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[44] *Pseudomonas aeruginosa* Biofilm Sensitivity to Biocides: Use of Hydrogen Peroxide as Model Antimicrobial Agent for Examining Resistance Mechanisms

By DANIEL J. HASSETT, JAMES G. ELKINS, JU-FANG MA, and TIMOTHY R. McDERMOTT

Introduction

In nature, bacterial biofilms are complex, yet highly structured communities that are often composed of multiple genera. Biofilms are ubiquitous, found on various substrata, and can be problematic in environmental, industrial, and clinical settings. When occupying such diverse niches, one unifying hallmark of biofilms is their relative resistance to a myriad of biocides and antibiotics, especially when compared to planktonic organisms. In industrial and environmental settings, oxidizing agents such as hydrogen peroxide (H_2O_2) and sodium hypochlorite ($NaOCl$) are used for biofilm control. However, killing of biofilm organisms with these agents is typically incomplete, with regrowth commonly occurring rapidly. As *Pseudomonas aeruginosa* is often associated with biofilms, it has been used by various laboratories as a model organism for studying biofilm resistance to these oxidizing biocides.

If available, *P. aeruginosa* preferentially uses molecular oxygen (O_2) as a terminal electron acceptor. However, aberrant electron flow occurring in respiratory metabolism can lead to the production of reactive oxygen intermediates (ROIs). These include superoxide (O_2^-), H_2O_2 , and the hydroxyl radical ($HO\cdot$). Although such apparent metabolic infidelity is common in aerobic organisms, ramifications of ROI production include cell damage, mutations, or death. Relief of ROI stress is provided by antioxidant defenses, including superoxide dismutase (SOD), catalase, peroxidase, DNA repair enzymes, and free radical scavenging agents.^{1,2}

Pseudomonas aeruginosa is well equipped to deal with oxidative stress. The organism possesses an iron- and a manganese-cofactored SOD,^{3,4}

¹ W. Beyer, J. Imlay, and I. Fridovich, *Prog. Nucleic Res.* 40, 221 (1991).

² J. Imlay and S. Linn, *J. Bacteriol.* 169, 2967 (1987).

³ D. J. Hassett, W. A. Woodruff, D. J. Wozniak, M. L. Vasil, M. S. Cohen, and D. E. Ohman, *J. Bacteriol.* 175, 7658 (1993).

⁴ D. J. Hassett, H. P. Schweizer, and D. E. Ohman, *J. Bacteriol.* 177, 6330 (1995).

whose function is to remove $O_2^{-\cdot}$.⁵ To dispose of H_2O_2 , *P. aeruginosa* possesses three catalases, designated KatA,^{6,7} KatB,⁶ and KatC.⁸ KatA is the predominant catalase and is produced during all growth stages.^{6,7} In planktonic cells, KatB has not been detected in exponential phase cells, but is observed at very low levels in stationary phase cells. However, when *P. aeruginosa* is exposed to exogenous H_2O_2 or the redox-cycling antibiotic paraquat, KatB levels are increased greatly due to the transcriptional activation of *katB*. Unlike KatA and KatB, little is known about KatC or its biological role in *P. aeruginosa*. Together, however, these catalases arguably constitute the major first-line defense mechanism in protecting *P. aeruginosa* against H_2O_2 .

Because our knowledge of this organism's response to oxidative stress is reasonably well grounded genetically and physiologically,^{3,4,7,9-11} we are in a position to begin asking specific questions regarding mechanisms by which biofilm bacteria resist oxidizing biocides. In studying bacterial biofilm behavioral responses to these antimicrobials, one can gain insight into the physiological processes of biofilm bacteria and how they differ from planktonic cells. This article describes methods for measuring biofilm responses to H_2O_2 in terms of cell survival and defense mechanisms. The basic method described for the quantification of cell resistance to H_2O_2 can be applied to study the effects of any biofilm biocide. To demonstrate basic utility of the approach in terms of assessing biofilm physiology, we also include spectrophotometric and activity gel-staining techniques for measuring catalase and SOD.

Growth of Planktonic Cultures and Biofilms

Planktonic cultures and biofilms are grown aerobically in a medium of choice. In the methods describe here we used either a defined media such

⁵ J. M. McCord and I. Fridovich, *J. Biol. Chem.* **244**, 6049 (1969).

⁶ S. M. Brown, M. L. Howell, M. L. Vasil, A. Anderson, and D. J. Hassett, *J. Bacteriol.* **177**, 6536 (1995).

⁷ D. J. Hassett, L. Charniga, K. A. Bean, D. E. Ohman, and M. S. Cohen, *Infec. Immun.* **60**, 328 (1992).

⁸ The *Pseudomonas* Genome Project, Pathogenesis Corporation and the Cystic Fibrosis Foundation, <http://www.pseudomonas.com>.

⁹ D. J. Hassett, P. Sokol, M. L. Howell, J.-F. Ma, H. P. Schweizer, U. Ochsner, and M. L. Vasil, *J. Bacteriol.* **178**, 3996 (1996).

¹⁰ D. J. Hassett, M. L. Howell, P. A. Sokol, M. Vasil, and G. E. Dean, *J. Bacteriol.* **179**, 1442 (1997).

¹¹ D. J. Hassett, M. L. Howell, U. Ochsner, Z. Johnson, M. Vasil, and G. E. Dean, *J. Bacteriol.* **179**, 1452 (1997).

as *Pseudomonas* basal mineral (PBM) medium¹² or complex media such as L-broth or Trypticase® soy broth (TSB) diluted 1:10 for batch planktonic cultures (1:10 TSB) or 1:100 for biofilms (1:100 TSB). The temperature used to culture planktonic cells should match that used for biofilms, and in the experiments described in this article, all cultures were grown at 25°. Planktonic cultures are grown to stationary phase (overnight) with shaking at 300 rpm and used to inoculate biofilm coupons or are collected for the preparation of cell extracts (described later).

Biofilms are grown in a multiple chamber drip flow reactor as described previously.¹³ Briefly, medium is dripped over sterile coupons held in parallel polycarbonate chambers.¹³ Each coupon (1.3 × 7.6 cm; composed of material relevant to the environment or substratum of interest), resting horizontally in the polycarbonate chamber, is inoculated aseptically with 1 ml of stationary phase culture and 10 ml of fresh media. The reactor cover is closed and bacteria are allowed to attach in a static environment for a 6- to 18-hr period. Longer attachment incubations will allow for better colonization of the coupon, resulting in cell replication and greater biofilm biomass. The entire reactor is then inclined at 10° and the nutrient flow (50 ml/hr) initiated. The medium drips onto the coupon at the raised edge, flows down lengthwise over the coupon, and then out an effluent port at the chamber base. Biofilms are grown for 24–72 hr, depending on the relative richness of the medium and required biofilm thickness.

Comparison of Planktonic Cell and Biofilm Cell H_2O_2 Resistance

Planktonic bacteria are grown overnight at room temperature and then diluted to the desired extent in the same medium. A sample is then diluted serially in phosphate-buffered saline, pH 7.0 (PBS), with appropriate dilutions spread onto R2A agar media (Difco, Franklin Lakes, NJ). The remaining cell suspension is treated with H_2O_2 at experimentally relevant concentrations (50 mM H_2O_2 in the experiments described here). After predetermined exposure periods, samples of the cell suspension are again diluted serially, except the dilution blanks contain 0.2% (w/v) sodium thiosulfate to neutralize the H_2O_2 . Aliquots of several dilutions are plated immediately on R2A agar medium. Colony-forming units (cfu) are enumerated after a 24- to 48-hr incubation at 37°, with the log₁₀ reduction in viability calculated as the comparison of final viable counts determined after H_2O_2 treatment versus initial viable cell counts of cells taken just prior to H_2O_2 exposure.

¹² R. M. Atlas, in "Handbook of Microbiological Media" (R. M. Atlas and L. C. Parks, ed.), 2nd ed., p. 1143. CRC Press, Ann Arbor, 1997.

¹³ C.-T. Huang, K. D. Xu, G. A. McFeeters, and P. S. Stewart, *Appl. Environ. Microbiol.* **64**, 1526 (1998).

Biofilms are treated by continuous flow of media containing H_2O_2 . The H_2O_2 concentration and exposure period are again varied relative to the planktonic culture treatment(s) and according to experimental interests. Following H_2O_2 treatment, biofilms on coupons are scraped into 50 ml of PBS containing 0.2% (w/v) sodium thiosulfate (to neutralize H_2O_2) and homogenized using a Brinkman homogenizer (Model PT 10/35, Brinkman Instruments, Westbury, NY). Homogenized biofilms are analyzed for viable bacteria by serial dilution and plating as for planktonic cultures. The suspended biofilms are also analyzed for total cell numbers by 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes, Inc., Eugene, OR) direct counts. The same assays are performed on untreated control biofilm coupons to verify the accuracy and precision of the direct count method. $\log_{10}$ reduction of viable biofilm bacteria is calculated based on the survival fractions defined as the ratio of colony forming units to direct microscopic counts with DAPI-stained cells. This approach factors out the detachment of cells occurring in biofilm experiments that is not a true measurement of disinfection. All experiments are repeated at least three times.

Measurement of Catalase and Superoxide Dismutase

Preparation of Cell Extracts

Following H_2O_2 or control treatments, planktonic cultures are collected by centrifugation at 5000g for 10 min at 4°, washed twice in ice-cold 50 mM potassium phosphate, pH 7.0 (KP_i), resuspended in 0.5 ml of the same buffer, and transferred to an Eppendorf tube for sonication. Biofilms are scraped and homogenized as described earlier. Bacteria are collected by centrifugation (5000g for 10 min at 4°), washed, suspended in 0.5 ml of ice-cold KP_i , and transferred to an Eppendorf tube for sonication as for planktonic cells. Cells are disrupted by sonication at an appropriate power setting (consult manufacturer guidelines) using a microtip. Thorough cell disruption is achieved by 5- to 30-sec sonications in an ice water bath. The sonicate is centrifuged at 13,000g for 10 min at 4°, and the supernatant is removed for protein estimates and enzyme assays. Protein concentration in the cell-free extracts is estimated by the method of Bradford¹⁴ using bovine serum albumin (BSA) fraction V (Sigma, St. Louis, MO) as standard.

Spectrophotometric Measurement of Superoxide Dismutase and Catalase

Catalase activity is monitored by following the decomposition of 18 mM H_2O_2 in KP_i at 240 nm at 25°. ^{6,9,15} Briefly, an appropriate amount of

¹⁴ M. M. Bradford, *Anal. Biochem.* **72**, 248 (1976).

¹⁵ R. F. Beers, Jr., and I. W. Sizer, *J. Biol. Chem.* **195**, 133 (1952).

cell-free extract is added to 3 ml of KP_i/H_2O_2 in a quartz cuvette, mixed thoroughly, and H_2O_2 decomposition followed for 1 min. Specific activity is calculated as in Eq. (1).

$$\text{Specific activity} = \frac{\Delta OD_{240}/\text{min} \times 3000}{43.6 (M \epsilon) \times \text{mg protein}} \quad (1)$$

Superoxide dismutase activity is monitored by following the autoxidation of pyrogallol at 320 nm,¹⁶ a modification of the original method described by Marklund and Marklund.¹⁷ This method is considerably less tedious and reagent intensive than the original SOD-inhibitable cytochrome c reduction assay described by McCord and Fridovich.⁵ A 10 mM solution of pyrogallol is prepared in 10 mM HCl. To a 1-ml quartz cuvette, add 1 ml of 50 mM Tris-HCl/1 mM EDTA (pH 8.2) that has been kept on a stir plate with constant stirring. Add 5 μ l of pyrogallol solution and mix thoroughly. Record the change in absorbance at 320 nm for 1 min. Adjust the volume of pyrogallol added until the change in OD_{320} is 0.02 ± 0.002 . The amount of cell-free extract that causes a 50% reduction in pyrogallol autoxidation (e.g., $\Delta OD_{320} = 0.01 \pm 0.001$) constitutes 1 U of activity. Specific activity is then calculated as U/mg protein.

Measuring Superoxide Dismutase and Catalase Activity in Native Gels

For detection of SOD activity in native gels, cell-free extracts (typically 40–80 μ g protein) are loaded onto 10% polyacrylamide minigels and separated by electrophoresis at 140 V (constant) at 4°. As soon as the bromophenol blue-tracking dye reaches the bottom of the gel, the gel is removed and submerged in a small tray containing 80 ml of 50 mM potassium phosphate (pH 7.8), 0.1 mM EDTA, 2 mg riboflavin, and 16 mg nitro blue tetrazolium (NBT). Finally, 400 μ l of TEMED is added to initiate NBT reduction and the gel is incubated in the dark for 30–45 min at 37°. The gel is removed, placed on a sheet of Saran wrap, and laid on a fluorescent light box. Within a few minutes, the gel turns purple, indicative of O_2 catalyzed reduction of NBT, whereas SOD activity bands are white. Once the color has developed sufficiently, the gel is wrapped in Saran wrap surrounded by aluminum foil to prevent further light-catalyzed NBT reduction and is photographed or scanned as desired.

For catalase activity gel staining, 5% polyacrylamide gels are used because of the larger size of the *P. aeruginosa* catalases (170 and 228 kDa relative to SODs (42 and 44 kDa). Before loading the samples, add 0.1

¹⁶ J. R. Prohaska, *J. Nutr.* **113**, 2048 (1983).

¹⁷ S. Marklund and G. Marklund, *Eur. J. Biochem.* **47**, 469 (1974).

mg/ml sodium thioglycolate to the top buffer and prerun the gel at 20 mA constant current for 30 min at 4°. Thioglycolate prevents APS-catalyzed radical formation that can cause catalase activity banding artifacts. Because *P. aeruginosa* possesses such high catalase activity (~500–4000 U/mg^{6,7}), load only 10–20 µg of cell-free extract per lane. As little as 5 µg can be used from organisms pretreated with H₂O₂. Load 50–100 µg of cell-free extract if working with organisms that produce significantly less catalase activity (e.g., *Escherichia coli*, 8–10 U/mg). Apply a constant current of 10–20 mA until the bromphenol blue reaches the bottom of the gel. At this point, the gel is ready to be removed from the casings. Use extreme care in dislodging the gel from the glass casings as 5% polyacrylamide gels are extremely fragile. After the top gel plate is removed, the gel and the bottom plate are placed in a small tray of distilled water. Using a flat metal spatula, carefully insert the spatula between the gel and the plate in short rapid motions. Slowly, the gel will become dislodged from the bottom plate. The water in the tray is removed by aspiration and replaced with 100 ml of distilled water containing 4 mM H₂O₂. The gel is incubated at room temperature for 10 min, the H₂O₂ solution is removed by aspiration, and the gel is washed with 100 ml of distilled water. The gel is then soaked in a solution containing 1% (w/v) ferric chloride and 1% (w/v) potassium ferricyanide. As soon as the gel turns a dark green, the ferric chloride/potassium ferricyanide solution should be removed and the gel rinsed with a light stream of distilled water to prevent overdevelopment. Finally, once all the dye is removed, the gel can be photographed immediately or, if necessary, stored in a tray of distilled water for a few days at 4° prior to photography.

Experimental Examples and Comments

As discussed earlier, one of the important features of biofilms is their innate resistance to biocides. The results depicted in Fig. 1 illustrate a typical response of biofilm *P. aeruginosa* to H₂O₂; H₂O₂ is significantly less effective at killing biofilm bacteria than highly vulnerable planktonic cells (Fig. 1A). In these experiments, planktonic bacteria were exposed to a single dose of 50 mM H₂O₂ for 60 min, while biofilms were exposed to a constant stream of 50 mM H₂O₂ for 60 min. Even after prolonged exposure, biofilm cell viability is high, assuring biofilm survival and regrowth subsequent to biocide treatment. These results suggest that biofilm bacteria are well equipped to resist H₂O₂.

As mentioned earlier, *P. aeruginosa* has two major catalases, KatA and KatB, that are expressed differentially, depending on exposure to oxidative stress, and two SODs, Fe-SOD and Mn-SOD. To illustrate the application

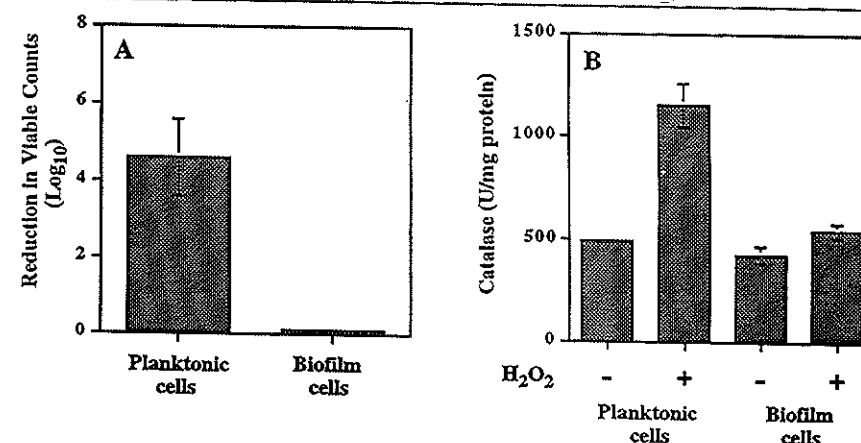


FIG. 1. (A) Sensitivity of planktonic cells and biofilm cells of *P. aeruginosa* wild-type strain PAO1 to H₂O₂. Data are shown as the log₁₀ reduction of viable cells after exposure to 50 mM H₂O₂ for 1 hr. (B) Catalase-specific activity of planktonic and biofilm cells without (-) and with (+) H₂O₂ treatment. Both cell types were grown in PBM prior to H₂O₂ treatment.

of the previous methods in the detection and measurement of these different enzymes, experiments were performed with planktonic and biofilm bacteria. As measured with spectrophotometric enzyme assays, overall total catalase levels in planktonic cells grown in PBM medium are maintained at approximately 500 U/mg, but increase significantly after only a brief exposure to an external oxidizing agent such as H₂O₂ (Fig. 1B). Under these conditions, catalase activity in biofilm bacteria treated with H₂O₂ is only about half of that observed with planktonic cells, inducing poorly in response to H₂O₂ treatment. Therefore, catalase activity per se may not explain the increased resistance of biofilm bacteria to H₂O₂.

As can be seen in Fig. 2, native gel analysis can be used to show that KatA is expressed constitutively in both cell types, serving to detoxify H₂O₂ synthesized during normal aerobic metabolism, and upregulated weakly in cells exposed to H₂O₂ (Fig. 2). Small amounts of KatB are seen in stationary phase planktonic cells, but the complement of this isozyme increases dramatically after treatment with H₂O₂. KatB is rarely observed in untreated *P. aeruginosa* biofilms and its induction in response to H₂O₂ is significantly less than with planktonic cells (Fig. 2).

Superoxide dismutase expression in *P. aeruginosa* is at least in part governed by iron availability.^{3,4,9-11} When iron is not limiting, only Fe-SOD is expressed, but under conditions of iron starvation, Mn-SOD is induced. This type of regulation is not coincidental as Fe-SOD requires iron for its active site, as Mn-SOD requires manganese. Therefore, *P. aeruginosa* has

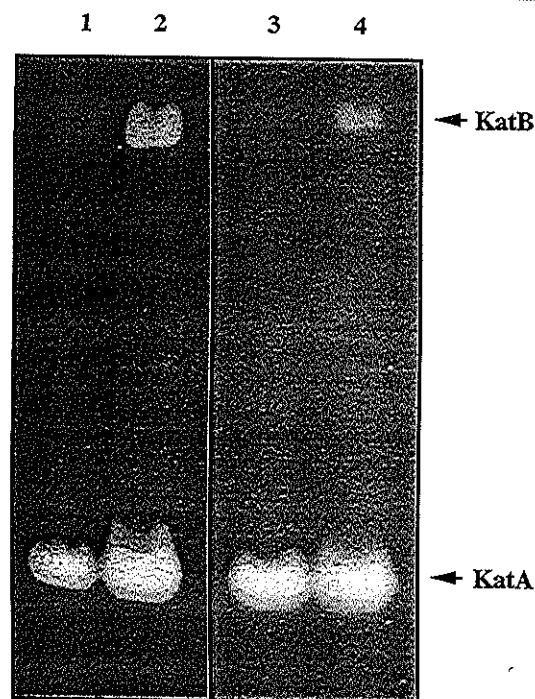


FIG. 2. Native gel catalase activity staining of cell-free extracts of *P. aeruginosa* wild-type strain PAO1 before and after treatment with H₂O₂; 15 μ g protein loaded per lane. (Left panel) Extracts from a 24-hr batch planktonic culture: Lane 1, no H₂O₂ treatment; and lane 2, six doses of 1 mM H₂O₂ every 10 min for 1 hr. (Right panel) Extracts from 72-hr biofilms: Lane 3, no H₂O₂ treatment; and lane 4 after constant exposure to 50 mM H₂O₂ for 1 hr. Gels were scanned using a Hewlett-Packard ScanJet IIcx, composed with Adobe Photoshop 3.0 for Macintosh, and printed using an Epson Stylus Color 600 printer.

evolved a mechanism to guarantee synthesis of SOD and thus protection against ROIs, regardless of micronutrient availability. An analysis of SOD activity in the different cell types grown in TSB medium reveals that SOD expression appears dissimilar. In planktonic cells, Fe-SOD is expressed constitutively, whereas Mn-SOD activity is induced only after exposure to the hydrophobic iron-specific chelator 2,2'-dipyridyl (Fig. 3). In contrast, biofilm bacteria express both SODs without the addition of an iron chelator (Fig. 3), suggesting either that biofilm cells have an iron requirement in excess of planktonic cells, which is met by the iron in the medium used, or that the *fatA-fumC-orfX-sodA* operon is subject to other regulatory mechanisms that are imposed during the biofilm mode of growth. This is

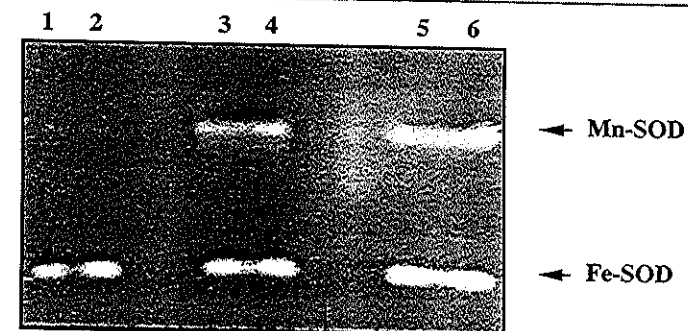


FIG. 3. Native gel Mn- and Fe-SOD activity stains of protein extracts of *P. aeruginosa* wild-type strain PAO1. All results are shown in duplicate, with 60 μ g protein loaded per lane. Lanes 1 and 2: protein extracts from planktonic cultures grown in 1:10 TSB without the iron-specific chelator 2,2'-dipyridyl; lanes 3 and 4: protein extracts from planktonic cultures grown in 1:10 TSB with 500 μ M 2,2'-dipyridyl; and lanes 5 and 6: protein extracts from biofilm cells grown in 1:100 TSB without 2,2'-dipyridyl. Gels were scanned using a Hewlett-Packard ScanJet IIcx, composed with Adobe Photoshop 3.0 for Macintosh, and printed using an Epson Stylus Color 600 printer.

one of few examples that demonstrate physiological differences between biofilms and their planktonic counterparts.

Summary

The biofilm mode of bacterial growth may be the preferred form of existence in nature. Because of the global impact of problematic biofilms, study of the mechanisms affording resistance to various biocides is of dire importance. Furthermore, understanding the physiological differences between biofilm and planktonic organisms ranks particularly high on the list of important and necessary research. Such contributions will only serve to broaden our knowledge base, especially regarding the development of better antimicrobials while also fine-tuning the use of current highly effective antimicrobials. Using H₂O₂ as a model oxidizing biocide, we demonstrate the marked resistance of biofilm bacteria relative to planktonic cells. Because many biocides are good oxidizing agents (e.g., H₂O₂, HOCl), understanding the mechanisms by which genes involved in combating oxidative stress are activated is important in determining the overall efficacy of such biocides. Future studies will focus on determining mechanisms of oxidative stress gene regulation in bacterial biofilms.

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