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Effect of Catalase on Hydrogen Peroxide Penetration into *Pseudomonas aeruginosa* Biofilms

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The penetration of hydrogen peroxide into biofilms formed by wild-type and catalase-deficient *Pseudomonas aeruginosa* strains was measured using microelectrodes. A flowing stream of hydrogen peroxide (50 mM, 1 h) was unable to penetrate or kill wild-type biofilms but did penetrate and partially kill biofilms formed by an isogenic strain in which the *katA* gene was knocked out. Catalase protects aggregated bacteria by preventing full penetration of hydrogen peroxide into the biofilm.

Bacteria in biofilms are protected from killing by antimicrobial agents (1, 4, 7). One mechanism of reduced biofilm susceptibility is failure of the antimicrobial agent to penetrate the biofilm fully. For example, direct measurements of penetration of hypochlorite (HOC1) (3, 5, 15) and hydrogen peroxide (H_2O_2) (9) into model biofilms have revealed significantly retarded or incomplete penetration of both antimicrobials.

There does not appear to be a generic barrier to antimicrobial mobility within biofilms. The effective diffusion coefficients of solutes the size of HOC1 and H_2O_2 within biofilms are about half their respective values in pure water (13). Biofilm penetration failure likely depends instead upon a neutralizing reaction between the antimicrobial and some constituent of the biofilm. The antimicrobial agent is reactively neutralized in the surface layers of the biofilm faster than it can diffuse into the biofilm interior (12, 14).

H_2O_2 in conjunction with a matched pair of bacterial strains that either carry or lack catalases, enzymes that disproportionate and neutralize H_2O_2, forms a convenient model system to investigate the role of reaction-diffusion interactions in mediating reduced biofilm susceptibility. In a previous article, the protective role of *Pseudomonas aeruginosa* catalases was described but the mechanism of protection was not defined (6). The purpose of the work reported here was to investigate the role of catalases in preventing effective penetration of H_2O_2 into biofilms of *P. aeruginosa*. We hypothesized that H_2O_2 would not fully penetrate catalase-positive biofilms and these would resist killing, whereas catalase-negative biofilms would be penetrated and would be susceptible to H_2O_2-mediated killing.

Experiments were performed using pure cultures of wild-type *P. aeruginosa* PAO1 and isogenic *katA* (10), *katB* (2), and *katA katB* (8a) mutants. Each mutant was generated via insertional interruption of the *kat* genes with gentamicin and/or tetracycline resistance cassettes and double crossover events evoked by sucrose counterselection.

Biofilms were grown in continuous flow reactors on a glucose minimal medium for 72 h at room temperature (25°C) as described elsewhere (6, 8). Antibiotic selection was not maintained during biofilm growth, as the mutants are stable. To measure H_2O_2 penetration, a biofilm-covered stainless steel slide was removed from the growth reactor and transferred to an open-channel rectangular conduit designed for microelectrode access (9). A stainless steel mesh with a 1.5-mm grid was laid on top of the biofilm to prevent sloughing of biomass. A gentle flow of medium was initiated at a mean fluid velocity of approximately 0.8 cm s^{-1}. Two amperometric microelectrodes sensitive to H_2O_2 (9) were positioned in the system. The tip of one microelectrode was set approximately 2 mm above the biofilm in the bulk flow, and the tip of the second electrode was positioned near the base of the biofilm approximately 10 μm from the substratum. To initiate a penetration experiment, the fluid flow was changed from growth medium to 50 mM H_2O_2 in the same medium. The extent of penetration was quantified by reporting the concentration measured at the base of the biofilm after 20 or 60 min divided by the applied bulk fluid concentration.

Biofilm susceptibility was measured by exposing biofilms to 50 mM H_2O_2 in the same reactors in which they were grown by simply switching the flow from minimal medium to medium containing 50 mM H_2O_2. After 1 h, biofilms were scraped from slides into 50 ml of phosphate buffer containing 0.1% sodium thiosulfate as a neutralizer. The suspension was homogenized, serially diluted, and plated on R2A agar to enumerate surviving bacteria (8). Killing was reported as the log reduction in viable cell counts.

Killing of planktonic bacteria by 50 mM H_2O_2 was measured in bacterial suspensions with an initial cell density of 10^7 CFU ml^{-1}. This cell density was low enough that the H_2O_2 concentration was maintained throughout the 1-h treatment period. Residual H_2O_2 was neutralized with sodium thiosulfate, and surviving microorganisms were enumerated by plating on R2A agar.

Means of data groups (normalized hydrogen peroxide concentration at the base of the biofilm or log reduction in viable counts) were compared for statistical significance by using a two-sample, two-sided *t* test assuming unequal variances. Because of the noise inherent in the penetration measurements,

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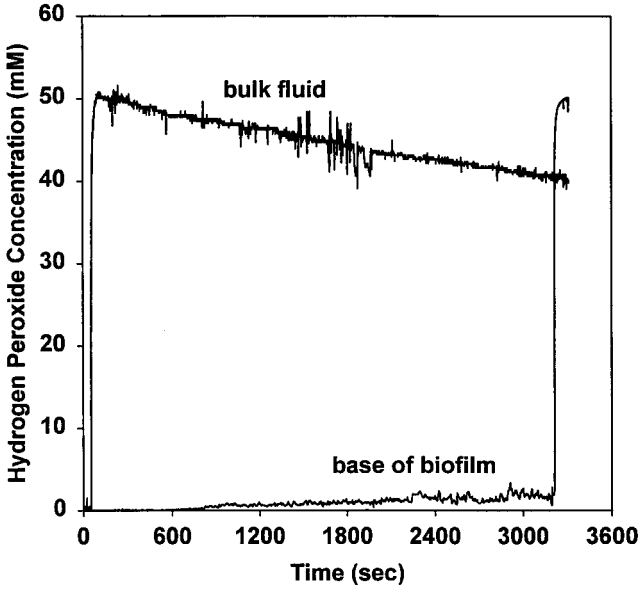

FIG. 1. Failure of H_2O_2 to penetrate a wild-type *P. aeruginosa* biofilm. The microelectrode corresponding to the data set designated base of biofilm was withdrawn from the biofilm into the bulk fluid after approximately 3,200 s. The spike in the signal at this time indicated that the electrode was still sensitive to H_2O_2.

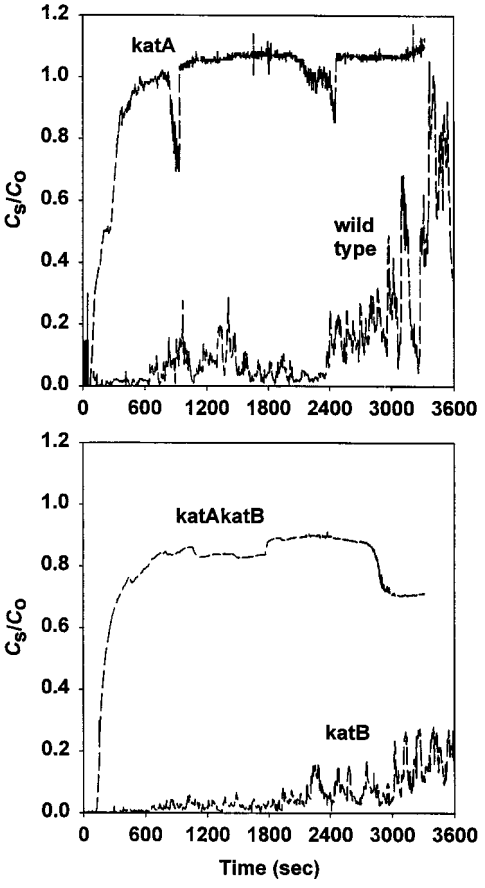

FIG. 2. Comparison of H_2O_2 penetration into wild-type and catalase mutant *P. aeruginosa* biofilms. The ratio plotted on the *y* axis, C_S/C_o, is the concentration of hydrogen peroxide measured within approximately 10 μm of the substratum divided by the concentration of hydrogen peroxide in the bulk fluid.

normalized hydrogen peroxide concentration data at 20 and 60 min were grouped for the purposes of statistical comparisons.

H_2O_2 failed to penetrate to the bottom of biofilms formed by wild-type *P. aeruginosa*, even when they were exposed to a continuously flowing solution of 50 mM H_2O_2 for 1 h (Fig. 1). These biofilms and those formed by the catalase mutant strains were approximately 100 μm thick. The concentration of H_2O_2 at the base of wild-type biofilm was only a small fraction of the bulk fluid concentration during the exposure period (Table 1), and this ratio was not statistically significantly different from zero ($P = 0.19$).

H_2O_2 was able to penetrate *katB*, *katA*, and *katA katB* mutant biofilms to respectively increasing degrees (Fig. 2). Biofilms formed by the *katB* mutant were poorly penetrated by H_2O_2. The extent of penetration in the *katB* mutant biofilm was not statistically significantly different from that in the wild-type biofilm ($P = 0.39$). H_2O_2 penetrated the *katA* mutant biofilm (Fig. 2), and the extent of penetration was significantly higher than that measured for the wild-type biofilm ($P = 0.012$). Biofilms formed by the *katA katB* strain were readily penetrated by H_2O_2 (Fig. 2). The H_2O_2 concentration at the base of the double mutant biofilm attained 90% of the bulk fluid concentration within 20 minutes (Fig. 2; Table 1). Penetration of H_2O_2 into the double mutant biofilm was significantly greater than that for the wild-type biofilm ($P = 0.001$).

Copious effervescence was noted during treatment of wild-type and *katB* biofilms. The noise evident in measuring penetration in these two biofilms may have been due to oxygen bubbles clinging transiently to the microelectrode tip. Gas bubbles were not evolved during the treatment of *katA* and *katA katB* biofilms.

Biofilms of all strains resisted killing by H_2O_2 compared to planktonic cells (Table 1). For example, wild-type planktonic cells exposed to 50 mM H_2O_2 experienced a 4.6-log-unit reduction in viable cell numbers, while the same treatment of

TABLE 1. Hydrogen peroxide penetration and killing of wild-type and catalase mutant *P. aeruginosa* biofilms[a]

Strain	Biofilm thickness (μm)	C_S/C_o[b] at:		Biofilm LR[c] at:		Planktonic LR at 60 min
		20 min	60 min	20 min	60 min	
PAO1	130 ± 20	0.04 ± 0.03	0.24 ± 0.23	0.31 ± 0.10	0.26 ± 0.15	4.6 ± 1.0
katA mutant	90 ± 10	0.61 ± 0.24	0.83 ± 0.17	1.1 ± 0.2	2.6 ± 0.3	6.4 ± 0.1
katB mutant	170 ± 20	0.15 ± 0.12	0.50 ± 0.24	0.01 ± 0.08	0.13 ± 0.10	5.5 ± 0.5
katA katB mutant	130 ± 20	0.9 ± 0.05	0.92 ± 0.08	0.8 ± 0.4	1.9 ± 0.2	ND[d]

[a] All values are means and standard errors of the means for three or four replicates.
[b] C_S/C_o, concentration of hydrogen peroxide measured within approximately 10 μm of the substratum divided by concentration of hydrogen peroxide in the bulk fluid.
[c] LR, log reduction in viable microorganisms.
[d] ND, not determined.

biofilm yielded only a 0.26-log-unit reduction. This difference was statistically significant ($P = 0.048$). Biofilms formed by the *katA* and *katA katB* strains were more susceptible to 1 h of exposure to H_2O_2 than were wild-type biofilms (Table 1), and these differences were statistically significant ($P = 0.022$ and 0.005, respectively), while *katB* biofilms were equally resistant ($P = 0.54$). Biofilms formed by the *katA* mutant were, however, significantly less susceptible to H_2O_2 than were planktonic cells of this strain ($P = 0.009$).

The major housekeeping catalase KatA is important in the protection of *P. aeruginosa* biofilms against killing by H_2O_2. Biofilms formed by KatA-positive strains were incompletely penetrated by 50 mM H_2O_2 and suffered scarcely any loss in viability. Biofilms formed by the *katA* mutant were penetrated by H_2O_2 and were partially killed. Interestingly, even the *katA* mutant, whose biofilms were fully penetrated by H_2O_2, was significantly less susceptible in the biofilm than planktonic cells of the same strain. This indicates that some protective mechanism other than incomplete penetration is operative in *P. aeruginosa* biofilms treated with H_2O_2. KatB was not essential for protection of *P. aeruginosa* biofilms under the conditions of our experiments. KatB is expressed only when bacteria have been subjected to prior exposure to H_2O_2 or paraquat (2, 6). However, KatB could likely contribute to the protection of biofilms against H_2O_2 if they were challenged during growth with a suitable inducing agent.

These results show that when bacteria aggregate in the form of a biofilm, catalases are extremely effective in protecting bacteria from damage by H_2O_2, a conclusion that reinforces the oft-cited work of Ma and Eaton (11). Our measurements further demonstrate that the mechanism of this protection can be largely attributed to failure of H_2O_2 to fully penetrate the biofilm due to a reaction-diffusion interaction.

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