cells grown in MGLP (Fig. 6a). Interestingly, although N16961 and WK10 grown in MGLP cultures presented similar specific catalase activity levels, the WT cells were about 50–100-fold more resistant to H2O2 challenge than the mutant ones (Fig. 6a). These results and those presented before (Fig. 3) highlight a close relationship between total catalase activity and oxidative stress response in V. cholerae O1 N16961. They also indicate that extracellular Pi levels regulate katG and katB expression in a PhoB/PhoR-independent manner in this strain. However, catalases are

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**Fig. 4.** β-Galactosidase-specific activity in N16961 and O395 cells harbouring pKBN16 or pKBO395. Cells were grown until exponential (OD600 = 0.5) or stationary (OD600 > 2.5) phase in LB, at 37 °C, under aeration and β-galactosidase-specific activity was measured in their lysates. Data represent the mean of four experiments with standard deviation as error bars.

**Fig. 5.** Specific catalase activity in V. cholerae N16961 and WK10 cells cultivated under Pi-rich and Pi-limiting conditions. WT N16961 and phoB/phoR mutant WK10 cells were grown in MG medium supplemented with Pi at high (HP) or low (LP) concentration, at 37 °C, under aeration, for 10 h (stationary phase). (a) Total catalase activity was determined in cell lysates. Columns represent the mean of five individual experiments with standard deviation as error bars. (b) V. cholerae catalase isozymes observed by non-denaturing polyacrylamide gel. Whole-cell lysates (100 µg) were resolved on a 4–8% gradient non-denaturing polyacrylamide gel, which was specifically stained for catalase activity.
not the only factor contributing to N16961 resistance to H₂O₂. The PhoB/PhoR system directly or indirectly contributes to ROS resistance in *V. cholerae* O1, confirming previous results from our group (Lery et al., 2013; von Kruger et al., 2006).

**PhoB/PhoR system is essential to control cytoplasmic ROS accumulation in *V. cholerae* O1 strain N16961 under Pi limitation**

In order to investigate how the PhoB/PhoR system protects N16961 cells against oxidative stress, we determined intracellular ROS levels in WT N16961 and *phoB/phoR* mutant WK10 cells grown in MGHP or MGLP for 10 h (Fig. 6b). Similar ROS levels were detected in the WT cells from both cultures and in the mutant WK10 cultivated in MGHP (Fig. 6b). In contrast, WK10 cells grown in MGLP presented about sevenfold more ROS levels than in MGHP (Fig. 6b), indicating that the PhoB/PhoR system, in an unknown way, protects *V. cholerae* O1 against ROS accumulation. These findings could help us to understand the phosphate starvation response of the *phoB* mutant WK3, in comparison to its parental strain from the classical biotype 569B of *V. cholerae* O1 described previously (von Krüger et al., 1999, 2006). WK3 differentially expressed many genes involved in stress resistance and survival in comparison to 569B under Pi limitation. These included genes of detoxification enzymes, such as the alkylhydroperoxide reductase subunit C, which is a common scavenger of peroxides in bacteria, and a spermidine/putrescine-binding protein that has been associated with DNA protection from oxidative damage (von Kruger et al., 2006). Our results indicate that the PhoB/PhoR system plays a role in orchestrating detoxifying mechanisms other than catalases to protect cells against oxidative stress.

**Concluding remarks**

In this study, it is shown for the first time a relationship between Pi levels and *katG* and *katB* expression in pandemic strains of *V. cholerae* O1. Although the PhoB/PhoR system is not required for full expression of catalase genes, it is involved in *V. cholerae* O1 response against oxidative stress. This conclusion is based on our findings that, besides catalases, a functional PhoB/PhoR system is critical for hydrogen peroxide resistance in *V. cholerae* O1, especially under Pi limitation. Therefore, this study reveals new roles for PhoB/PhoR, a system that has already been related to many physiological processes and pathogenicity in *V. cholerae* O1 (Lery et al., 2013; Pratt et al., 2009, 2010; Sultan et al., 2010; von Kruger et al., 2006).

**ACKNOWLEDGEMENTS**

We thank Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro, Conselho Nacional de Desenvolvimento Científico e Tecnológico and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior for financial support. We are grateful to Mr E. Camacho for technical support and to Dr Rodrigo Fortunato for his assistance in intracellular ROS determination.

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Edited by: G. H. Thomas and Y. Li