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Characterization of a catalase gene from *Aeromonas veronii*, the digestive-tract symbiont of the medicinal leech

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The catalase gene *katA* of the medicinal leech symbiont *Aeromonas veronii* bv. sobria was cloned, sequenced, and functionally characterized. Southern hybridization, using an *A. veronii katA*-specific hybridization probe, suggested the presence of a single gene copy in many *Aeromonas* species. *A. veronii katA* consisted of 1446 nt encoding a protein with a high degree of similarity to the small-subunit group III bacterial catalases. A catalase-null mutant (JG186) was constructed through gene-replacement mutagenesis. In the parent strain (HM21R), catalase activity was only detected in extracts of cells grown to early exponential phase following H_2O_2 induction, in which the ability to induce activity was inversely related to optical density. In contrast, induced JG186 cells were very sensitive to oxidative stress, with survival being affected even at low H_2O_2 concentrations. In contrast to the findings of previous reports of other symbiotic systems, the catalase mutant was not defective in its ability to competitively colonize or persist within its host, in both co-inoculation and sole-colonization assays. This body of evidence suggests either that oxidative stress, in the form of H_2O_2 exposure, is not encountered by the microbial partner under the examined symbiotic conditions or that compensatory mechanisms exist. The data suggest that although many colonization factors reoccur, each symbiotic system has also evolved specific mechanisms that affect symbiont–host dynamics.

INTRODUCTION

Physiological adaptations typically reflect the selection pressures encountered within ecological niches. For example, catalases are responsible for the decomposition of hydrogen peroxide to water and oxygen ($2H_2O_2 \rightarrow 2H_2O + O_2$), and have presumably evolved to protect against cellular damage arising from exposure to H_2O_2. The two main factors that influence the expression of catalase gene(s) in bacteria are the exposure to sublethal H_2O_2 levels during exponential growth (Barnes *et al.*, 1999) and entry into stationary phase (reviewed by Loewen, 1997). Reactive oxygen species (ROS) may also be powerful microbicidal weapons implemented by eukaryotic cells during infection. Highly diffusible oxidants, such as H_2O_2, enter membranes and can damage a variety of cell targets (Franzon *et al.*, 1990; Hassett & Cohen, 1989), including DNA, RNA, proteins and lipids, leading to the destruction of microbes. In addition, ROS are generated continuously during aerobic growth by the electron transport chain, leading to the production of H_2O_2 (Cabiscol *et al.*, 2000; Gonzalez-Flecha & Demple, 1995). The toxicity of H_2O_2 necessitates that aerobic microbes or those whose life cycle relies on host infection encode a functional catalase within their genomes.

Aeromonas veronii is a facultative anaerobe which has a propensity to colonize the digestive tracts of a variety of hosts, including humans, leeches and mosquitoes, with manifestations of infection ranging from pathogenesis to mutualism (Graf *et al.*, 2006; Janda & Abbott, 1998). Recently culture-independent analysis of the digestive-tract microbiota of the medicinal leech (*Hirudo verbana*, one of three species within the *Hirudo medicinalis* complex; Hirudinea: Arhynchobdellida: Hirudinidae) (Apakupakul *et al.*, 1999; Siddall & Burreson, 1998; Siddall *et al.*, 2001; Trontelj & Utevsky, 2005) has revealed the presence of a currently uncultured *Rikenella*-like species (Worthen *et al.*, 2006; Kikuchi & Graf, 2007). These two symbionts are the dominant members of the leech digestive-tract microbiota.

Abbreviations: CI, competitive index; ROS, reactive oxygen species.

The GenBank/EMBL/DDBJ accession no. for the *A. veronii katA* sequence is EF028076.

A figure showing the results of transformation of *E. coli* ZK918 with purified plasmid DNA from the *A. veronii* genomic library, after plating on MacConkey agar, is available as supplementary data with the online version of this paper.

Putative functions for the *Aeromonas* symbiont within the medicinal leech include: (i) aiding in the digestion of the blood meal; (ii) providing essential nutrients lacking in the exclusive blood diet, such as B-complex vitamins; (iii) providing 'colonization resistance', in which *A. veronii* prevents colonization by other potentially harmful microorganisms, thus preventing the putrefaction of blood and permitting long-term storage; and (iv) priming the microenvironment for the obligate anaerobic *Rikenella*-like symbiont (Worthen *et al.*, 2006; reviewed by Graf, 2002).

Analogous to vertebrate innate immune responses, the introduction of Gram-negative bacteria and their by-products into leech wounds activates an inflammatory response involving the infiltration of macrophage-like cells (de Eguileor *et al.*, 1999, 2000a, b). During phagocytosis or following stimulation with a wide variety of agents, macrophages undergo respiratory bursts that are characterized by the production and release of ROS into the extracellular milieu (Forman & Torres, 2002; Park, 2003). ROS production is essential for the increased bactericidal capability of stimulated macrophages (Johnston & Kitagawa, 1985). Bacteria can protect themselves against host-produced ROS by upregulating the genes encoding protective enzymes, such as superoxide dismutase, peroxidase and catalase. While the importance of these protective enzymes is well established for pathogens (Franzon *et al.*, 1990; Mandell, 1975; Zheng *et al.*, 1992), they have also been shown to be crucial for the successful colonization of the light organ of the Hawaiian bobtail squid *Euprymna scolopes* by its extracellular, mutualistic symbiont *Vibrio fischeri* (Visick & Ruby, 1998), and for the regulation of the infection of root nodules by the nitrogen-fixing *Sinorhizobium meliloti* (Santos *et al.*, 2001).

We report the cloning and functional characterization of the *A. veronii* gene encoding the antioxidant enzyme catalase *katA*. We further examine the presence of *katA* homologues in other *Aeromonas* species. Implementing a targeted mutant analysis approach, the importance of *katA* for *A. veronii* survival following exposure to damaging oxidative-stress conditions, and for proliferation and persistence within the medicinal leech, was evaluated.

METHODS

Bacterial strains, plasmids and growth conditions. The sources and properties of the bacterial strains and plasmids used in this study are listed in Table 1. The bacteria were grown at 200 r.p.m. in Luria–Bertani (LB) medium at 37 and 30 °C for *Escherichia coli* and *Aeromonas* species, respectively (Sambrook & Russell, 2001). The growth rates were determined in LB containing rifampicin (Rf) at 30 °C.

The *A. veronii* strain HM21R is a spontaneous Rf^r mutant derived from HM21, an isolate from the leech digestive tract (Graf, 1999). HM21RS was derived from HM21R by plating 10^8 cells from an overnight culture on LB [Rf, streptomycin (Sm)] plates. Where appropriate, antibiotics were added at the following concentrations: ampicillin (Ap), 100 μg ml^{-1}; chloramphenicol (Cm), 1 μg ml^{-1} for *A. veronii* and 10 μg ml^{-1} for *E. coli*; kanamycin (Km), 100 μg ml^{-1}; Sm, 100 μg ml^{-1}; and Rf, 100 μg ml^{-1} for *Aeromonas* selection and 10 μg ml^{-1} for maintenance.

Animals. The medicinal leeches used in this study were obtained from LeechesUSA and Zaug GmbH (Biebertal, Germany). The animals were starved for at least 3 months prior to delivery and maintained without feeding in leech tanks at 25±1 °C (Graf, 1999).

Isolation of genomic DNA. Genomic DNA was isolated using a modification of the cetyltrimethylammonium bromide (CTAB) method (Nelson & Selander, 1994) by preheating the samples to 65 °C for 5 min prior to lysis with SDS. For large-scale DNA isolation, the volume was increased 30-fold, samples were preheated to 65 °C for 10 min, and the precipitated DNA was isolated using a glass Pasteur pipette.

Construction of *A. veronii* genomic library. Genomic DNA was partially digested with *Sau*3A and separated by electrophoresis in low-melting-point agarose. DNA fragments (~6.5–7.6 kb) were purified by phenol extraction and cloned into the dephosphorylated *Bam*HI site of pBSIIKS+. The ligations were transformed into *E. coli* XL-1 Blue MRF′ super competent cells (Stratagene) through heat shock at 42 °C.

Complementation of the *E. coli rpoS* mutant. Purified plasmid DNA from the *A. veronii* genomic library was transformed into calcium-competent *E. coli rpoS* mutant ZK918 (Sambrook & Russell, 2001). Transformants were plated on MacConkey agar (Ap, Km) and screened for red colonies. Strains exhibiting this phenotype were picked and patched onto MacConkey agar plates, where the colour phenotype was reassessed. The colonies were additionally examined for the production of catalase by observing the release of O_2 bubbles following the addition of 3 % H_2O_2 (Supplementary Fig. S1).

Characterization of the complementing plasmids. The complementing plasmids were isolated using Plasmid Mini or Plasmid Midi kits (Qiagen) and sequenced using a combination of subcloning and primer walking (Central DNA Sequencing Facility, Department of Clinical Research, University of Berne, and the Biotechnology Center, University of Connecticut). The DNA sequences were aligned using Vector NTI (Invitrogen) and compared to the databases using the BLAST 2.2.13 algorithm (Altschul *et al.*, 1997).

Southern analysis. DNA (*Aeromonas hydrophila* ATCC 14715, *Aeromonas salmonicida* CDC 0434-84, *Aeromonas caviae* ATCC 15468, *Aeromonas media* CDC 0862-83, *Aeromonas eucrenophila* ATCC 23309, *Aeromonas sobria* CIP 7433, *A. veronii* bv. sobria CDC 0437-84, *Aeromonas veronii* bv. sobria HM21, *A. veronii* bv. veronii ATCC 35624, *Aeromonas schubertii* ATCC 43700 and *Aeromonas allosaccharophila* LMG 140549) was digested with *Pst*I for 6 h and separated by agarose gel electrophoresis. The loading of an equivalent amount of DNA in the various lanes was confirmed by ethidium bromide staining. The DNA was transferred onto a nylon membrane through overnight capillary transfer and fixed to the membrane with 0.4 M NaOH (Sambrook & Russell, 2001).

A 743 bp hybridization probe was PCR-amplified from HM21 DNA using PkatF, 5′-TCG ACA ACA ACA ACA GCC TCA C-3′, and PkatR1, 5′-CAC CTG CAC ATA GAC TTT CCA GC-3′, as described below, and subsequently labelled using the Amersham ECL Direct System (Amersham Life Science). The membrane was prehybridized at 42 °C in the Amersham ECL direct hybridization buffer. After 1 h, the probe was added and allowed to hybridize overnight. The membrane was washed twice for 20 min at 42 °C in the primary wash buffer and twice for 5 min in the secondary wash buffer. The

Table 1. Strains and plasmids used in this study

Designation	Relevant characteristics	Source or reference
***A. veronii* bv. sobria strains**		
HM21	Wild-type, isolated from *H. medicinalis*	Graf (1999)
HM21R	Spontaneous Rf^r derivative of HM21	Graf (1999)
HM21RS	Spontaneous Sm^r derivative of HM21R	This study
CDC 0437-84	*A. veronii* bv. sobria	
JG186	HM21R, *katA1* (*katA* : : pJG58), Rf^r, Km^r	This study
Other *Aeromonas* species		
LMG 140549*	*A. allosaccharophila*	
ATCC 15468*	*A. caviae*	
ATCC 23309*	*A. eucrenophila*	
ATCC 14715*	*A. hydrophila*	
CDC 0862-83	*A. media*	
CDC 0434-84	*A. salmonicida*	
ATCC 43700	*A. schubertii*	
CIP 7433*	*A. sobria*	
ATCC 35624*	*A. veronii* bv. veronii	
***E. coli* strains**		
ZK918	W3110 Δ*lacU169 tna-2 rpoS* : : km^r *bol* : : *lacZ*	Bohannon *et al.* (1991)
XL-1 Blue MRF'	Cloning strain, Δ*mcrA*, Δ(*mcrCB-hsdSMR-mrr*)*173*, *endA1*	Stratagene
Plasmids		
pBSKS+	Ap^r, ColE1, high-copy-number cloning vector	Stratagene
pEP2	pBSKS+ with a 6.4 kb *A. veronii Sau*IIIA fragment, containing the complete coding sequence of *katA*	This study
pJG54	pBSIIKS+ containing Δ*katS* in the *Sma*I site, Ap^r	This study
pJG55	pBSIIKS+ containing Δ*katL* in the *Sma*I site, Ap^r	This study
pJG57	pKAS46 containing Δ*katS*, Ap^r, Km^r	This study
pJG58	pKAS46 containing Δ*katL*, Ap^r, Km^r	This study
pKAS32	oriR6K, *oriT*, *rpsL*, Ap^r, Km^r	Skorupski & Taylor (1996)
pMA2	pBSKS+ with a 6.4 kb *A. veronii Sau*IIIA fragment, containing the complete coding sequence of *katA*	This study
pMA5	pBSKS+ with a 6.4 kb *A. veronii Sau*IIIA fragment, containing the complete coding sequence of *katA*	This study
pMA6	pBSKS+ with a 6.4 kb *A. veronii Sau*IIIA fragment	This study
pMA7	pMA5 with a *Pst*I deletion of 3.2 kb	This study
pMA8	pMA5 with a *Sal*I deletion of 4.5 kb	This study
pMA9	pMA5 with a *Sac*I deletion of 6.1 kb	This study
pMA10	pMA7 with a Km^r : : *katA* fusion	This study
pRRJG5	pCR 2.1-*katA*	This study
pRRSR1	pMMB207-*katA*	This study

*Type strain.

membrane was incubated with detection reagents 1 and 2 for 1 min and exposed to X-ray film.

Construction of the catalase mutants. For the construction of the catalase mutants, two internal fragments of *katA* were amplified by PCR using PkatF and either PkatR1 (amplifying a 743 bp product and resulting in Δ*katA*S) or PkatR2, 5′-AGG AGA AGA GTC GCC CTT G-3′ (amplifying a 966 bp product and resulting in Δ*katA*L). Single amplicons were obtained for each of the two primer sets. The PCR products were blunt-ended using T4 DNA polymerase and ligated into *Sma*I-digested pBSII, yielding pJG54 (containing the shorter PCR product, Δ*katA*S) and pJG55 (containing the longer PCR product, Δ*katA*L). The cloned fragments were introduced into the π-dependent R6K origin of replication suicide vector pKAS32, using *Xba*I–*Eco*RI ends, resulting in pJG57 (Δ*katA*S) and pJG58 (Δ*katA*L). The suicide plasmids were transformed into S17-1 λ*pir* and introduced into HM21R by conjugation with shaking (Simon *et al.*, 1983). Donor (5×10^7 c.f.u. between 0.4 and 0.8 OD_{600}) and recipient (2×10^8 c.f.u. between 0.25 and 0.4 OD_{600}) cells were harvested and spotted on an LB agar plate. Following an overnight incubation at 30 °C, transconjugants were selected on LB agar (Rf, Km).

Zymography. Whole-cell lysates were prepared from the cells as described in Methods, Sensitivity to H_2O_2, below. The cells were lysed by sonication (three bursts, 15 s) and centrifuged at 4 °C for 30 min (12 000 ***g***). Supernatants were loaded onto a 10 %, w/v, acrylamide gel and separated at 150 V for 45 min. The gel was washed extensively in double-distilled (dd) H_2O and for 10 min in 0.0155 M H_2O_2.

Subsequently, the gel was stained in a solution containing 1 %, w/v, ferric chloride and 1 %, w/v, potassium ferric cyanide for the visualization of catalase activity (Barnes *et al.*, 1999).

Analysis of catalase activity. Paired 5 ml aliquots of cultures were removed at various optical densities and either induced with 0.05 mM H_2O_2 or not induced. The aliquots were incubated for an additional 30 min before being placed on ice. A crude cell extract was prepared by sonication. Specific catalase activity was determined by measuring the removal of H_2O_2 from the crude cell extract for 60 s and dividing by the amount of protein, as determined by a Lowry protein assay (Lowry *et al.*, 1951; Beers & Sizer, 1952; Visick & Ruby, 1998). Briefly, the cell pellet was resuspended in phosphate buffer, pH 7.0, containing 5 mM EDTA, 10 %, v/v, glycerol and 25 μM PMSF. The change in A_{240} was monitored four consecutive times (15 s per period) following the addition of 0.5 volumes of 59 mM H_2O_2 to 1 volume of crude extract. The mean rates were converted to U mg^{-1} min^{-1}.

Reverse transcription of *katA*. Total RNA was isolated from 2.5×10^8 *A. veronii* (JG186 and HM21R) cells grown to stationary or early mid-exponential phase, employing the Qiagen RNeasy protocol for total RNA isolation from bacteria. An aliquot of the mid-exponential-phase cells was exposed to a sublethal dose of H_2O_2 (0.05 mM) for 30 min at 30 °C. The optional on-column RNase-free DNase I (Qiagen) was used to remove contaminating DNA. After RNA isolation, traces of contaminating DNA were further eliminated with an RNase-free DNase I treatment. Random hexamer primers, iScript reverse transcriptase (Bio-Rad) and 0.5 μg total RNA were utilized for first-strand cDNA synthesis. Subsequent PCR was performed with *katA*F′, 5′-GAC AAG ACC CTG CAC AGC-3′, and *katA*R′, 5′-CGC TCA TTG GCG TCG TTG-3′. The absence of DNA contamination was verified by PCR using RNA template lacking a reverse-transcription step.

Sensitivity to H_2O_2. The sensitivity of the *katA* mutant JG186 and its parent strain HM21R to H_2O_2 was determined for cells prepared in three different ways. The cells were grown either to stationary or to early exponential phase. A portion of the early exponential-phase cells was exposed to a sublethal dose of H_2O_2, as described above. Cells from both strains and at each growth condition were diluted to the same density (5×10^6 c.f.u. ml^{-1}), challenged with various H_2O_2 concentrations for 30 min at 30 °C, serially diluted and plated. The percentage survival was determined by dividing the count obtained following the exposure to H_2O_2 of each strain by the count obtained prior to challenge.

Complementation of JG186. A 1.8 kb amplicon that contained *katA* and its promoter and termination regions was PCR-amplified using katAFullF′, 5′-GAC CAC ATC ACC GTT CTC CA-3′, and katAFullR′, 5′-TGC TCG CAA TAG AAA ACG GG-3′. The amplicon was cloned into pCR 2.1 (Invitrogen), resulting in pRRJG5. The cloned 1.8 kb fragment was moved into the broad-host-range vector pMMB207 (Morales *et al.*, 1991) from pRRJG5 by utilizing the *Eco*RI sites. The resulting plasmid, pRRSR1, was electroporated into *E. coli* DH5α λ *pir* cells. The presence of the insert was verified through DNA sequencing and restriction digests. pRRSR1 and pMMB207 were each introduced into HM21R and JG186 through conjugation. The resulting strains were induced and challenged with 0.8 and 1.0 mM H_2O_2, respectively, as described above.

Colonization assay. The competition assay used in this study, which compared the ability of JG186 and a competitor strain to colonize the medicinal leech, was similar to the assay we described previously (Graf, 1999), except that the two strains were simultaneously fed to the leech in a single blood meal. Briefly, leeches were fed heparinized sheep blood (25 U ml^{-1}, Sigma) that had been stored for 18–24 h at 23 °C. Cells (500 c.f.u. ml^{-1} of JG186 and HM21RS) were added to blood that had been preheated to 56 °C, which inactivates the antimicrobial properties of the complement system, which can kill sensitive bacteria (Indergand & Graf, 2000). The animals were fed 5 ml blood and individuals were subsequently incubated at 23 °C. At 6 and 18 h post-blood meal, animals were sacrificed and the intraluminal fluid was collected from the crop of the digestive tract. Serially diluted samples were plated on LB plates containing the appropriate antibiotics and incubated overnight at 30 °C, allowing for comparative counts of the introduced microbiota. The limit of detection was 10 c.f.u. ml^{-1}. Similarly, sole-colonization assays were performed to rule out the possibility that the *katA* mutation was circumvented by the endogenous catalase of HM21RS. For the sole-colonization assay, the inoculum was between 200 and 500 c.f.u. ml^{-1} of either JG186 or HM21R, and animals were sacrificed 18 h post-blood meal to determine colonization levels. For each time point, at least three animals were used.

Statistical analysis. The data were analysed using Graph Pad Prism 2.01. A competitive index (CI) was calculated in order to compare test strain concentrations in the intraluminal fluid following their introduction. The absolute and relative levels of each strain were monitored over time by sacrificing animals at predetermined time points and plating serial dilutions on antibiotic-containing plates. CI was calculated as follows:

$$CI = (Mutant_{output}/Wild\text{-}type_{output})/(Mutant_{input}/Wild\text{-}type_{input})$$

A CI of 1 indicates that the mutant colonizes to the same level as the competitor strain, while CI <1 indicates that the mutant has a colonization defect. A two-tailed, one-sided t test was used to test whether the CI differed from 1. Student's t test was performed to determine whether colonization levels significantly differed for mutants introduced in sole-colonization assays versus competition assays. Significant differences ($P \leqslant 0.05$) are reported.

RESULTS

Isolation of plasmids that complement the *rpoS* mutant ZK918

The initial goal of this study was to isolate a functional *rpoS* homologue from a genomic library of *A. veronii* bv. sobria by complementing the *E. coli rpoS* mutant ZK918 (Bohannon *et al.*, 1991). This mutant has two phenotypes that can be easily screened for and that have been complemented in other studies by *rpoS* homologues from *Vibrio cholerae* and *Pseudomonas putida* (Ramos-Gonzalez & Molin, 1998; Yildiz & Schoolnik, 1998). One of these phenotypes is the red colouration of colonies on MacConkey agar due to lactose fermentation that is dependent on the transcription of the σ^S-dependent promoter fusion *bolAp1–lacZ*. The second phenotype is the release of O_2 bubbles by colonies after the addition of H_2O_2 due to the expression of the σ^S-dependent HPII catalase *katE*.

Competent ZK918 were transformed with a genomic library from *A. veronii*. Of the 9000 transformants screened, three exhibited the red colouration and release of O_2 after the addition of H_2O_2. The complementing plasmids pMA2, pMA5 and pEP2 were isolated and reintroduced into the *rpoS* mutant ZK918. All three plasmids again complemented ZK918, indicating that

complementation was due to the plasmids and not to a secondary chromosomal mutation in the transformed strain. Analysis of these plasmids by restriction endonuclease digestion was consistent with all three plasmids carrying similar chromosomal regions and was confirmed by sequencing the ends of the inserted genomic DNA. Because all three plasmids complemented ZK918 and contained essentially the same chromosomal region, we focused on pMA5 in our subsequent analysis.

The region of pMA5 that was responsible for complementing the *E. coli rpoS* mutant was identified by deleting regions of the inserted DNA, transforming the subcloned plasmids into ZK918, and testing for red colony colour as well as the release of O_2 bubbles following H_2O_2 exposure (Fig. 1a). Interestingly, transformation with pMA10 resulted in a red colour but failed to restore the ability of ZK918 to produce O_2 bubbles (Supplementary Fig. S1), suggesting that two different loci may actually be responsible for the two phenotypes.

DNA sequence analysis

The complementing region was sequenced in both directions using a combination of primer walking and subcloning. Database searches with the sequence of the cloned *Aeromonas* DNA using BLASTN and BLASTX showed no similarity to any sigma factors, suggesting that the inserted DNA does not encode a sigma factor. Further sequence analysis revealed one large ORF (ORF1) and two smaller ORFs (ORF2 and ORF3) that are transcribed in the opposite direction (Fig. 1b).

ORF1 consisted of 1446 nt encoding 482 amino acids with a predicted molecular mass of 54 kDa and isolectric point (pI) of 5.98. Putative −10 and −35 regions for the *E. coli* housekeeping sigma factor were found 27 and 52 bp, respectively, upstream of the presumptive start codon. A potential Shine–Dalgarno sequence (AGGAGA) was also detected 7 bp upstream of the presumptive start codon. Downstream of the stop codon a GC-rich interrupted dyad symmetry followed by a run of Ts was found, consistent with the stem–loop structure of a rho-independent terminator.

Comparison of the nucleotide sequence from ORF1 with the public databases using BLASTX revealed sequence similarity to the group III small-subunit bacterial catalases (Klotz *et al.*, 1997), including HktE from *Pasteurella multocida* (70 % identity and 81 % similarity), a putative catalase from *Photobacterium profundum* (71 % identity and 79 % similarity) and the catalases of *Bordetella pertussis*, *Bordetella parapertussis* and *Bordetella bronchiseptica* (69 % identity and 83 % similarity). Furthermore, the deduced amino acid sequence was conserved along the entire protein length with a slightly lower degree of sequence identity at the carboxyl terminus. In addition, a PROSITE search revealed that the two regions conserved in functional catalases are also present in *A. veronii* protein. Catalase region 1 is responsible for haem binding and contains a conserved tyrosine residue (RLFSY.DTQ), while catalase region 2, a section of the catalytic site, contains a conserved histidine residue (F.R....ER..H..GSG) (Fig. 1c). In accordance with the high sequence identity, the retention of conserved residues and the release of bubbles

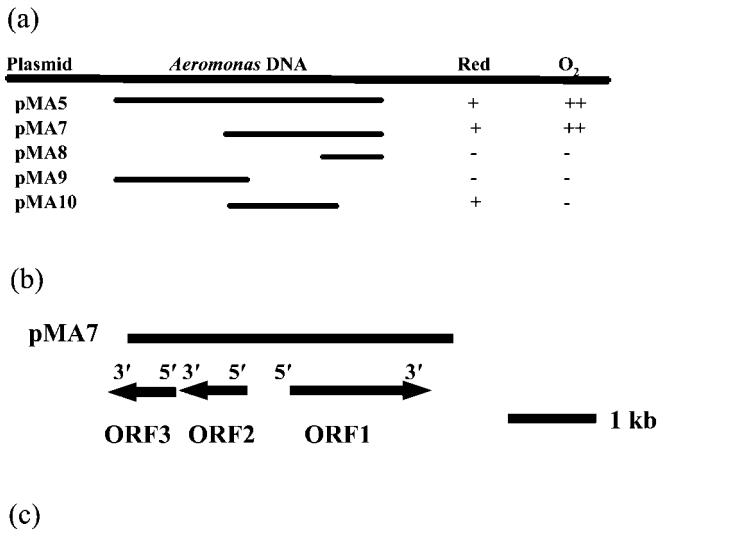

Fig. 1. (a) The region of pMA5 responsible for complementing the *E. coli rpoS* mutant was identified by deleting regions of the inserted DNA, transforming the subcloned plasmids into ZK918 and testing for red colony colour as well as the release of O_2 bubbles following exposure to H_2O_2. The smallest plasmid that restored both phenotypes of ZK918 was pMA7. (b) Genetic map of the *katA* region of pMA7. Sequence analysis revealed one large ORF (ORF1) encoding *katA*, and two smaller ORFs (ORF2 and ORF3). Large arrows indicate the locations and directions of transcription of the identified genes. (c) Deduced amino acid sequence of *A. veronii katA*. The boxed region denotes catalase region 1, and the conserved tyrosine residue is in white on black type. The underlined region denotes catalase region 2, part of the catalytic site that contains a conserved histidine residue (asterisked).

following the addition of H_2O_2 in the complemented *rpoS* mutant ZK918, we designated the gene as *katA* (accession no. EF028076).

ORF2 had sequence similarity to a putative acetyltransferase of *Bacillus subtilis*, whereas ORF3 was homologous to a DNA gyrase inhibitor. A comparison of the *A. veronii katA* locus with the *A. hydrophila* genome revealed conservation in genetic organization (Seshadri *et al.*, 2006).

Presence of catalase genes in *Aeromonas* species

We were interested in determining whether other *Aeromonas* species possess a similar catalase gene. An internal fragment of *katA* was amplified and used as a probe for Southern analysis of nine *Aeromonas* species (Fig. 2). With the exception of *A. schubertii*, all of the *Aeromonas* species examined revealed one band that hybridized to the *katA* probe, suggesting that these eight species possess a similar catalase gene. Interestingly, the *Aeromonas* leech isolate HM21 *katA* band co-migrated with that of *A. veronii* bv. veronii rather than that of *A. veronii* bv. sobria.

Construction of *katA* mutants

Deletion mutants were constructed by introducing internal fragments of *katA*, located on a suicide vector, through homologous recombination into the *A. veronii* chromosome. This results in two copies of *katA* with each having a deletion in either the 5′ or the 3′ region. The presumptive mutants were screened for inability to release air bubbles after the addition of H_2O_2, and the disruption of the *katA* gene in four of the Km^r and catalase-minus strains, JG185 and JG186 (Δ*katA*L derived) and JG183 and JG184 (Δ*katA*S derived), was confirmed by Southern blotting using a *katA*-specific probe. A shift in the molecular mass of the bands that hybridized with the *katA* probe was observed, indicating that the plasmid had incorporated into the *katA* locus (data not shown). We chose JG186 for further functional characterization. The growth rate of JG186 in LB did not differ from that of HM21R and HM21RS under similar conditions.

Detection of catalase activity

Facultative or obligate aerobic bacteria can possess multiple catalases. Our Southern analysis suggested the presence of only one catalase, but we wanted to verify catalase activity independently of sequence similarity through zymography. Whole-cell lysates of the parent strain and JG186 were obtained from cells grown to early exponential phase and stationary phase, and from early exponential-phase cells that were exposed to a sublethal concentration of H_2O_2 for 30 min (induced). Catalase activity was detected only from lysates of induced early exponential-phase HM21R cells (Fig. 3). No other samples exhibited catalase activity, further supporting the presence of only one *A. veronii* catalase that is inducible by oxidative stress. Furthermore, these results also demonstrated that JG186, the *katA* mutant, does not produce detectable levels of catalase.

The specific catalase activity (U mg^{-1} min^{-1}) of the parent strain and JG186, with and without exposure to sublethal levels of H_2O_2, was monitored over a range of cell densities (Fig. 4 and data not shown). No catalase activity above background was ever detected for JG186. Interestingly, the ability of the wild-type strain to induce the catalase activity

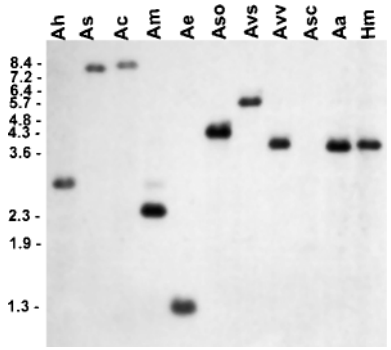

Fig. 2. Southern blot analysis of *Aeromonas* species DNA hybridized with a 743 bp *A. veronii katA* fragment. Ah, *A. hydrophila* ATCC 14715; As, *A. salmonicida* CDC 0434-84; Ac, *A. caviae* ATCC 15468; Am, *A. media* CDC 08262-83; Ae, *A. eucrenophila* ATCC 23309; Aso, *A. sobria* CIP 7433; Avs, *A. veronii* bv. sobria CDC 04374-84; Avv, *A. veronii* bv. veronii ATCC 35624; Asc; *A. schubertii* ATCC 43700; Aa, *A. allosaccharophila* LMG 140549; Hm, *A. veronii* bv. sobria HM21.

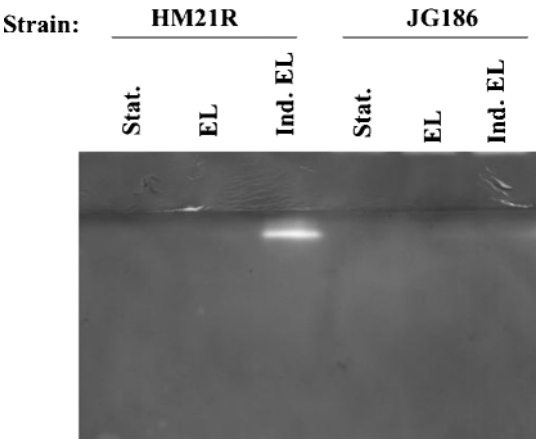

Fig. 3. Zymogram analysis of catalase activity of HM21R and JG186 through cell growth. Catalase activity was specifically detected from HM21R early exponential-phase lysates that had been induced and subsequently challenged with H_2O_2. No other samples demonstrated catalase activity. Stat., stationary; EL, early exponential; Ind. EL, induced early exponential.

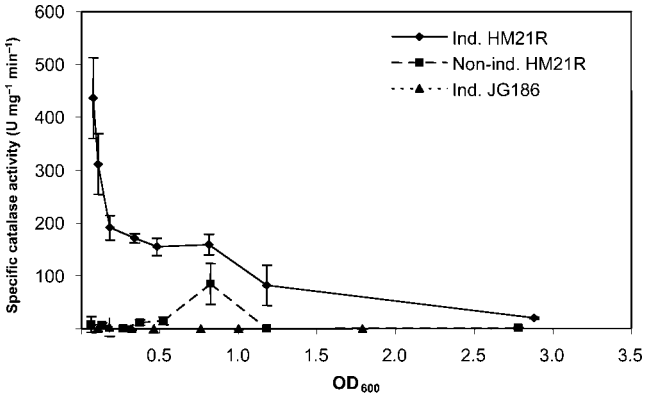

Fig. 4. Catalase activity (U mg^{-1} min^{-1}) of HM21R and JG186 over a range of cell densities (OD_{600} 0.1–2.7). Catalase activity was determined following the addition of H_2O_2, with and without prior exposure to sublethal levels. Error bars show SD, unless smaller than the symbols. Ind., induced; Non-ind, non-induced.

was inversely related to the optical density of the culture. It is possible that the specific catalase activity from cells at low optical density can be elevated by an underestimation of total protein level. Reverse-transcriptional analysis further confirmed *katA* expression by H_2O_2-induced HM21R (data not shown).

Sensitivity to H_2O_2

The sensitivity and protective response of the wild-type strain (HM21R) to oxidative stress was determined by exposing the cells to increasing concentrations of H_2O_2 (0.01–10 mM) and monitoring their survival. The cells were grown to early exponential phase or stationary phase, and subsequently a portion of the early exponential-phase cells was exposed to a sublethal concentration of H_2O_2 (induced cells). Non-induced HM21R early exponential-phase cells (Fig. 5a) were less resistant to increasing H_2O_2 concentrations than induced (Fig. 5b) and stationary-phase (Fig. 5c) cells. Both induced and stationary-phase HM21R were adversely affected commencing at 1 mM H_2O_2, with survival rates of 88 and 70 %, respectively (Fig. 5b, c).

We then determined the importance of *katA* in providing oxidative stress protection by exposing JG186 to similar concentrations of H_2O_2. Interestingly, no survival differences between JG186 and HM21R were detected in stationary-phase cells (Fig. 5c). In contrast to induced HM21R, induced JG186 cells were much more sensitive to H_2O_2, with survival negatively affected at concentrations as low as 0.1 mM. These results demonstrated that *katA* is critical in providing an inducible protection against exogenous H_2O_2 *in vitro*. However, in a similar manner to HM21R, non-induced early exponential-phase JG186 cells were less resistant than their induced counterparts, with survival dropping to 2 % (Fig. 5a) in comparison to

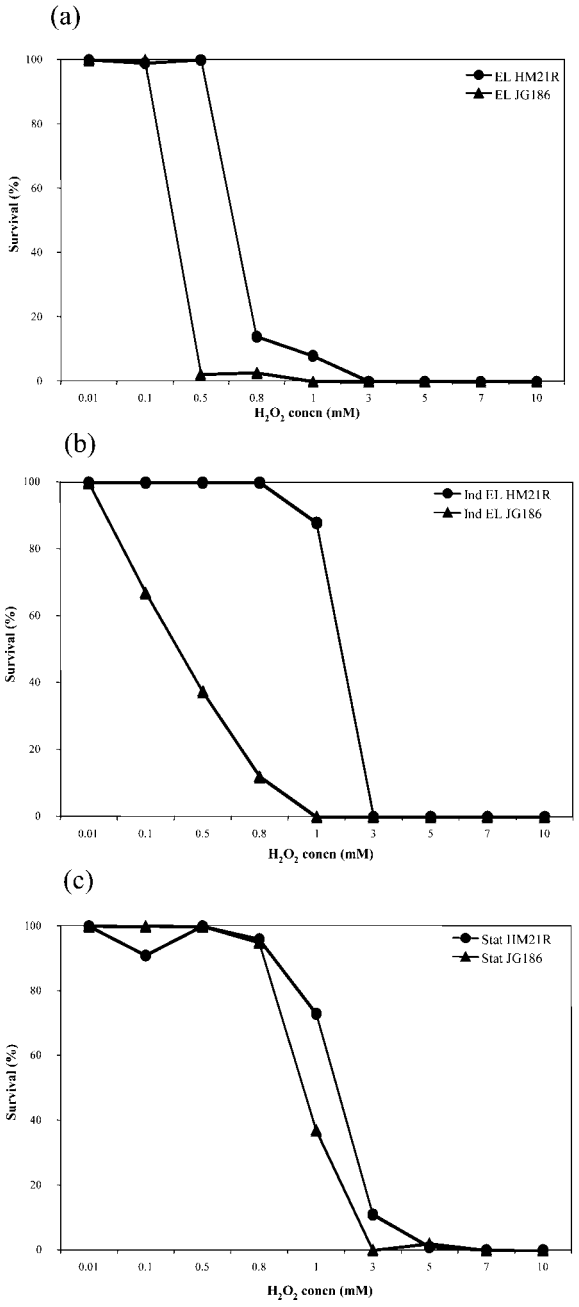

Fig. 5. Representative percentage survival of (a) early exponential-phase HM21R and JG186, (b) induced early exponential-phase HM21R and JG186, and (c) stationary-phase HM21R and JG186, following exposure to various H_2O_2 concentrations (0.01–10 mM), based on three independent trials. EL, early exponential; Ind. EL, induced early exponential; Stat., stationary.

38 % (Fig. 5b) at 0.5 mM H_2O_2. These results suggest that *A. veronii* bv. sobria retains another inducible mechanism in addition to *katA* to safeguard against oxidative stress, and that during stationary phase, protection is provided through catalase-independent means.

Colonization of the medicinal leech

In symbiotic relationships, mechanisms that control the spatial (anatomical) localization and the proliferation of the symbiotic flora are critical for the maintenance of homeostasis (Rio *et al.*, 2006). One common mechanism employed to control infections of pathogenic bacteria is to create an environment of oxidative stress that kills sensitive bacteria. Catalase has been shown to be required for the normal symbiotic competence of *V. fischeri* and *Eu. scolopes*, being induced by both oxidative stress and the approach to stationary phase (Visick & Ruby, 1998). Accordingly, we were interested to determine whether the loss of *katA* would lead to a reduced ability of *A. veronii* to colonize *H. medicinalis*. Using the competition assay, no significant differences in the c.f.u. ml^{-1} of JG186 and HM21RS at 6 h (a two-tailed, one-sided *t* test was used to test whether the CI differed from 1, with $P=0.1827$) following introduction were detected, suggesting that at least under these conditions, the loss of *katA* was not essential for symbiotic establishment (data not shown). Furthermore, no significant differences in the c.f.u. ml^{-1} of JG186 and HM21RS at 18 h following feeding (a two-tailed, one-sided *t* test was used to test whether the CI differed from 1, with $P=0.3762$) were detected, indicating that symbiotic persistence remains unaltered following the loss of catalase activity (data not shown). Additionally, a sole-colonization assay indicated no difference in JG186 colonization levels in comparison to HM21R at 18 h, suggesting that the mutant did not rely on the catalase activity of the competitor strain for colonization ($P=0.27$) (data not shown).

DISCUSSION

The *A. veronii katA* belongs to the group III catalases, which are predominantly found in proteobacteria, many of which lead a parasitic lifestyle (Klotz *et al.*, 1997). It is interesting to note that bacteria which harbour a group III catalase and inhabit a restricted environment typically contain only a single catalase isozyme (Klotz *et al.*, 1997), supporting our finding of only one catalase gene, *katA*, within the *A. veronii* genome. Similarly, the *A. hydrophila* genome contains only one *katA* gene (Seshadri *et al.*, 2006), and zymographic analysis of *A. salmonicida* also reveals only one catalase (Barnes *et al.*, 1999). Although this study found no definitive role for *A. veronii katA* in the medicinal leech symbiosis, it is likely that *katA* has functional importance to counter ROS produced as by-products during aerobic growth (Loewen, 1997). The lack of a hybridization signal from *A. schubertii* strain ATCC 43700 suggests either that the *katA* homologue is undetectable with the probe utilized due to the evolutionary distance from *A. veronii* or possibly that a homologue is lacking (Yanez *et al.*, 2003; Soler *et al.*, 2004). The differences observed in *katA* hybridization patterns provide a promising tool for distinguishing the two *A. veronii* bvs (sobria versus veronii) that could previously only be differentiated using an array of biochemical assays (Graf, 1999) or by DNA sequencing of *gyrB* and *rpoD* (Yanez *et al.*, 2003; Soler *et al.*, 2004).

Significant protection against oxidant killing was provided by *katA* during H_2O_2 challenges, particularly during exponential growth. Correspondingly, catalase activity was detected only from early exponential-phase HM21R cell lysates that had been induced with a sublethal concentration of H_2O_2. These induced cells were more protected against oxidative stress when subsequently challenged with even greater H_2O_2 concentrations. Similar challenges proved detrimental to non-induced HM21R and the *katA* mutant JG186, supporting the importance of both catalase activity and an adaptive antioxidant response that facilitates protection against subsequent lethal doses. Interestingly, the induction of *katA* was detected only in cells during early exponential-phase growth, differing from that reported for *A. salmonicida*, in which induction during mid-exponential and early stationary phase provides the greatest protection against H_2O_2 challenge (Barnes *et al.*, 1999). Also, *A. salmonicida* is protected against higher levels of H_2O_2 (Barnes *et al.*, 1999), although this could be due to differences in experimental design. In addition to the protection provided by KatA, a catalase-independent defensive mechanism against exogenous H_2O_2 was observed in stationary-phase *A. veronii* cells. This additional protection may be due to the upregulation of other antioxidant enzymes, such as the glutathione and thiol peroxidases that are present in the *A. hydrophila* genome (Seshadri *et al.*, 2006). If such peroxidases are also present in the *A. veronii* genome, they could contribute to the protection detected during the stationary phase. Additionally, the low metabolic activity during stationary phase reduces the generation of ROS during respiration, and thus even basal levels of antioxidant enzymes may provide sufficient protection against oxidative stress (Barnes *et al.*, 1999). The expression of *A. veronii katA* from pRRSR1 was unable to restore the wild-type phenotype. However, a polar effect of the *katA* mutation is unlikely, given that following the rho-independent terminator, according to the *A. hydrophila* genome, the downstream gene, a conserved hypothetical protein, is transcribed in the opposite direction. Our *A. veronii* draft genome sequence supports conservation in the genetic organization of the *katA* locus.

Oxygen and its reactive derivatives have been demonstrated to play significant roles in promoting the efficiency and specificity of non-digestive-tract symbiotic systems, such as those between *S. meliloti* and leguminous plants, photosynthetic algae and marine invertebrates, and *V. fischeri* and *Eu. scolopes* (reviewed by Ruby & McFall-Ngai, 1999). Unlike catalase mutants in these systems, the *A. veronii* catalase mutant was not defective in its ability to colonize or persist within its host digestive tract, at least under the conditions tested. This suggests either that oxidative stress, in the form of H_2O_2 exposure, is not encountered by the

microbial partner during the colonization of the leech digestive tract or that other compensatory mechanisms exist, such as the previously mentioned peroxidases. Examining whether catalase is crucial for *A. veronii* infection in other associations, such as the cooperative zebrafish digestive tract, and whether virulence is attenuated in the mouse model may provide further insights. Similarly, *B. pertussis* does not require catalase for persistence within human polymorphonuclear leukocytes (PMNs), although phagocytosis is associated with a respiratory burst that involves the generation of O_2 and H_2O_2 (DeShazer *et al.*, 1994). It is possible that O_2 introduced with the fresh blood meal is removed rapidly in the leech digestive tract by the aerobic metabolism of *Aeromonas*, generating an environment that does not permit the generation of ROS and is suitable for the anaerobic *Rikenella*-like symbiont that likely does not possess antioxidant enzymes. Synergistic interactions between the two microbes resulting in enhanced mixed-species, polysaccharide-embedded microcolony formation have been suggested to occur within the leech digestive tract (Kikuchi & Graf, 2007). The apparent lack of oxidative stress suggests that other mechanisms must be responsible for ensuring the specificity, establishment and maintenance of this unusually simple digestive-tract community.

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