KatA, the Major Catalase, Is Critical for Osmoprotection and Virulence in Pseudomonas aeruginosa PA14

Ji-Sun Lee, Yun-Jeong Heo, Jeong K. Lee, and You-Hee Cho*

Department of Life Science, Sogang University, Seoul 121-742, Korea

Received 3 November 2004/Returned for modification 7 January 2005/Accepted 24 February 2005

We demonstrate that among the three monofunctional catalases of Pseudomonas aeruginosa PA14, KatA and, to a lesser extent, KatB, but not KatE, are required for resistance to peroxide and osmotic stresses. KatA is crucial for adaptation to H$_2$O$_2$ stress and full virulence in both Drosophila melanogaster and mice. This dismantling of catalase roles represents a specialized catalytic system primarily involving KatA in responses to adverse environmental conditions.

Catalases are central components of the enzymatic detoxification pathways that prevent the formation of the highly reactive hydroxyl radical (HO$_\bullet$) by decomposing hydrogen peroxide (H$_2$O$_2$) and contribute to a variety of physiological processes involving adaptation and survival mechanisms. Because the toxicity of H$_2$O$_2$ released by phagocytes has been implicated in the host innate immune responses, bacterial pathogens exploit catalytic enzyme systems to survive the host environment. Although the involvement of catalases in virulence mechanisms has been demonstrated in many bacterial pathogens, little is known on the roles of catalases in the pathogenesis of Pseudomonas aeruginosa, an opportunistic human pathogen that is frequently associated with the alveolar surface and is most likely subjected to oxidative stresses within the pulmonary airways (1).

P. aeruginosa is a unique bacterium that has three differentially evolved monofunctional catalase genes, katA, katB, and katE, but no bifunctional catalase (catalase-peroxidase) gene on its genome (25). Like other clade 3 monofunctional catalases, the major catalase, KatA, is H$_2$O$_2$ inducible (4, 13, 18). The expression of a second H$_2$O$_2$-inducible catalase, KatB, that belongs to clade 1 is noteworthy (2, 14). The third catalase, KatE, is one of the clade 2 catalases that are highly conserved among most bacterial species (3, 14). With the exception of a Streptomyces coelicolor catalase (CatB) that plays important roles in osmoprotection and differentiation (5), little else is known about the physiological role of clade 2 catalases.

The purpose of this work is to systematically determine the roles of the three differentially evolved monofunctional catalases in stress responses and survival mechanisms of P. aeruginosa strain PA14 (20). We used nonpolar, unmarked deletion mutants of each catalase and investigated their resistance and adaptation in response to stress conditions in vitro and their virulence in Drosophila melanogaster and mice. We demonstrate that KatA plays an important role in virulence and oxidative and/or osmotic stress responses. In combination with the previously published studies (2, 10, 11, 18, 19, 26), these results suggest the specialized roles of P. aeruginosa catalases in response to environmental stresses and pathogenic interactions as well.

Construction of catalase mutants of P. aeruginosa PA14 and their resistance to H$_2$O$_2$. The genome sequence of the P. aeruginosa reference strain PA01 reveals three monofunctional catalase genes, katA, katB, and katE, of different evolutionary origins, clade 3, clade 1, and clade 2, respectively (13, 14). No homologue of bifunctional catalases has been found in P. aeruginosa thus far. Isolation of the gene encoding a manganese-containing nonheme pseudocatalase from lactic acid bacteria (12) and compilation of its homologous sequences from the bacterial genome sequence databases revealed a homologous gene (PA2185 or katM after Mn-catalase) on the 12th variable segment of the PA01 genome (21).

Most P. aeruginosa strains, including PA14, do not harbor a katM gene (28; Heo and Cho, unpublished). As summarized in Table 1, we have created seven unmarked deletions (katA, katB, katE, katAB, katAE, katBE, and katABE) in strain PA14 to systematically address the potential role of catalases in stress responses and virulence (15).

We have verified all the catalase mutants through genetic structure analyses by PCR and Southern hybridization (Fig. 1) (15), expression profiles by total catalase activity staining (15), and growth inhibition on plates containing 100 μM H$_2$O$_2$ (Fig. 2A). All the mutants exhibited doubling times similar to that of the wild type (15). The growth of the katA and to a lesser extent katB but not katE mutants was inhibited by H$_2$O$_2$. The contribution of KatB to H$_2$O$_2$ resistance was more evident in the katAB mutant (Fig. 2A).

The H$_2$O$_2$ sensitivity of the katA mutant was completely restored by introducing the pUCP18 (22)-derived plasmid containing appropriate full-length catalase constructs (Fig. 2B). In contrast, multicopy expression of the katB gene only partially restored the H$_2$O$_2$ resistance of the katA mutant. We conclude that the KatA is most critical in H$_2$O$_2$ resistance, whereas resistance mediated by KatB was only discernible when KatA expression was abolished. In contrast, katE does not affect H$_2$O$_2$ resistance.

KatA is required for adaptation to H$_2$O$_2$ in P. aeruginosa PA14. In an attempt to investigate the roles of catalases in the adaptation to H$_2$O$_2$ stress, we examined the sensitivity of PA14 to H$_2$O$_2$ in liquid culture. Mid-logarithmic PA14 cells were
pretreated with a nonlethal level of H$_2$O$_2$ (1 mM) for 30 min before being exposed to the killing concentration of H$_2$O$_2$ (100 mM). A 30-min treatment time was chosen to investigate the steady-state response rather than the early and acute responses. The viability of cells was determined at 5-min intervals. Less than 0.1% of the unadapted or naive cells remained viable 10 min after exposure to 100 mM H$_2$O$_2$. In contrast, when cells were pretreated with 1 mM H$_2$O$_2$, survival was enhanced more than 1,000-fold (Fig. 3A). The sublethal pretreatment affected the cells’ growth and/or survival compared to the control (~60% survival as shown in Fig. 3B) and is slightly harsher than those previously described in other bacteria (7, 8, 9, 17).

We analyzed all the catalase mutants in the adaptation experiment to determine whether catalases participate in the adaptive response to H$_2$O$_2$. The katA mutant was more sensitive to 1 mM H$_2$O$_2$ than the wild type was. Moreover, the katAB mutant was even more sensitive ($<10^{-4}$ viability) to the pretreatment. Therefore, the residual survival (~25%) of the katA mutant bacteria by the pretreatment may be attributed to KatB, which is in a good agreement with the results on solid agar culture (Fig. 2A). The H$_2$O$_2$ pretreatment enhanced the cells’ resistance and viability against the killing concentration of H$_2$O$_2$, which was completely abolished in the katA mutant.

**FIG. 1.** Creation of catalase mutants. Based on the PAO1 sequences, PCR deletions of each monofunctional catalase were generated and used to create three single (katA, katB, and katE), three double (katAB, katAE, and katBE), and a triple (katABE) mutant in wild-type PA14 (WT) via homologous recombination followed by sacB-dependent segregation as summarized in Table 1. Multiplex PCR using three sets of primers was used to verify the predicted genetic structures of the mutants. The PCR product sizes of the intact genes (designated by the solid arrowhead on the left) for katA, katB, and katE were 2.1, 2.8, and 2.5 kb, respectively, whereas those of deletions (designated by the empty arrowhead on the right) were 1.5, 2.1, and 0.4 kb, respectively. Because the PCR products from the intact katA gene and the deleted katB gene are almost the same size, only two bands were observed for katB and katBE (lanes 3 and 7, respectively).

**FIG. 2.** Oxidative stress resistance of catalase mutants. (A) Cells were grown in LB broth at 37°C to an optical density at 600 nm of 0.5. Five 10-fold serial dilutions of the cells in LB broth were spotted onto an LB agar medium containing 100 μM H$_2$O$_2$. (B) Complementation of H$_2$O$_2$-sensitive phenotype of the katA mutant was performed by introducing pUCP18-derived plasmids containing full-length fragments of katA (pUCP-KatA), katB (pUCP-KatB), and katE (pUCP-KatE). Cells were diluted as in A and spotted onto LB agar medium containing 100 μM H$_2$O$_2$ and 200 μg/mL carbenicillin. The numbers (from 10$^6$ to 10$^2$) indicate the CFU of the cell spots.

Killing of the pretreated katB and katE mutant bacteria by 1 mM H$_2$O$_2$ was discernible (Fig. 3B and data not shown). This result suggests that the basal and/or inducible expression of KatA, but not KatB, is responsible for the adaptation to H$_2$O$_2$, despite the rapid induction of katB by H$_2$O$_2$ in the presence of functional KatA (19) (data not shown).

It is clear, however, that KatA and KatB have overlapping but distinct roles in oxidative stress responses, since the multicyclic KatB failed to fully compensate for the absence of KatA in terms of H$_2$O$_2$ resistance and adaptation (data not shown). The catalytic functions involving both KatA and KatB during normal growth and oxidative stress remain to be further deciphered by combining this result with detailed and systematic gene expression analyses in each catalase mutant background with or without oxidative challenge.

**KatA is preponderantly required for osmoprotection in *P. aeruginosa* PA14.** A minor catalase (CatB) from the actinomycete *S. coelicolor* is known to be required for resistance to osmotic stress and differentiation (5). We tested whether *P. aeruginosa* catalase mutants are susceptible to osmotic stresses. As shown in Fig. 4A, KatA was critical in the resistance to KCl treatments (0.8 M and 0.9 M), whereas deletion of katB or katE had no significant effect on salt resistance. However, the different KCl sensitivities of the katA and katAB mutants, depending on the KCl concentration, suggest that KatB may play a minor role in osmoreistance as in H$_2$O$_2$ resistance (Fig. 2).

Since KCl increases ionic strength as well as osmotic strength, we used a nonionic osmolyte, sucrose, with comparable amounts of KCl (23). As shown in Fig. 4B, sucrose treatments at 32% (~0.89 M) and 34% (~0.94 M) exhibited similar results as observed in KCl treatments, uncovering the involvement of KatA in sucrose resistance, although the responses to the two different concentrations were more subtle than those in the KCl treatments, especially in the katA and katAB mutants, indicating the minor role of KatB in this condition.

**TABLE 1. *P. aeruginosa* strains used in this study**

| Genotype | Strain name | Relevant characteristics | Reference or source |
|----------|-------------|-------------------------|---------------------|
| Wild type | PA14 | Prototrophic, virulent burn wound isolate | 20 |
| KatA | PRL700 | PA14 ΔkatA (0.60-kb deletion of katA) | This study |
| KatB | PRL800 | PA14 ΔkatB (0.72-kb deletion of katB) | This study |
| KatE | PRL900 | PA14 ΔkatE (2.10-kb deletion of katE) | This study |
| KatAB | PRL780 | PRL700 ΔkatB (0.72-kb deletion of katB) | This study |
| KatAE | PRL790 | PRL780 ΔkatE (2.10-kb deletion of katE) | This study |
| KatBE | PRL800 | PRL790 ΔkatB (0.72-kb deletion of katB) | This study |
| KatABE | PRL798 | PRL790 ΔkatB (0.72-kb deletion of katB) | This study |

Downloaded from http://iai.asm.org on April 30, 2019 by guest
The sensitive phenotype of the katA mutant was restored by trans complementation with the pUCP18-derived plasmid expressing KatA (Fig. 4C). Unlike H₂O₂ sensitivity, however, multicopy KatB could not restore growth of the katA mutant on salt-containing media, which may imply differential functions and/or regulations of KatA and KatB in response to osmotic stress.

It is intriguing that the cell-free culture supernatant from the wild-type culture in the stationary growth phase could restore the KCl sensitivity of the katA mutant, although we were not sure whether or not the supplied activities absent in the culture supernatant of the katA mutant were working extracellularly. Further experimentation is needed to unravel how catalases such as P. aeruginosa KatA and S. coelicolor CatB protect against osmotic stresses. Considering that the general stress responses likely require alternative sigma factors (3, 6), it will be of special interest to analyze the gene expression in response to specific and general stress conditions.

KatA is required for virulence in P. aeruginosa PA14. The in vitro oxidative and osmotic stress phenotypes of catalase mutants are most likely related to the survival pathways, and therefore likely implicated in virulence due to unfavorable conditions.
conditions, *P. aeruginosa* may encounter in the host environment. We examined whether the *P. aeruginosa* catalases play a role in host infection using the *D. melanogaster* model, since it was a simple alternative model host to evaluate *P. aeruginosa* virulence potentials, as measured by fly mortality and in vivo proliferation of *P. aeruginosa* (16, 27).

*D. melanogaster* infection was performed by pricking 2- to 5-day-old adult flies with 50 to 200 CFU of PA14 cells as described previously (16). Mortality was monitored at 25°C for up to 54 h postinfection (Fig. 5). Four catalase mutants (*katA*, *katAB*, *katAE*, and *katABE*) commonly deficient in KatA exhibited significant virulence attenuations in terms of delayed death kinetics (by more than 10 h) and lower mortality, whereas the remaining three mutants (*katB*, *katE*, and *katBE*) were as virulent as the wild type (Fig. 5A). Reintroduction of the full-length *katA* gene restored the attenuated virulence of the *katA* mutant to the wild-type level, whereas *katA* mutant cells harboring a multicopy plasmid expressing either KatB or KatE were still avirulent (Fig. 5B).

The virulence attenuation of the *katA* mutant was verified by bacterial proliferation in *D. melanogaster* (Fig. 6). PA14 cells proliferate almost exponentially in flies, as described by Lee et al. (16), where the linear regression analyses from the 57 data points (from live flies) gave a slope of 0.1734, which is statistically significant ($r^2 = 0.897$). The slope corresponds to a doubling time of 1.736 h. However, not all *katA* cells proliferate exponentially in flies, unlike the wild type. The bacterial proliferations from 78 live flies were delayed about 6 h, and some infected flies completely cleared the bacteria (Fig. 6B).

The involvement of KatA in virulence was further verified in mammalian hosts, using the mouse peritonitis model as described previously (24). The mice were monitored from 6 to 64 h after intraperitoneal challenge with $5 \times 10^6$ CFU of *P. aeruginosa* and regarded as dead when they displayed ruffled fur, evidence of dehydration, and nonresponsiveness to stimuli. More than 90% of the mice that had been infected with the wild-type cells died within 36 h in our experimental conditions (Fig. 7). As in *D. melanogaster*, the *katA*, *katAB*, and *katAE* mutants are shown as open symbols. Symbols: $\times$, wild type; ○ and ●, single mutants; ○ and ●, double mutants; ■, triple mutant.

![FIG. 5. Virulence of catalase mutants in *D. melanogaster*. Fly mortality was determined using groups of 100 flies. Flies were infected with 50 to 200 CFU of the wild-type or mutant bacteria that had been grown in LB broth to an optical density at 600 nm of 3.0 and kept at 25°C. Flies that died within 12 h postinfection were excluded from mortality determination. Mortality studies were repeated at least five times with similar results. Mutants with *katA* deleted are indicated with solid symbols and those with intact *katA* are shown as open symbols. Symbols: $\times$, wild type; ○ and ●, single mutants; ○ and ●, double mutants; ■, triple mutant.](image1)

![FIG. 6. Bacterial proliferation in *D. melanogaster*. Batches of 10 flies were infected either with the wild type (A) or the *katA* mutant (B) as described for Fig. 5. Homogenates of individual infected flies were collected every 6 h up to 48 h postinfection and plated on LB agar to determine the CFU per fly. The CFU determined from live (open symbols) and dead (solid symbols) flies are shown in a log scale, showing a statistically significant linearity (+) only for the wild-type bacteria. The results are representative of three independent experiments. Symbols: $\times$, wild type; ○ and ●, single mutants; ○ and ●, double mutants; ■, triple mutant.](image2)

![FIG. 7. Virulence of catalase mutants in mice. LB broth-grown cells (optical density at 600 nm of 3.0) were harvested and washed twice with phosphate-buffered saline (150 mM NaCl, 20 mM phosphate, pH 7.0), and appropriately diluted to reach $5 \times 10^6$ CFU in 100 µl of phosphate-buffered saline containing 1% mucin, which helps to induce infection in naive mice as an adjuvant. Groups of 10 anesthetized BALB/c mice (4 to 6 weeks old) were infected intraperitoneally with 100 µl of bacterial suspension. Percentages of survivors over the indicated time points are shown. Each point represents the mean of three independent. Mutants with *katA* deleted are indicated by solid symbols and those with intact *katA* are shown as open symbols. Symbols: $\times$, wild type; ○ and ●, single mutants; ○ and ●, double mutants; ■, triple mutant.](image3)
katAE, and katABE mutants were less virulent in the mouse peritonitis model, with ~40% mice surviving the infection, exhibiting delayed killing (by more than 20 h).

Conclusion. These phenotypic analyses of the three monofunctional catalases (KatA, KatB, and KatE) in *P. aeruginosa* PA14 suggest that the catalytic system of KatA is crucial for oxidative and osmotic stress responses. KatA is also required for adaptation to peroxide stress and for virulence of this bacterium, which is intuitively understandable in that it is critical for stress responses as well as adaptations in vitro that may resemble unfavorable host environments. It is also explainable in part by the regulation of katA, which involves quorum-sensing circuits (11).

The pivotal roles of KatA in virulence mechanisms can be further authenticated and generalized, by investigating its involvement in virulence of other *P. aeruginosa* strains such as PA01, since the multifactorial nature of virulence pathways is related with the genetic backgrounds that accounts for different virulence potentials, and its expression and regulation in conjunction with related enzymes and regulators such as RpoS and OxyR.

We are grateful to Gee Lau for helpful comments.

This work was supported by grants from the 21C Frontier Microbial Genomics and Applications Center (MG05-0104-05-0) and the Korea Research Foundation (2004-015-C00505) and by a Special Research Grant from Sogang University (2002-3010) to Y.-H. Cho.

REFERENCES

1. Bodey, G. P., R. Bolivar, V. Fainstein, and L. Ladeja. 1983. Infections caused by *Pseudomonas aeruginosa*. Rev. Infect. Dis. 5:279–313.

2. Brown, S. M., M. L. Howell, M. L. Vasil, A. J. Anderson, and D. J. Hassett. 1995. Cloning and characterization of the katA gene of *Pseudomonas aeruginosa* encoding a hydrogen peroxide-inducible catalase: purification of KatA, cellular localization, and demonstration that it is essential for optimal resistance to hydrogen peroxide. J. Bacteriol. 177:6536–6544.

3. Cho, Y.-H. 1999. Gene expression and the role of catalases in *Streptomyces coelicolor* A3(2). Ph.D. thesis. Seoul National University, Seoul, Korea.

4. Cho, Y.-H., and J.-H. Roe. 1997. Isolation and expression of the catA gene encoding the major vegetative catalase in *Streptomyces coelicolor* Muller. J. Bacteriol. 179:4049–4052.

5. Cho, Y.-H., E.-J. Lee, and J.-H. Roe. 2000. A developmentally regulated catalase required for proper differentiation and osmoprotection of *Streptomyces coelicolor*. Mol. Microbiol. 35:150–160.

6. Cho, Y.-H., E.-J. Lee, B.-E. Ahn, and J.-H. Roe. 2001. SigB, an RNA polymerase sigma factor required for osmoprotection and proper differentiation of *Streptomyces coelicolor*. Mol. Microbiol. 42:205–214.

7. Christman, M. F., R. W. Morgan, F. S. Jacobson, and B. N. Ames. 1985. Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*. Cell 41:753–762.

8. Demple, B., J. Halbrook, and S. Linn. 1983. *Escherichia coli* th mutants are hypersensitive to hydrogen peroxide. J. Bacteriol. 153:1079–1082.

9. Dowds, B. C., P. Murphy, D. J. McConnell, and K. M. Devine. 1987. Relationship among oxidative stress, growth cycle, and sporulation in *Bacillus subtilis*. J. Bacteriol. 169:5771–5775.

10. Hassett, D. J., E. Alsabbagh, K. Parvatiyar, M. L. Howell, R. W. Wilmott, and U. A. Ochsner. 2000. A protease-resistant catalase, KatA, released upon cell lysis during stationary phase is essential for aerobic survival of a *Pseudomonas aeruginosa* oxyR mutant at low cell densities. J. Bacteriol. 182:4557–4563.

11. Hassett, D. J., J. F. Ma, J. G. Elkins, T. R. McDermott, U. A. Ochsner, S. E. West, C. T. Huang, J. Fredericks, S. Burnett, P. S. Stewart, G. McFeters, L. Passador, and B. H. Igleswki. 1999. Quorum sensing in *Pseudomonas aeruginosa* controls expression of catalase and superoxide dismutase genes and modulates biofilm susceptibility to hydrogen peroxide. Mol. Microbiol. 34:1082–1093.

12. Igarashi, T., Y. Kono, and K. Tanaka. 1996. Molecular cloning of manganese catalase from *Lactobacillus plantarum*. J. Biol. Chem. 271:29521–29524.

13. Klotz, M. G., and P. C. Loewen. 2000. Role of the *Pseudomonas aeruginosa* *rpoS* gene in oxidative stress defenses and DNA repair. *OxyR-dependent regulation of katA-ankB, ahpF, and atbp-ahpF*. J. Bacteriol. 182:4533–4544.

14. Klotz, M. G., G. R. Klassen, and P. C. Loewen. 1997. Phylogenetic relationships among prokaryotic and eukaryotic catalases. Mol. Biol. Evol. 14:951–958.

15. Lee, J.-S. 2005. Phenotypic analyses of multifunctional catalases in *Pseudomonas aeruginosa*. M.S. thesis. Sogang University, Seoul, Korea.

16. Lee, J.-S., S.-H. Kim, and Y.-H. Cho. 2004. Dithiothreitol attenuates the pathogenic interaction between *Pseudomonas aeruginosa* and *Drosophila melanogaster*. J. Microbiol. Biotechnol. 14:367–372.

17. Lee, J.-S., Y.-C. Hah, and J.-H. Roe. 1993. The induction of oxidative enzymes in *Streptomyces coelicolor* upon hydrogen peroxide treatment. J. Gen. Microbiol. 139:1013–1018.

18. Ochsner, U. A., M. L. Vasil, E. Alsabbagh, K. Parvatiyar, and D. J. Hassett. 2000. Role of the *Pseudomonas aeruginosa*oxyR-recG operon in oxidative stress defense and DNA repair: OxyR-dependent regulation of katA-ankB, ahpF, and atbp-ahpF. J. Bacteriol. 182:4533–4544.

19. Palma, M., D. Deluca, S. Worgall, and L. E. Quadri. 2004. Transcriptome analysis of the response of *Pseudomonas aeruginosa* to hydrogen peroxide. J. Bacteriol. 186:248–252.

20. Rahme, L. G., E. J. Stevens, S. F. Wolford, J. Shao, R. G. Tompkins, and F. M. Ausubel. 1995. Common virulence factors for bacterial pathogenicity in plants and animals. Science 268:1899–1902.

21. Robbe-Saule, V., C. Cynaual, M. Ibanez-Ruiz, D. Hermant, and F. Norel. 1991. Identification of a non-haem catalase in *Salmonella* and its regulation by RpoS (*rpoS*). Mol. Microbiol. 39:1533–1545.

22. Schweizer, H. P. 1991. *Excherichia-Pseudomonas* shuttle vectors derived from pUC18/19. Gene 107:99–121.

23. Shortridge, V. D., A. Lazdunski, and M. L. Vasil. 1992. Osmoprotectants and phosphate regulate expression of phospholipase C in *Pseudomonas aeruginosa*. Mol. Microbiol. 6:863–871.

24. Sonneletnier, E., S. Hagens, F. Rosenau, S. Wilhelm, A. Habel, K. E. Jager, and U. Blasi. 2003. Reduced virulence of a *hbq* mutant of *Pseudomonas aeruginosa* O1. Microb. Pathog. 35:217–228.

25. Stover, C. K., X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrener, M. J. Ohman. 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1. J. Bacteriol. 182:1057–1073.

26. Vodovar, N., C. Acosta, B. Lemaître, and F. Bocard. 2004. *Drosophila*: a polyvalent model to decipher host-pathogen interactions. Trends Microbiol. 12:235–242.

27. Wolfgang, M. C., B. R. Kulasekara, X. Liang, D. Boyd, K. Wu, Q. Yang, C. G. Miyada, and S. Lory. 2003. Conservation of genome content and virulence determinants among clinical and environmental isolates of *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA 100:8484–8489.

Editor: V. J. DiRita