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Legionella pneumophila Catalase-Peroxidases: Cloning of the *katB* Gene and Studies of KatB Function

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***Legionella pneumophila*, the causative organism of Legionnaires' pneumonia, is spread by aerosolization from man-made reservoirs, e.g., water cooling towers and air conditioning ducts, whose nutrient-poor conditions are conducive to entrance into stationary phase. Exposure to starvation conditions is known to induce several virulence traits in *L. pneumophila*. Since catalase-peroxidases have been extremely useful markers of the stationary-phase response in many bacterial species and may be an avenue for identifying virulence genes in *L. pneumophila*, an investigation of these enzymes was initiated. *L. pneumophila* was shown to contain two bifunctional catalase-peroxidases and to lack monofunctional catalase and peroxidase. The gene encoding the KatB catalase-peroxidase was cloned and sequenced, and *lacZ* fusion and null mutant strains were constructed. Null mutants in *katB* are delayed in the infection and lysis of cultured macrophage-like cell lines. KatB is similar to the KatG catalase-peroxidase of *Escherichia coli* in its 20-fold induction during exponential growth and in playing a role in resistance to hydrogen peroxide. Analysis of the changes in *katB* expression and in the total catalase and peroxidase activity during growth indicates that the 8- to 10-fold induction of peroxidase activity that occurs in stationary phase is attributable to KatA, the second *L. pneumophila* catalase-peroxidase.**

Legionella pneumophila is an intracellular parasite and the causative organism of Legionnaires' pneumonia, a disease spread by aerosolization of the pathogen from environmental reservoirs. The reservoirs from which *L. pneumophila* is most frequently aerosolized are nutrient sparse: showerheads, respirators, air conditioning ducts and water cooling towers (10, 29, 30). These epidemiological considerations indicate that *L. pneumophila* must survive starvation conditions between periods of replication in a suitable host. It has recently been shown that several *L. pneumophila* virulence traits, including cytotoxicity, infectivity, and sodium sensitivity, are absent from exponentially growing cultures but are expressed in response to starvation. These observations led to the model where the stationary phase is a necessary prerequisite to the acquisition of virulence by legionella and not merely a stress state to be endured between rounds of intracellular multiplication (5). Stationary-phase gene expression has been associated with acquisition of virulence traits in other bacterial pathogens, e.g., *Salmonella* species, *Pseudomonas aeruginosa*, and *Yersinia enterocolitica* (2, 8, 18, 23). Therefore, genes in stationary-phase pathways of *L. pneumophila* may be tools for identifying genes in pathways leading to virulence.

In many bacterial species, genes controlling the antioxidant response play important roles in the stationary phase. The *Escherichia coli* KatE catalase is a hallmark of the stationary phase and is part of the cross-resistance to stress that accompanies starvation. *E. coli katG*, encoding a catalase-peroxidase, and *xth*, encoding the DNA repair enzyme exonuclease III, are other antioxidant enzymes that are expressed in the stationary phase under the control of RpoS, the stationary-phase sigma factor (14, 19, 21). We showed that the stationary-phase viability of a *katG* null mutant in *Caulobacter crescentus* is reduced by 6 orders of magnitude compared to that of the wild type, which is indicative of the importance of that catalase-peroxidase in stationary-phase survival (32).

Little is known about the *L. pneumophila* stationary-phase response. We began an investigation of catalase-peroxidases in *L. pneumophila* because in other bacterial species these genes have been extremely useful tools for studying the stationary-phase response. We report here that *L. pneumophila* contains two bifunctional catalase-peroxidases and no monofunctional catalases or peroxidases. We cloned the *katB* gene encoding one of the catalase-peroxidases, constructed *lacZ* fusion and null strains, and identified a role for *katB* in H_2O_2 resistance of the free-living organism and in infection of human macrophage lines.

MATERIALS AND METHODS

Media and growth conditions. *E. coli* DH5α was used for cloning. The liquid and solid media for culturing *E. coli* were Luria Bertani medium (31) with the following antibiotics at the indicated final concentrations: sodium ampicillin (100 μg/ml), kanamycin sulfate (50 μg/ml), gentamicin sulfate (5 μg/ml), and chloramphenicol (25 μg/ml). The culture media for *L. pneumophila* were AYE broth (17) and CYE plates (9) with the following antibiotic concentrations: kanamycin sulfate (25 μg/ml), gentamicin sulfate (5 or 10 μg/ml), and chloramphenicol (5 μg/ml). The temperature for culturing was 37°C. The parental *L. pneumophila* strain for genetic constructions was the wild-type strain JR32 (36) (Table 1).

Southern blotting. Restriction digests of 4 μg of genomic DNA (32, 33) were electrophoresed overnight in 0.8% agarose and blotted by capillary transfer to nitrocellulose (BA85; Schleicher and Schuell). Hybridization was performed at 62°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate. Washing was performed at 55°C in 2× SSC–0.1% sodium dodecyl sulfate.

Cloning of *L. pneumophila katB*. A *Bam*HI genomic blot of wild-type *L. pneumophila* DNA was hybridized with an *E. coli katG* probe to identify 6.4- and 5.7-kb bands. The probe was a PCR fragment of *E. coli katG* from the third nucleotide of the ATG start codon through the codon for the penultimate amino acid, L725 (34). The 5.7-kb band was gel purified and ligated into the *Bam*HI site of pUC12, producing a library of ≈600 Ap^r colonies, which were patched to grids. Blots of *Bam*HI-digested plasmid DNA from progressively smaller pools were hybridized with the *E. coli katG* probe. By this approach a plasmid containing the 5′ end of *L. pneumophila katB* was isolated. To isolate an overlap containing the 3′ end, a 9-kbp fraction was gel purified from an *Eco*RI/*Hin*dIII genomic digest of strain JR32 and ligated into pUC12, producing a library which was screened as described above. In this cloning, the probe was a 2.1-kb *Hin*dIII/*Bam*HI fragment containing the 5′ end of *katB* and some 5′ upstream sequence. The overlap fragment contained ≈7 kb beyond the *Bam*HI site in the 5.7-kbp fragment initially cloned.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
L. pneumophila		
JR32	Homogeneous salt-sensitive isolate of AM511 (Philadelphia-1 Sm^r r^-m^+)	H. Shuman (36)
PB102	JR32 *katB*$^+$ *katB*::*lacZ* (*katB* fusion strain)	This work
PB117	JR32 *katB*::ΩCm (*katB* null mutant)	This work
E. coli		
UM383	HB101 *katG17*::Tn*10*(Tet)*katE*::Tn*10*(Kan)	P. Loewen
UM383(pMMB207αB::*katB*)	UM383 with a PCR-amplified *katB* gene in the *Hin*d/*Pst* site of pMMB207αB	This work
Plasmids		
pJBZ280	*lacZ* with polylinker upstream for constructing translational fusions; ColE1 *ori* (Kan^r)	M. R. K. Alley (3)
pNPTS138	Derivative of pLITMUS 38 cloning vector with *nptI*, RK2 *ori*T, and *Bacillus subtilis sacB*; Kan^r	27
pHP45 ΩCm	pBR322 derivative containing the Cm^r gene with transcriptional and translational terminators and a polylinker at each end of the antibiotic cassette (Amp^r)	28
pMMB207αB	RSF1010 derivative; IncQ *lacI*^{q} Gen^r P*tac ori*T; multiple cloning site α complementation	36
pMMB207αB::*katB*	pMMB207αB with *Hin*d/*Pst* fragment containing PCR-amplified *katB* gene in *Hin*d/*Pst* site	This work
pNPTS138 *katB*::ΩCm	pNPTS138 with *katB* null allele (*katB*::ΩCm) in *Bam*HI site	This work

Construction of the chromosomal *katB*::*lacZ* translational fusion. The 5.7-kb *Bam*HI fragment containing the 5′ end of *katB* and upstream sequence was subcloned into the *Bam*HI site of pJBZ280 (3) (Table 1) to create an in-frame translational fusion. *E. coli* colonies harboring the construct were blue on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) plates, indicating that the *Legionella* promoter was recognized in *E. coli*. This construct was transformed by electroporation into strain JR32, and Kan^r transformants were selected. Three cycles were performed, in which cultures were grown overnight without kanamycin and then plated without kanamycin and isolated colonies were patched to identify Kan^r strains. Southern blotting demonstrated that the fusion had integrated into the chromosome adjacent to *katB*. The fusion strain, PB102, was blue with X-Gal overlay (36). A *L. pneumophila* chromosomal integrant derived from pJBZ280 with *katB* in the wrong orientation with respect to *lacZ* was white with X-Gal and showed no significant LacZ activity in assays of liquid cultures.

Construction of *L. pneumophila katB* null mutant. A *katB* null mutant was made by allelic exchange with sucrose counterselection (27, 32, 33, 36). The 5.7-kb *Bam*HI fragment containing the 5′ end of *katB* in pUC12 was cleaved at the unique *Apa*I site. This site was filled in with Klenow fragment and blunt-end ligated with the ΩCm cassette excised from pHP45 ΩCm (28) with *Hin*dIII and blunted. The resulting 8.5-kb fragment containing the null allele was excised from pUC12 with *Bam*HI and subcloned into the sucrase vector pNPTS138 (27). Wild-type *L. pneumophila* JR32 was transformed by electroporation with pNPTS138::*katB*::ΩCm. Individual Cm^r transformants were streaked on CYE–chloramphenicol–2% sucrose to identify Cm^r Suc^r Kan^s strains (33, 36). Allelic-exchange mutants were identified by Southern blotting, catalase and peroxidase enzyme assays, and activity staining of gels.

Enzymatic assays. Peroxidase activity was assayed at pH 6.4, monitoring the oxidation of dianisidine at 460 nm (ε_M = 11.3 mM^{-1} cm^{-1} [6]). Catalase activity was assayed at pH 7.2, monitoring the decomposition of H_2O_2 at 240 nm (ε_M = 39.4 M^{-1} cm^{-1} [1]). One unit of activity equals 1 μmol of H_2O_2 decomposed per min. Catalase activity was visualized as clear zones in nondenaturing polyacrylamide gels via inhibition of diaminobenzidine oxidation after permeation of the gel with a mixture of horseradish peroxidase, H_2O_2, and diaminobenzidine. Peroxidase activity was visualized as brown zones by omitting horseradish peroxidase from the protocol (7, 13). β-Galactosidase was assayed with *o*-nitrophenyl-β-D-galactoside as the substrate; activity was expressed in Miller units (22).

Measurement of resistance to hydrogen peroxide. Zone of inhibition tests were performed by using overnight cultures in AYE with the appropriate antibiotic (0.1 ml in 3 ml of 0.8% agar without nutrients on CYE plates without antibiotics). Whatman 3MM disks (7-mm diameter) received 10 μl of freshly diluted H_2O_2. The plates were incubated for 48 h.

Growth of *L. pneumophila* in cultured THP-1 macrophage cells. THP-1 cells were maintained and prepared for infection as previously described (33). *L. pneumophila* cells from overnight cultures in AYE were diluted in RPMI 1640 supplemented with 10% fetal calf serum, 1% glutamine, and 20% normal human serum and then added to the THP-1 cells (see Fig. 4 for further details). Aliquots removed daily were plated on CYE plates without added antibiotics.

PCR methodology. PCR was used to amplify a fragment of 2.7 kb, beginning 304 nucleotides (nt) upstream of the *katB* ATG translational start and ending 234 nt downstream of the *katB* translational stop. The PCR mixture contained 40 ng of *Hin*dIII-digested JR32 genomic DNA, 20 pmol each of 5′ and 3′ primers (Fig. 1), 2 mM each deoxynucleoside triphosphate dNTP and 2.5 U of *Taq* DNA polymerase in 100 μl. The amplification program was 1 cycle of 1 min at 94°C, 1 min at 55°C, and 2.5 min at 72°C; 25 cycles of 1 min at 94°C, 1 min at 55°C, and 4 min at 72°C; and a final cycle of 1 min at 94°C, 1 min at 55°C, and 5 min at 72°C. Following digestion with *Hin*dIII and *Pst*I, the PCR fragment was ligated into pMMB207αB (Table 1).

Nucleotide sequence accession number. The GenBank accession number for the *katB* gene is AF078110.

RESULTS

Cloning the *katB* catalase-peroxidase gene of *L. pneumophila*. Genomic Southern blots were hybridized with probes derived from *E. coli katE* and *katG*, encoding a monofunctional catalase and bifunctional catalase-peroxidase, respectively. *Bam*HI and *Pst*I genomic digests each showed two bands with the *katG* probe and no significant hybridization with the *katE* probe.

Bands of 6.4 and 5.7 kbp were observed in the *Bam*HI digest. The 5.7-kbp *Bam*HI band was cloned by hybridization. The sequence at one end was highly homologous to that at the 5′ end of bacterial catalase-peroxidase genes and the amino-terminal sequences of the encoded enzymes. The nucleotide sequence of the entire catalase-peroxidase gene was determined by isolating a fragment containing the 3′ end (Fig. 1). This catalase-peroxidase gene was named *katB*. Sequences upstream and downstream of *katB* were not homologous to any sequences in the databases. The 6.4-kbp band has been shown to contain a second catalase-peroxidase gene, *katA* (4).

KatB catalase-peroxidase sequence and enzyme activity. The *katB* open reading frame (ORF) encoded a protein of 721 amino acids highly homologous to bacterial catalase-peroxidases over its entire amino acid sequence: it was 65% identical to the catalase-peroxidase of *Bacillus stearothermophilus* and

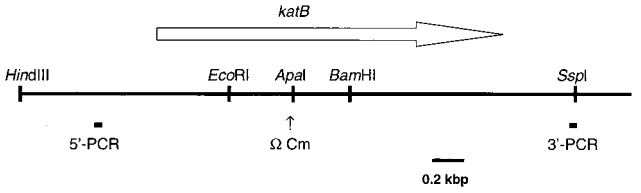

FIG. 1. Restriction map of *L. pneumophila katB* region. The thick horizontal line depicts the region sequenced (3,865 nt). Every nucleotide reported was identified in two to five sequencing runs, each with a different sequencing primer. Beneath the line are indicated the locations of the 5′ (TGATGCAGGCCTGCAGTTCACCAGTCAGCAGAGCG) and 3′ (GGTTTACAAAGCTTAGATGAGACTTACAGAAACG) PCR primers (5′-PCR and 3′-PCR) and the site of insertion of the ΩCm cassette (ΩCm). The underlined regions are *Pst*I and *Hin*dIII sites, respectively, that replaced 6 nt in the genomic *L. pneumophila* sequence. The arrow indicates the direction of transcription and the position of the *katB* open reading frame.

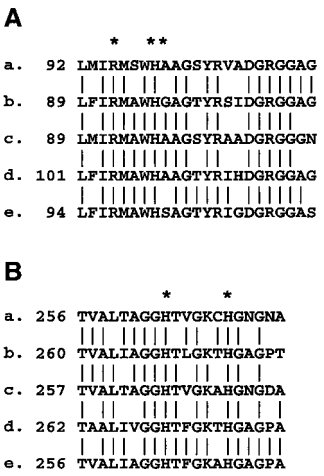

FIG. 2. Conserved active site residues in bacterial catalase-peroxidases. The asterisks mark the conserved residues that are homologous to residues of known function in yeast CCP. *L. pneumophila* KatB (a), *E. coli* KatG; (b), *R. capsulatus* (c), *M. tuberculosis* ATCC 25618 (d), and *B. stearothermophilus* (e) sequences are shown. (A) Distal side of heme. Residues marked by asterisks correspond to those forming the binding site for peroxide in CCP (R-48, W-51, and H-52 in the CCP sequence). (B) Proximal side of heme. Residues marked by asterisks correspond to CCP residues which form the fifth coordination position of the heme (H-175) and a site of hydrogen bonding to a heme propionate (H-181).

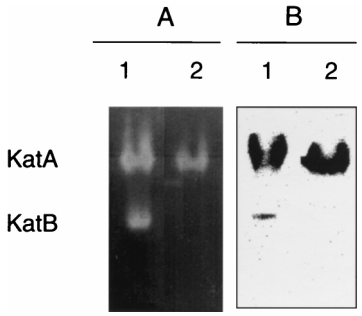

FIG. 3. Zymogram analysis for catalatic and peroxidatic activities. Cell extracts of overnight AYE cultures (500 μg of protein per lane) were electrophoresed under nondenaturing conditions, and the gel was stained to visualize catalatic (A) or diaminobenzidine-peroxidase activity (B). The two catalase-peroxidases in *L. pneumophila* are indicated. Lanes 1, strain JR32 (wild type); lanes 2, strain PB117 *katB*::ΩCm (*katB* null).

57 to 60% identical to catalase-peroxidases of *E. coli*, *Mycobacterium tuberculosis*, and *Rhodobacter capsulatus*. In the absence of the three-dimensional structure data for catalase-peroxidases, putative heme ligands and active site residues have been identified by sequence homologies with the monofunctional cytochrome *c* peroxidase (CCP) from *Saccharomyces cerevisiae* (35). Residues forming the peroxide binding site on the distal side of the heme (Fig. 2A) and residues which bind the proximal side of the heme (Fig. 2B) in CCP are conserved in *L. pneumophila* KatB. In addition, the adjacent sequences were highly homologous with those in other catalase-peroxidases. *L. pneumophila* KatB showed no homologies to monofunctional catalases or to monofunctional peroxidases from fungi or bacteria that use nonheme cofactors, e.g., manganese peroxidase, NADH flavoprotein peroxidase, or the lignin glycoprotein peroxidases with bound calcium.

The *katB* ORF plus 304 nt of upstream sequence and 234 nt of downstream sequence was amplified by PCR from *L. pneumophila* genomic DNA and cloned into the broad-host-range vector pMMB207αB, which is maintained in *E. coli* and *L. pneumophila*. This construct, pMMB207::*katB*, was transformed into strain UM383, an *E. coli katE katG* null mutant. Cell extracts of the transformant contained catalase and dianisidine peroxidase activities of 1.2 and 0.003 U/mg of cell protein, respectively. No detectable catalase or dianisidine peroxidase activity was found in extracts of untransformed strain UM383. These data indicate that *L. pneumophila katB* encodes a functional catalase-peroxidase which can be expressed in *E. coli* and that *katB* is not a pseudogene.

Construction of a *katB* null strain. An ΩCm cassette (26, 28), which interrupts transcription and translation, was cloned into the *Apa*I site within the Pro-280 codon of KatB. Exchange of wild-type *katB* for *katB*::ΩCm was accomplished by using sucrose counterselection and confirmed by Southern blotting (data not shown). A nonfunctional gene product was expected because residues essential for enzymatic function, i.e., a Trp proposed as the site of free radical formation and an Asp which stabilizes a histidine ligand of the heme (12), lie carboxyl terminal to the site in cytochrome *c* peroxidase, which is homologous to *L. pneumophila* Pro-280.

Cell extracts of wild-type and *katB* null strains were electrophoresed under nondenaturing conditions, and the acrylamide gels were stained for catalatic and diaminobenzidine peroxidatic activities. With the wild type (Fig. 3A and B, lanes 1), each enzyme stain visualized two bands. The coincidence of catalatic and peroxidatic staining for the two activities suggested that *L. pneumophila* expressed two catalase-peroxidases under the growth conditions used. With null strain PB117 *katB*::ΩCm, the band with greater mobility was absent (Fig. 3A and B, lanes 2). These data demonstrated that *katB* encoded the catalase-peroxidase with faster mobility and confirmed that strain PB117 was a functional *katB* null mutant. The slower catalase-peroxidase band was labeled KatA, as it is encoded by the second catalase-peroxidase gene implicated in genomic blots (4). Our data show that KatA and KatB account for the total catalatic and peroxidatic activity observed under the growth conditions used.

Characterization of the *katB* null mutant. (i) Growth in media and sensitivity to hydrogen peroxide. The *katB* null mutation had no effect on exponential growth. The doubling times of wild-type strain JR32 and null strain PB117 were identical in AYE broth: 170 ± 14 and 180 ± 12 min, respectively. Similarly, there was no difference in survival in the stationary phase. During 4 days in the stationary phase the titer of *katB*-null and wild-type *L. pneumophila* decreased similarly. This contrasted with the 10^4- to 10^6-fold decrease in survival of a *L. pneumophila sodC* null mutant (33).

Although the null mutant was no different from the wild type in growth and survival under normal aerobic culture conditions, it was more sensitive to an imposed H_2O_2 stress (Table 2). A second, independently isolated *katB* null strain showed an identical phenotype, consistent with the H_2O_2 phenotype

TABLE 2. Resistance to hydrogen peroxide[a]

Strain[b]	Diam of clearing (cm) at H_2O_2 concn (M) of:			
	1	2	5	10
Wild type	2.95 ± 0.05	3.4	4.15 ± 0.05	4.9
katB null	3.2	3.65 ± 0.05	4.4	5.13 ± 0.03

[a] Overnight cultures in top agar; 10 μl of solution at the indicated H_2O_2 concentration were applied to the disk. Where no error is listed, the duplicate determinations gave identical numerical values.

[b] Wild type, strain JR32; *katB* null, PB117 *katB*::ΩCm.

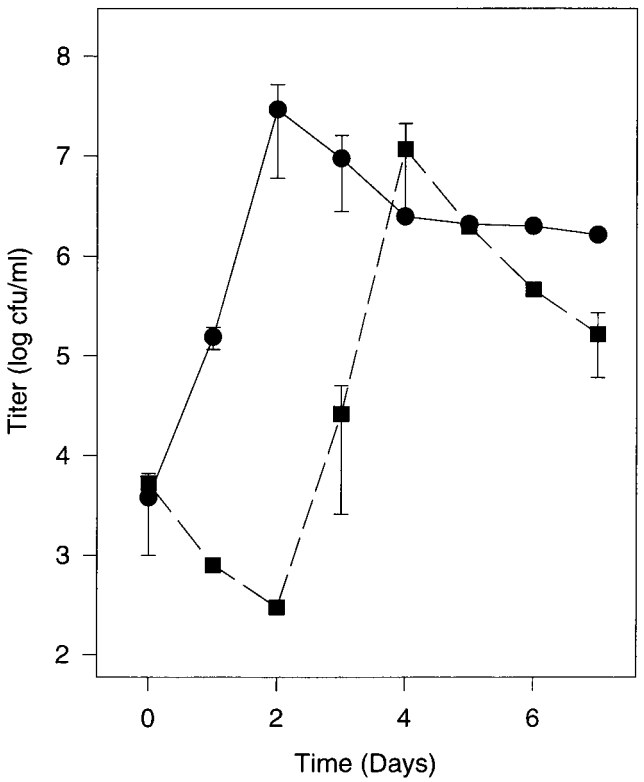

FIG. 4. Growth of *L. pneumophila* in cultured macrophage cells. Adherent THP-1 cells (3×10^5 per well) were infected on day 0 with 1×10^3 to 5×10^3 cells of the wild-type strain JR32 (●) or the *katB* null strain PB117 (■) from overnight cultures in AYE medium. Error bars indicate standard deviations.

being attributable to the *katB* null mutation and not to a spontaneous mutation elsewhere in the chromosome.

(ii) Growth in THP-1 macrophage line. Infection of cultured macrophage-like cell lines has been the model for *L. pneumophila* infection of pulmonary macrophages in Legionnaires' disease (30, 33, 36). *L. pneumophila* fails to replicate in most tissue culture media. Increases in the *L. pneumophila* titer in the medium are therefore attributable to release of the bacterium following invasion, intracellular replication, and lysis of a macrophage host. Wild-type *L. pneumophila* infected the THP-1 macrophage-like line as previously observed by us and others (33, 36) (Fig. 4). For *katB* null mutant PB117, the time course of the infection was reproducibly delayed by 2 days compared to that of the wild type. Further studies are necessary to discern if the delay is due to differences in entry, intracellular growth, or lysis. A similar delay was observed when cultured HL-60 macrophage-like cells were infected with PB117 *katB*::ΩCm.

An independently isolated *katB* null mutant exhibited an identical delay in infection of THP-1. This supports the contention that the above-mentioned infection phenotype is attributable to inactivation of *katB* and not to a spontaneous mutation in another gene.

Construction and characterization of a *katB*::*lacZ* fusion strain. A translational fusion in a Kan^r ColE1 plasmid was constructed with 4.6 kbp of upstream sequence and 1.1 kbp from the 5′ end of the *katB* ORF. Capitalizing on the generally poor maintenance of ColE1 plasmids in *L. pneumophila*, a cointegrate was isolated by selecting for Kan^r transformants of wild-type *L. pneumophila* and then screening for Kan^r after repeated culturing in the absence of kanamycin. The translational fusion strain, PB102, was blue by X-Gal overlay on CYE plates. Southern blotting (data not shown) confirmed that the fusion integrated adjacent to chromosomal *katB*. This strategy appears not to have been used previously with *L. pneumophila* and may be of general use in the construction of chromosomal fusions.

Exponential cultures of strain PB102 in AYE (optical density at 600 nanometers, 0.4 to 1.0) were treated with single additions of H_2O_2 to 15 or 60 μM. No change in LacZ activity was observed from 0.5 to 3 h after H_2O_2 challenge. These results suggested that the KatB catalase-peroxidase of *L. pneumophila* is relatively inert to hydrogen peroxide induction. In contrast, *katG* catalase-peroxidase was induced 20-fold when exponential cultures of *C. crescentus* were treated with the same range of H_2O_2 concentrations (32).

Expression of the *katB*::*lacZ* fusion increased about 30-fold during exponential growth and then decreased by about 25% in the stationary phase (Fig. 5A). A similar induction was seen for *E. coli katG* and was attributed to an increase in the number of respiratory centers during exponential growth, leading to increased H_2O_2 production per cell (11). Catalatic and peroxidatic activity during the transition from exponential to stationary phase was measured for wild-type *L. pneumophila* (Fig. 5B). Peroxidase activity in wild-type *L. pneumophila* increases

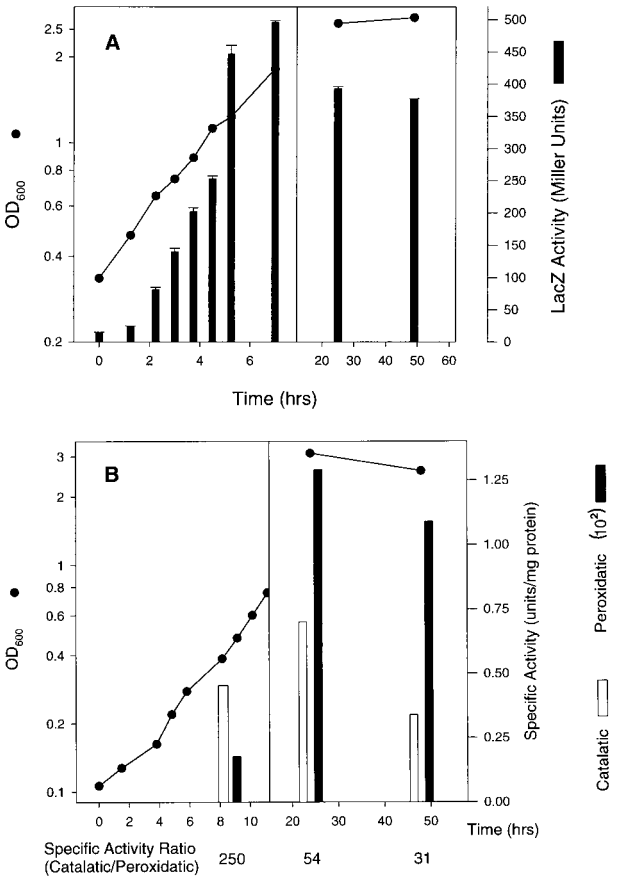

FIG. 5. Expression of catalatic and peroxidatic activities during growth. (A) Growth stage induction of the *katB*::*lacZ* fusion in the chromosomal fusion strain PB102. (B) Catalatic and dianisidine peroxidase specific activities of wild-type strain JR32 in mid-exponential and stationary phases and activity ratio (below abscissa). Error bars indicate standard deviations. OD_{600}, optical density at 600 nm.

about six- to eightfold in the stationary phase, in agreement with data in the literature (24).

DISCUSSION

Studies of *L. pneumophila* virulence and intracellular multiplication have outpaced studies of its physiology and metabolism. Recent observations linking stationary-phase gene expression with the acquisition of *L. pneumophila* virulence traits (5) suggest that the study of stationary-phase physiology is an avenue for identifying virulence genes. We initiated studies of catalase-peroxidases in *L. pneumophila* because such enzymes are frequently linked with the stationary-phase stress response. The most recent studies of catalases and peroxidases in *L. pneumophila*, over a decade ago, reported a single bifunctional catalase-peroxidase, based on chromatographic separation of activities in a cell extract (24, 25). In the present study, we established that in fact two catalase-peroxidases are present, KatA and KatB. The discrepancy may be due to cochromatography of the two in the prior study or to strain differences, although both laboratories used the Philadelphia-1 strain or a derivative of it.

We cloned the gene for *L. pneumophila katB* catalase-peroxidase, encoding a typical bifunctional catalase-peroxidase, in polypeptide length and active site homologies with CCP. Functionally, KatB is similar to *E. coli* KatG catalase-peroxidase. The 30-fold induction of *katB* during exponential growth can be reasonably attributed to increased cellular production of H_2O_2 resulting from increased respiration and increased leakage of electrons to O_2, as proposed for *E. coli katG* (11). Although *L. pneumophila* and *E. coli* catalase-peroxidases both play roles in resistance to H_2O_2, only *E. coli katG* is induced by H_2O_2. If *katB* is induced during exponential growth, why isn't it induced by H_2O_2 addition? During exponential growth in *E. coli*, *katG* levels correlate with the rate of H_2O_2 production but not with the absolute level of H_2O_2 (11). If a similar mechanism operates in *L. pneumophila*, then a bolus addition of H_2O_2 may create a rate of increase in H_2O_2 concentration that is a poor mimic of physiological H_2O_2 production. In complex media, the doubling time of *L. pneumophila* is 3 h, compared to 30 min or less for that of *E. coli*. Consequently, respiration and respiratory generation of H_2O_2 during exponential growth are likely to occur at a lower rate in *L. pneumophila* than in *E. coli*, and *katB* induction may be tuned to smaller H_2O_2 gradients.

We and others have observed an 8- to 10-fold induction of peroxidatic activity in *L. pneumophila* stationary phase (24). Here we showed that this induction is largely, if not entirely, due to KatA, because expression of *katB* is reduced in the stationary phase relative to its maximum during exponential growth (Fig. 5A). We also showed that during growth the ratio of catalatic activity to peroxidatic activity changes substantially, from ≈250 in the exponential phase to ≈30 to 50 in the stationary phase (Fig. 5B). We directly determined the catalatic:peroxidatic activity ratio for KatB as ≈400 by expressing *katB* in a catalase and peroxidase mutant of *E. coli*. Therefore, the catalatic:peroxidatic activity ratio for KatA must be <30 to 50, as it is the major but not the exclusive catalase-peroxidase in the stationary phase (Fig. 3). A survey of the catalatic:dianisidine peroxidase activity ratios for purified bacterial catalase-peroxidases shows values ranging from 1,800 (*R. capsulatus* [16]) to 250 (*Klebsiella pneumoniae* [15]) to ≈100 (*M. tuberculosis* and *E. coli* [6, 20]). A ratio of less than 30, inferred for KatA, would be uncommonly low and indicative of an enzyme with a propensity towards peroxidatic versus catalatic activity.

In sum, our studies are the first molecular genetic investigation of catalase-peroxidases in *L. pneumophila*. We demonstrated a role for the KatB catalase-peroxidase in defense against H_2O_2 and in infection of macrophages. We also identified the KatA catalase-peroxidase as responsible for the stationary-phase increase in peroxidase activity and as a potentially useful marker for the stationary-phase gene expression that precedes virulence. In addition, KatA is predicted to have an uncommonly high peroxidatic activity relative to catalatic activity. Cloning of *L. pneumophila katA* and construction and characterization of *katA* fusion and null strains are in progress.

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