

Resistance of Biofilms to the Catalase Inhibitor 3-Amino-1,2,4-triazole

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Abstract: Consortia of catalase positive bacteria consisting of *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Klebsiella pneumoniae*, in both the planktonic form and as biofilms, disproportionate hydrogen peroxide into oxygen and water. The biofilm, however, continued to disproportionate the hydrogen peroxide in the presence of the catalase inhibitor, 3-amino-1,2,4-triazole, while the planktonic organisms did not. While the bacterial catalase–peroxidase–dismutase system was probably responsible for the disproportionation of hydrogen peroxide in both cases, biofilms resisted inhibition of this enzyme system. © 1998 John Wiley & Sons, Inc. *Biotechnol Bioeng* 59: 156–162, 1998.

Keywords: *Pseudomonas aeruginosa*; *Pseudomonas fluorescens*; *Klebsiella pneumoniae*; 3-amino-1,2,4-triazole; catalase; hydrogen peroxide

INTRODUCTION

One of the unique and puzzling features of biofilms is their unusually high resistance to antimicrobial agents (Costerton et al., 1987; Nichols, 1989; Brown and Gilbert, 1993). Although susceptibility of planktonic bacteria to biocides and antibiotics is well documented, these same microorganisms, when present as biofilms, require the same antimicrobial agents in concentrations hundreds or even thousands of times greater to kill them or modify their metabolic states. For example, 8 h of incubation with 50 µg/mL of tobramycin (an aminoglycoside antibiotic) was lethal to a planktonic culture of *Pseudomonas aeruginosa*. However, biofilms of *P. aeruginosa*, even after 12 h of contact with 1000 µg/mL of tobramycin, were still active (Nickel et al., 1985). In another example, it was found that biofilm bacteria were 150 to more than 3,000 times more resistant to free chlorine and 2 to 100 times more resistant to monochloramine than their planktonic counterparts (LeChevallier et al., 1988a).

This behavior is exhibited by biofilms in the presence of a variety of antimicrobial agents. It occurs with oxidizing biocides such as chlorine (LeChevallier et al., 1988a,b; De-

Beer et al., 1994), monochloramine (LeChevallier et al., 1988a,b; Chen et al., 1993a,b), various iodinated compounds (Brown and Grauthier, 1993; Pyle and McFeters 1990; Carson et al., 1978), and hydrogen peroxide (H₂O₂) (Wilson et al., 1990; Vincent et al., 1989). It also occurs with antibiotics (Amabile-Cuevas et al., 1995; Nickel et al., 1985; Morck et al., 1994).

Various hypotheses have been proposed to account for the resistance of sessile bacterial populations to antimicrobial agents. The behavior is so ubiquitous that specific targeted mechanisms are unlikely. One hypothesis invokes a reaction-diffusion model (Costerton et al., 1987; Hoyle and Costerton, 1991; Stewart, 1994; Stewart and Raqepas, 1995). It implies that antimicrobial agents are consumed, or deactivated, by intercellular polysaccharides or cellular constituents. This includes the possibility of being strongly adsorbed to biofilm components. In addition, reaction is retarded as the agents meet diffusional resistance while penetrating the biofilm.

Another hypothesis suggests that since growth rates in biofilm populations are lower than those seen in planktonic organisms, the reaction rates with antimicrobial agents will be lower as well (Brown et al., 1988; Gilbert et al., 1990; Stewart, 1994). Consequently, exposure times for comparable kills by these agents would be longer.

In these hypotheses there is no active, specific metabolic defense mechanism operating. Although they may explain resistance behavior to some antimicrobial agents, by no means do they cover all possibilities.

This study describes the resistance of a biofilm consortium of catalase-(+) bacteria to a specific catalase inhibitor, 3-amino-1,2,4-triazole (AT), in the presence of hydrogen peroxide (HP), and compares it to the behavior of the planktonic organisms under the same conditions. The literature is replete with examples where AT inhibits the activity of catalase in bacteria, and in so doing, reduces the cell's ability to protect itself against HP. The characteristics of AT, namely that it is a small, easily diffusible molecule that targets the catalase enzyme, will be used as a probe to understand the contrasting physiologies of biofilm and planktonic organisms.

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MATERIALS AND METHODS

Biofilm Development

The microorganisms used in the experiments were *P. aeruginosa*, *Pseudomonas fluorescens*, and *Klebsiella pneumoniae*, which were taken from the culture collection at the Center for Biofilm Engineering at Montana State University. In our study, 1-mL portions of stock cultures of *P. aeruginosa* (7.7×10^9 CFU/mL), *P. fluorescens* (4.8×10^{10} CFU/mL), and *K. pneumoniae* (7.2×10^{10} CFU/mL) were used to inoculate a reactor. A modified Scheusner's mineral salts nutrient solution (1.7 mM K_2HPO_4 ; 2.2 mM KH_2PO_4 ; 7.6 mM $(NH_4)_2SO_4$; 0.040 mM $MgSO_4 \cdot 7H_2O$; 0.22 mM glucose; 15 μ g/L yeast extracts) was used to grow biofilms. Aerobic biofilms were grown on a flat plate reactor. Figure 1 shows the experimental setup. The reactor was operated at a dilution rate of 3.2 h^{-1} . This dilution rate greatly exceeded the growth of planktonic cells in the reactor ($<0.15 \text{ h}^{-1}$) and thus ensured that the activity of biofilm microorganisms dominated that of suspended cells in the system.

HP and Dissolved Oxygen Microelectrodes

A glass-covered platinum wire was used as a hydrogen peroxide probe. The tip (10–20 μ m) of the electrode was coated with cellulose acetate membrane (5%, Sigma Chemical). The HP working solution used was at a concentration of 0.3%, prepared by using 30% HP (EM Science). This 30% solution was standardized by titrating with 0.1 N $KMnO_4$ (Fisher Scientific). When a potential of +0.8 volts is applied between the HP microelectrode and the SCE reference electrode, HP is oxidized to oxygen. This creates a

current that is proportional to the HP concentration in the solution surrounding the tip of the probe.

The dissolved oxygen microelectrode was constructed as described by Jorgensen and Revsbech (1983). When a potential of –0.8 volts is applied between the platinum cathode and the Ag/AgCl reference electrode, oxygen is reduced on the gold tip of the cathode. This creates a current that is proportional to the oxygen concentration in the solution surrounding the tip of the probe.

Measurement of HP and Dissolved Oxygen Concentration Profiles

The amperometric method was used to measure the HP and dissolved oxygen concentrations in the liquid and/or biofilms. A picoammeter/DC voltage source (Hewlett Packard) was used to apply a proper potential in the solution (+0.8 volts for HP probe, and –0.8 volts for dissolved oxygen electrode). A micromanipulator (World Precision Instruments) was used to move the microelectrodes. It was equipped with a stepper motor (Oriol) and manipulated by a computer controller (Oriol). The measured signal was directed to a computer containing a data acquisition system. HP and dissolved oxygen concentration profiles were measured using the above data-collection system in the described biofilm system. By setting up the steps, delay times, and collecting times, the DO and HP concentration profiles were collected either without HP treatment or at different times while being treated with hydrogen peroxide in the system.

Inhibition of Catalase Activity in Biofilm and Batch Cultures

To examine the catalase activity in the biofilm and in the suspended bacteria system, we used a specific catalase inhibitor, 3-amino-1,2,4-triazole (Margoliash et al., 1960; Heim et al., 1955) (Aldrich Chemical Co.), and pretreated either biofilms or planktonic bacteria. For the biofilm experiments we introduced a specific concentration of the inhibitor into the reactor and incubated for 1 h. Then we rapidly pumped 0.3% HP to the system. For the batch culture, we added the inhibitor to the cultures to reach a specific concentration of the inhibitor and mixed for 1 h. This was followed by mixing with the HP stock solution to reach a final working concentration of 0.3%. The dissolved oxygen profiles were collected again using the same data collection system as mentioned above to see the inhibitory effects on either the biofilms or batch cultures.

Catalase Activity Assay and the Effect of Aminotriazole on Catalase

Planktonic cells and biofilm samples were collected in 250-mL centrifuge bottles. These bottles were centrifuged in a RC5C centrifuge (Sorvall Instruments) for 20 min at 10,000 rpm at 4°C. The pellets were then resuspended using 10 mL

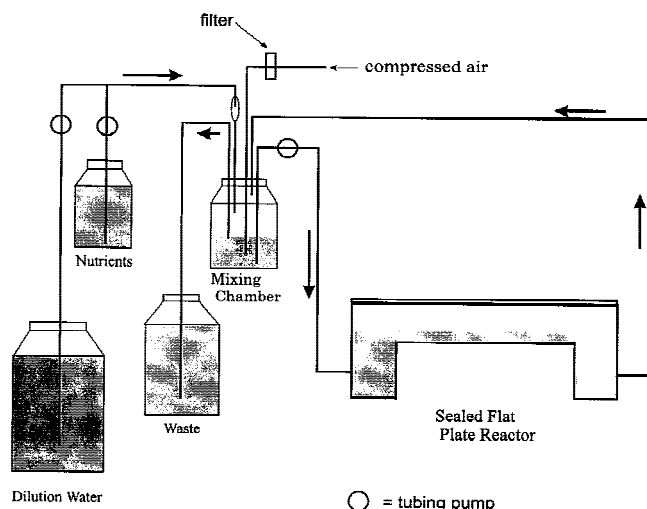


Figure 1. Experimental setup: Nutrients mixed with dilution water are pumped into the mixing chamber. The solution from the mixing chamber is rapidly recycled through the flat plate reactor. A waste effluent equal in flow to the nutrient mixture input is pumped from the mixing chamber to maintain constant volume.

of 0.1 M phosphate buffer in plastic test tubes. The cell suspensions were sonicated using a probe sonicator for 1 min on ice. After sonication, the samples were centrifuged again at 10,000 rpm for 20 min. The supernatants were collected and assayed for catalase activity and total protein. Catalase activity was determined according to Beers and Sizer (1952). Protein in the sample was determined by the method of Lowry et al. (1951) using a Lowry protein assay kit (Sigma Chemicals). The specific catalase activity was expressed as μmol of HP consumed/min/mg of protein.

RESULTS

HP Concentration Profiles in Biofilms Exposed to HP

Two control experiments were performed prior to measuring HP concentration profiles in the biofilms. First, the HP concentration in the biofilm in the absence of externally applied HP was determined. A flat response was observed, which indicated that no HP was produced spontaneously from the biofilm. In the second control experiment, HP concentration profiles were measured in a biofilm-free flat plate reactor with HP flowing through. In this experiment a flat curve was also observed, indicating that neither the HP solution itself nor the hydrodynamic characteristics of the reactor perturbed HP measurement by the microelectrode.

The experiments were performed using the HP microelectrode to measure transient HP concentration in the biofilm. A 0.3% HP (88.8 mM) solution was used. Several locations in biofilms of differing thickness were examined for HP penetration. For the biofilm in Fig. 2 the thickness

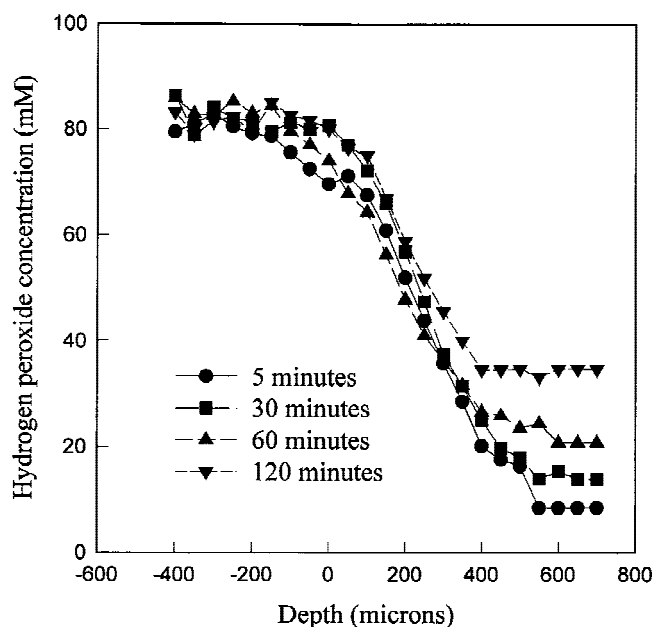


Figure 2. HP concentration profiles in biofilms, with 0.3% HP treatment. Negative numbers on the x-axis represent the bulk fluid, and zero corresponds the interface of bulk fluid and biofilm.

was approximately 700- μm thick. The HP concentration profiles were measured at 5, 30, 60, and 120 min into the experiments. Only after 120 min did the concentration of HP at the substratum reach 50% of the applied concentration. Penetration of HP into the biofilm, therefore, was a very slow process or else it was rapidly consumed by biofilm components.

Dissolved Oxygen Concentration Profiles in Biofilms Exposed to HP

Before and after taking the experimental dissolved oxygen concentration profiles, the dissolved oxygen probe was calibrated in air or medical oxygen saturated water and nitrogen saturated water. Interestingly, when HP was introduced into the biofilm system oxygen was produced. Figure 3 shows an S-shaped dissolved oxygen concentration profile typical of biofilms consuming oxygen in the absence of HP. However, when HP was introduced into the system, dissolved oxygen (DO) in the biofilm increased relative to controls (Fig. 4a). At the end of the experiment, commercial bleach (20%; Clorox Company) was used for about 4 to 5 h to kill the biofilm but not remove it. DO profiles, measured again in the presence of HP, resulted in flat, zero response curves, indicating that viable biofilms were required for oxygen generation. These experimental curves suggested that HP was degraded while oxygen was produced.

Inhibition of Catalase Activity in Biofilm and Planktonic Cultures

Catalase, a ubiquitous enzyme in prokaryote and eukaryote cells, disproportionates HP into water and oxygen. Since the three species of bacteria that were used are catalase positive,

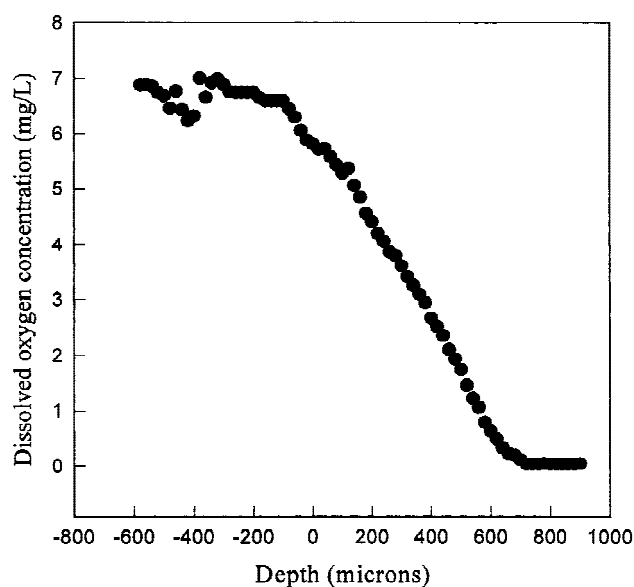


Figure 3. Dissolved oxygen concentration profiles, without HP treatment. Negative numbers on the x-axis represent the bulk fluid, and zero corresponds the interface of bulk fluid and biofilm.

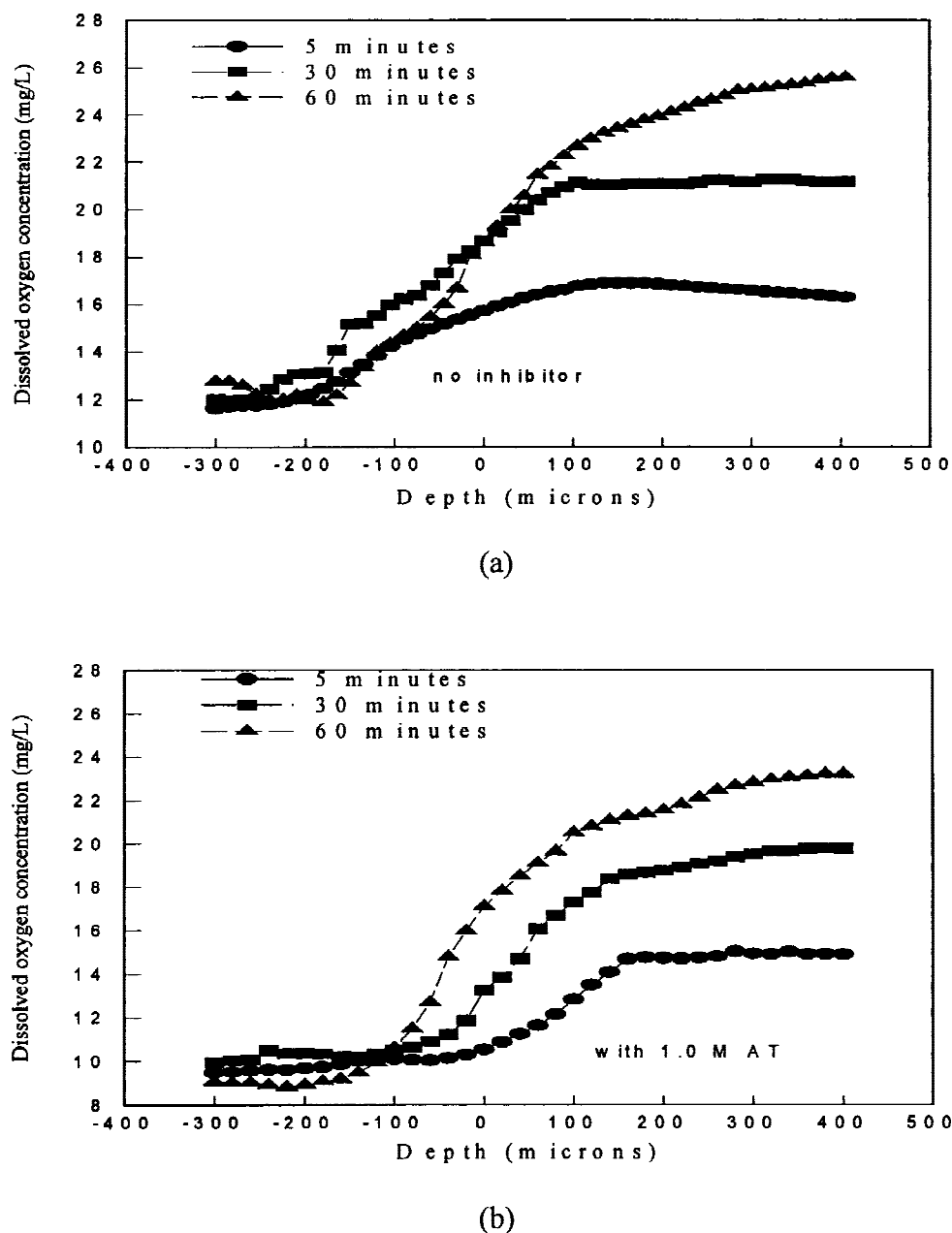


Figure 4. Dissolved oxygen concentration profiles in biofilm exposed to 0.3% HP, (a) with 1.0 M AT present and (b) without AT. Negative numbers on the x-axis represent the bulk fluid, and zero corresponds the interface of bulk fluid and biofilm.

the observed increase in oxygen may have resulted from the activity of bacterial catalase. To test this hypothesis, biofilms were pretreated with a specific catalase inhibitor, 3-amino-1,2,4-triazole (AT), for 1 h. This was followed by introduction of HP into the system. DO concentration profiles were measured and compared to oxygen profiles in an inhibitor-free, HP-treated biofilm of similar thickness. There were no significant differences between DO profiles for biofilms exposed to inhibitor concentrations of from 50 to 1,000 mM and profiles in an inhibitor-free system. Figure 4b show the case where biofilm was pretreated with 1,000 mM inhibitor. Compare this to Fig. 4a where no AT was present.

In another experiment using the three species (*P. aeruginosa*, *P. fluorescens*, and *K. pneumoniae*) in a planktonic batch culture, 3-amino-1,2,4-triazole, even at low concentrations, had a distinct dose-dependent inhibitory effect on the catalase activity of the suspended bacteria (Fig. 5). This was in marked contrast to the results obtained with biofilms.

Inhibition of Catalase Activity in Cell Extracts of Biofilm and Planktonic Cultures

The catalase and total protein assays were conducted on biofilm and planktonic organism extracts exposed to varying amounts of the catalase inhibitor, 3-amino-1,2,4-triazole

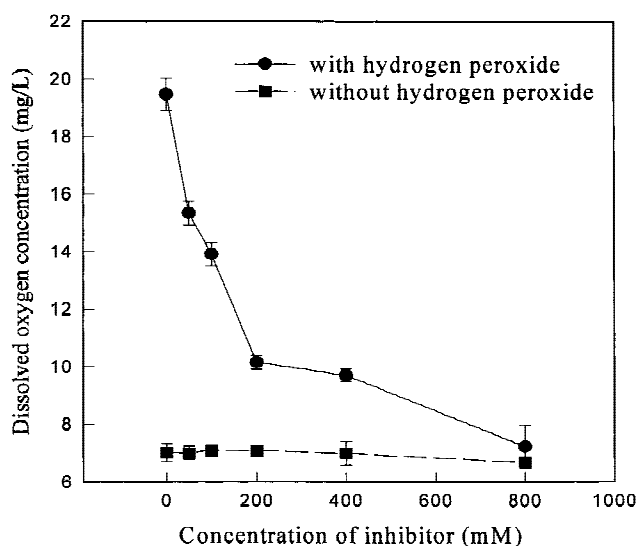


Figure 5. Inhibitory effect of AT on a planktonic three species culture. This was indicated by the change in concentration of dissolved oxygen in solution (error bars indicate standard errors for replicates of three experiments).

(AT). The supernatant samples were pretreated with AT for 1 h and then mixed with hydrogen peroxide where the change in concentration of hydrogen peroxide was monitored again to see if there was any inhibitory effect of aminotriazole on the activity of isolated catalase. Results are shown in Fig. 6.

DISCUSSION

In this study, catalase positive biofilms were shown to degrade HP and produce oxygen. A specific inhibitor of catalase activity, 3-amino-1,2,4-triazole (AT), did not inhibit HP degradation in biofilms, even though it was effective when applied to planktonic cell cultures.

During exposure of biofilms to HP, what was first perceived as penetration resistance was later attributed to a rapid consumption of HP. This penetration resistance in biofilms was characterized by a slow increase in HP concentration inside the biofilm (Fig. 2). This was unexpected considering that Van Stroe-Biezen et al. (1993) reported rapid diffusion of HP in a polymer matrix. For a biofilm approximately 600–700- μm thick, 2 h of exposure were required to reach 50% of ambient concentration at the substratum (Fig. 2). Assuming no reaction between HP and biofilms, the diffusion half-life of HP can be calculated from its Fourier number ($F_0 = D_{\text{HP}}t_{1/2}/r^2$), where F_0 is 2 (DeBeer et al., 1994; Perry and Chilton, 1973). The diffusion coefficient of HP (D_{HP}) in phosphate buffer solution is $1.43 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$ (25°C) (Van Stroe-Biezen et al., 1993). The path length (r) in our experiments is around 600–700 μm . Thus the ($t_{1/2}$) of diffusion should be about 8 to 11 min, less than 1/10 the observed penetration time. These results indicated that in this kind of multicolony biofilm, the effective penetration of HP was significantly retarded. Previous

study of chlorine penetration (Chen and Stewart, 1996) showed that chlorine penetrates relatively quickly (773- μm thickness, <30 min) into an artificial film consisting of agarose gel alone. However, when *P. aeruginosa* (1.44 g/L cell mass, 526- μm thickness) was added to the artificial film, chlorine penetration was also greatly retarded (after 3 h the chlorine concentration at the substratum was only 1/10 that in the bulk solution). Thus, the retardation of the HP in the multispecies biofilms was consistent with the experimental data from the chlorine penetration experiments. This kind of slow penetration can be explained by a mechanisms involving diffusion plus reaction (Stewart, 1994).

The reaction of HP can follow two general paths. It can either be consumed by oxidizable organic components of the bacteria, or, as in this study where catalase-(+) organisms are present, by catalyzed disproportionation into water and oxygen ($2\text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2\text{H}_2\text{O}$). The observed rapid increase in oxygen concentration in our biofilm (Fig. 4a) and planktonic cultures (data not shown) suggested that catalase catalyzed disproportionation was of primary importance.

Two other pieces of evidence support involvement of catalase in the degradation of HP by our three species consortium. First, when the biofilm was killed with bleach, leaving it intact on the substratum, a flat oxygen profile was measured across the layer. Second, it was observed that cell extracts of both biofilms and planktonic cells demonstrated comparable specific catalase activity (Fig. 6). Also, with the specific catalase inhibitor, AT, the cell extracts of both systems showed dose-dependent reductions in their HP disproportionation rates.

A remarkable difference in response was observed, however, when HP was added to AT-pretreated biofilms and AT-pretreated planktonic cultures, where both systems contained the same three catalase-(+) bacterial species. With the planktonic culture, a dose-dependent inhibition of catalase activity was found. The effective dose needed to reduce catalase activity, ED_{50} , was $\sim 150 \text{ mM}$ (Fig. 5). However, with the biofilm system, even a 1.0 M concentration of the inhibitor had no observable effect on the putative disproportionation reaction (compare Fig. 4a and 4b). Since the systems tested were pretreated with AT an hour prior to introducing HP, the difference in HP degradation rates reflected the difference in interaction of the organisms with AT. The biofilm, somehow, prevented AT-inhibition of the catalase. According to current thought, this may have been due to two possible mechanisms: (1) diffusional resistance of the inhibitor through the extracellular polymeric networks of the biofilm (reaction-diffusion hypothesis; Costerton et al., 1987; Hoyle et al., 1991; Stewart, 1994; Stewart and Raqepas, 1995), or (2) resistance to transport of AT across the cellular membranes of the biofilm organisms (reduced metabolic activity hypothesis; Brown et al., 1988; Gilbert et al., 1990; Stewart, 1994).

Some recent research suggests that an alternate physiological pathway may be triggered or expressed in the biofilm to produce the remarkable resistance to AT observed. It

has been reported that more than one form of catalase often exists in a single organisms. *K. pneumonia* (Goldberg and Hochman, 1989), *Pseudomonas putida* (Katsuwon and Anderson, 1992), *Escherichia coli* (Claiborne and Fridovich, 1979), and *P. aeruginosa* (Brown et al., 1979) are but some organisms producing multiple catalases.

The occurrences of different catalases in a single organism may be due to biochemical variations affecting a single gene product or may be separate enzymes produced from distinct genes (Katsuwon and Anderson, 1992). Katsuwon and Anderson (1992) found that the two catalase isozymes found in *P. putida* responded differently to the inhibitor, AT. Catalase A, found in the cytoplasm, was totally inhibited by AT, while catalase B, found in the membranes, was not.

These observations have direct bearing on the results obtained in our system. The ability of an organism to turn on specific genes in response to contacting a surface (Davies et al., 1993), or to being in close proximity to other organisms (quorum sensing; Fuqua et al., 1996), may hypothetically explain why our biofilm is resistant to AT inhibition of catalase while the same consortium of organisms in the planktonic state is not. *K. pneumonia* and *P. aeruginosa*, both members of the consortium tested in this study, have already been mentioned as producing multiple catalases. It is possible that at least one of these organisms possesses a surface- or proximity-promoted, AT resistant, catalase gene and can express that gene in the transition from planktonic to biofilm form.

If the above hypothesis were true then catalases isolated from the biofilm and planktonic systems should have had different responses to AT inhibition. And, while the catalases obtained from biofilm and planktonic organisms appeared to have the same activity in the presence of AT (Fig. 6), they were isolated without concern for two interesting possibilities. It is conceivable that membrane-bound cata-

lase was centrifuged down with other cell debris during enzyme isolation. It is also possible that the gene responsible for AT resistant catalase is only expressed in the presence of high concentrations of HP, and since the catalases were isolated in the absence of HP, this gene might not have been turned on. Further study will be needed to explain this phenomenon.

Because some bacteria attack cells that use HP as antimicrobial agents, it is reasonable to believe that they have developed enzymes such as catalase to thwart this defense. Likewise, it is also understandable that host or target cells have escalated their defenses by generating catalase-inhibiting agents (Almagor et al., 1983). In our study, the observed resistance of biofilms to AT may demonstrate a general defense mechanism against cellular antagonists that use HP in combination with catalase inhibitors. Regardless, this is another intriguing example of biofilms preserving their internal metabolic processes where their planktonic counterparts fail.

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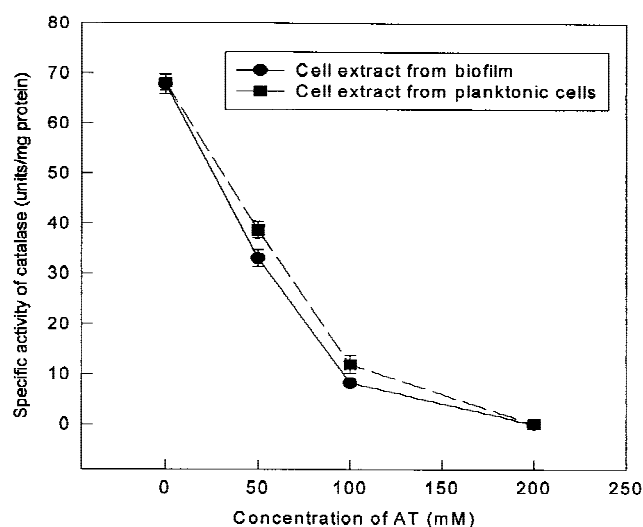


Figure 6. The decrease of specific catalase activity in the presence of AT from catalase isolated from planktonic cells and biofilms (error bars indicate standard errors for replicates of three experiments).

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