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Physiological Responses of Bacteria in Biofilms to Disinfection

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In situ enumeration methods using fluorescent probes and a radioisotope labelling technique were applied to evaluate physiological changes of *Klebsiella pneumoniae* within biofilms after disinfection treatment. Chlorine (0.25 mg of free chlorine per liter [pH 7.2]) and monochloramine (1 mg/liter [pH 9.0]) were employed as disinfectants in the study. Two fluorogenic compounds, 5-cyano-2,3-ditolyl tetrazolium chloride and rhodamine 123, and tritiated uridine incorporation were chosen for assessment of physiological activities. Results obtained by these methods were compared with those from the plate count and direct viable count methods. 5-Cyano-2,3-ditolyl tetrazolium chloride is an indicator of bacterial respiratory activity, rhodamine 123 is incorporated into bacteria in response to transmembrane potential, and the incorporation of uridine represents the global RNA turnover rate. The results acquired by these methods following disinfection exposure showed a range of responses and suggested different physiological reactions in biofilms exposed to chlorine and monochloramine. The direct viable count response and respiratory activity were affected more by disinfection than were the transmembrane potential and RNA turnover rate on the basis of comparable efficiency as evaluated by plate count enumeration. Information revealed by these approaches can provide different physiological insights that may be used in evaluating the efficacy of biofilm disinfection.

Methods to determine disinfection efficacy have relied on viability assays using conventional plate counting (PC) techniques for decades. One problem associated with this practice is that certain microorganisms are unable to form colonies on established media (3, 4, 35, 36). This is especially problematic when examining microbial communities with significant species diversity representing a wide range of physiological activities. Also, exposure to disinfectants under suboptimal conditions results in bacteria that become unable to form colonies on selective media because of reversible injury (18). This phenomenon in operating drinking water systems has been documented (20). Environmental exposure in the absence of disinfectants can also decrease culturability (28). These inadequacies are more acute when dealing with biofilms or microbial communities in which spatial heterogeneities are established and may be complex. Therefore, the traditional approach of describing microbial populations on the basis of colony-forming ability is inadequate for many systems since established culture media often cause underestimates of the actual bacterial population density. In addition, conventional methods provide no information on spatial heterogeneities within structured communities such as biofilms.

Numerous alternative approaches have been applied to obtain quantitative information on bacteria or their physiological activities within environmental systems. Direct examination employing traditional microscopy (16, 23, 27) and, more recently, scanning confocal laser microscopy (5) has made progress in visualizing attached microbial communities. Other approaches, including 16S rRNA analysis (22, 26, 35), flow cytometry (1, 11, 24, 25), and probes for expression of specific genes (32, 34), have also been used in such studies. However, most of these techniques require either complex preparation processes or sophisticated and expensive equipment. As a result, assessment of biofilm disinfection still relies mainly on the conventional approach of scraping and enumeration by PC techniques with their inherent problems.

In a previous paper, two rapid in situ methods for assessing the physiological activities of biofilm bacteria were presented (37); they employ the fluorogenic probes 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and rhodamine 123 (Rh 123). These compounds reflect cellular respiratory activity and membrane potential, respectively. RNA turnover rate is also a useful indicator of physiological activity since it has been used to determine the in situ growth rate and the response of bacteria to changes in environmental conditions (13). In this study, we followed changes in the physiological activities of attached bacteria with exposure to disinfectants without disrupting biofilm integrity by using CTC, Rh 123, and tritiated uridine incorporation (TUI). These methods provided more precise information describing the physiological consequences of biocides within bacterial biofilms than the conventional enumeration approach.

MATERIALS AND METHODS

Biofilm apparatus. Bacterial biofilms were grown in a simple reactor made from wide-mouth pint Mason jars (Kerr; 450 ml) as described elsewhere (6). Jars containing six stainless steel coupons, inoculated medium, and a magnetic stir bar were incubated on a thermally insulated magnetic stirrer with a constant speed (285 rpm). Preparation of coupons has been described in a previous report (38).

Bacterial strains and growth conditions. *Klebsiella pneumonia* Kp1, isolated from drinking water, was obtained from D. Smith, South Central Connecticut Water Authority, New Haven. The bacterial culture was grown in 1/10 Trypticase soy broth (TSB) (Difco) for 24 h at 35°C and then transferred (1%) to sterile medium (1/10 TSB) for another 24 h before being used as the inoculum for biofilm reactors. Biofilms were

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formed on 316 stainless steel (12 by 76 mm^2) coupons incubated at 25°C for 36 h (38).

Preparation of disinfectants. The disinfectants used were chlorine and monochloramine. The chlorine solution was prepared from sodium hypochlorite (30), and the monochloramine solution was made with sodium hypochlorite and ammonium chloride (14). The biofilm coupons were exposed to disinfectants in chlorine-demand-free sterile phosphate-buffered water (PBW) without $MgCl_2 \cdot 6H_2O$ (2) at pH 7.2 for chlorine and at pH 9.0 for monochloramine. The concentrations of chlorine and monochloramine were measured with a Hach DPD chlorine test kit and Hach digital amperometric titrator (Loveland, Colo.).

Disinfection of biofilms. Biofilm coupons were rinsed in sterile distilled water (38) and then transferred to an acid-washed quart wide-mouth Mason jar (Kerr; 900 ml) containing chlorine-demand-free phosphate-buffered water and a magnetic stir bar. Biofilm coupons were then treated with disinfectants (0.25 mg of chlorine per liter [pH 7.2] and 1 mg of monochloramine per liter [pH 9]) at 25°C. The concentrations of disinfectants were chosen to allow comparisons of the data with previously published results obtained by PC on TLY medium (19) and by direct viable count (DVC) (38). Coupons were removed after 5 and 10 min, and the disinfectant was neutralized by immersion in sodium thiosulfate (0.01%, final concentration). Surviving biofilm bacteria were enumerated by Rh 123 and CTC staining (37).

Fluorochromes and staining procedures. The fluorochromes utilized in this study were CTC (Polysciences, Inc.) and Rh 123 (Eastman Kodak Co.). Bacteria attached to coupons were rinsed (38) and transferred to a staining vessel containing 0.04% (approx. 4 mM) CTC in reagent-grade water or Rh 123 (5 μg/ml, final concentration) in phosphate-buffered saline (pH 7.2). Details of the CTC reduction assay and Rh 123 staining procedures are described elsewhere (37). Coupons were then air dried prior to examination by epifluorescence microscopy.

TUI assay. Uridine uptake was employed to determine the RNA turnover rate of biofilm bacteria. [5,6-^{3}H]uridine (40.1 Ci/mmol; 1,483.7 Tbq/mmol) (Dupont, NEN) was used in the TUI assays. Biofilm coupons withdrawn at timed intervals during disinfection were placed in a vessel containing 4 ml of uridine solution (0.2 μCi/ml) to allow incorporation. After 4 h of incubation at 25°C, coupons were retained in the vessel and attached bacteria were removed from coupons by scraping with a sterile rubber policeman, collected in a vial, and sonicated (Sonogen Automatic Cleaner; Branson Instruments Inc.) for 5 min to disperse aggregates. An aliquot of the suspension (100 μl) was added to an Eppendorf tube with 0.1 ml of lysis buffer (Tris HCl [50 mM], EDTA [10 mM], sodium dodecyl sulfate [4%] [pH 12.4]), vortexed for 5 min, and then transferred onto a Whatman GF/C glass fiber filter. The filter was washed three to five times with 5% cold trichloroacetic acid to precipitate bacterial RNA. The filter was washed again with cold 95% ethanol (4°C) to remove residual trichloroacetic acid and then dried in an oven (100°C) overnight before being transferred to a small vial with 3 ml of scintillation fluid (0.5% PPO [2,5-diphenyloxazole] in xylene). Quantitation of radioactivity was done with a Packard Tri-Carb 460 CD liquid scintillation counter. Quenching analysis was carried out with [5,6-^{3}H]uridine to determine the effects of lysis buffer and glass fiber filters. No significant quenching effect was detected from either the lysis buffer or the glass fiber filters.

Enumeration with epifluorescence microscopy. A Leitz Ortholux II microscope fitted with an epifluorescence illumination system and a 100-W mercury lamp was used. Different Leitz Pleomopak filter blocks appropriate for individual fluorochromes were used. The H filter block was used for visualizing attached bacteria stained with Rh 123, acridine orange, and intracellular CTC-formazan, whereas filter block B was used in the enumeration of total cell numbers stained with 4′,6-diamidino-2-phenylindole (DAPI) by epifluorescence microscopy. After a 2-h incubation at 35°C, respiring cells within Kp1 biofilms reduced soluble CTC to CTC-formazan deposits, which were visualized as intracellular red crystals in bacteria by epifluorescence microscopy. The barrier filter (LP 515) in the Leitz H filter block gave a distinctive color contrast on the biofilm coupons. Nonrespiring bacteria appeared green when stained with DAPI, whereas respiring cells were also green but contained red CTC-formazan crystals. Filter block N2.1 was used to visualize the red CTC-formazan crystals without DAPI fluorescence. The accumulation of Rh 123 in Kp1 cells within biofilms appeared green with filter block H. The results obtained by using the two different fluorescence stains were compared with the enumeration data from PC and in situ DVC methods (38).

Statistical analysis. Microscopic enumeration results in each experiment were obtained from counts of 10 microscopic fields. If the total cell number was <400, more than 10 fields were examined. Statistical analyses were performed on all data from three replicates by mathematical functions within SigmaPlot (Version 5.0 by Jandel Scientific) and one-way analysis of variance with InStat (Version 1.1 by GraphPAD) computer software. The level of significance was set at $P < 0.05$ for all comparisons.

RESULTS

Biofilm chlorination. The initial unexposed mean ± the standard error of the mean of total bacterial density on coupons was $(1.80 \pm 0.27) \times 10^4$ cells per mm^2 as determined microscopically by staining with DAPI. The mean number of viable cells, which was measured by colony formation on TLY plates after removing attached bacteria from coupons, was $(1.44 \pm 0.74) \times 10^4$ CFU/mm^2. The cell population demonstrating respiratory activity, determined by CTC reduction, was $(1.71 \pm 0.20) \times 10^4$ cells per mm^2. The initial total cell density obtained by acridine orange direct count enumeration on a different area of the same coupon was $(1.90 \pm 0.48) \times 10^4$ cells per mm^2, and the number of cells retaining transmembrane potential, which were stained by Rh 123, was $(1.64 \pm 0.26) \times 10^4$ cells per mm^2.

Free chlorine (0.25 mg/liter) was used to treat attached cells grown on stainless steel coupons. Biofilms of *K. pneumoniae* Kp1 were exposed to chlorine or monochloramine for timed intervals and assayed to determine the effects of these disinfectants on cellular respiration and membrane potential. Comparison of disinfection effects after disinfectant exposure for 5 and 10 min was determined by using log N/N_0 values. Each datum point is a log of the ratio of the number of surviving bacteria to the initial number of bacteria. During chlorine disinfection, the respiratory activity of attached cells on coupons decreased by 1.93 logs after 10 min (Fig. 1). However, the fraction of biofilm bacteria that retained membrane potential decreased less after chlorination, showing only a decline of 0.93 log at 10 min (Fig. 1). A larger decrease in the viability of biofilm bacteria as determined by the DVC method was observed (2.34 logs after 10 min). The largest decrease was seen in the PC results: bacterial viability declined 3.30 ± 0.50 logs, and the confidence interval became larger after 10 min of chlorination (Fig. 1). The differences among the four enumeration methods were statistically significant ($P < 0.05$).

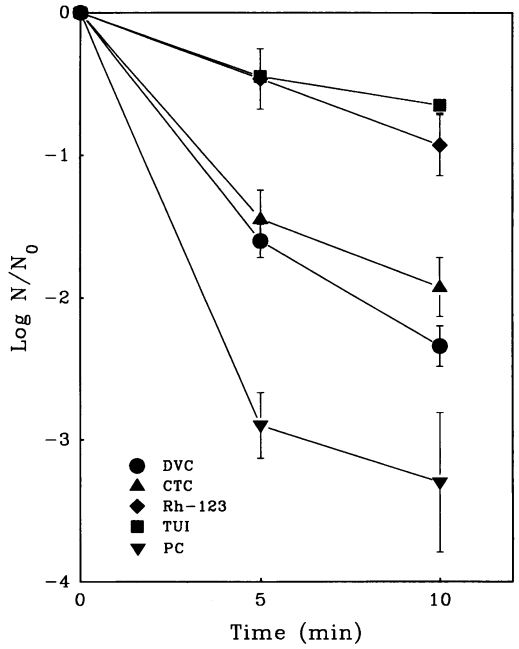

FIG. 1. Effect of chlorine treatment (0.25 mg/liter) (pH 7.2) on *K. pneumoniae* KP1 biofilms. Bacterial activity was assessed by five different methods (n = 3). Bars represent the standard errors of the means.

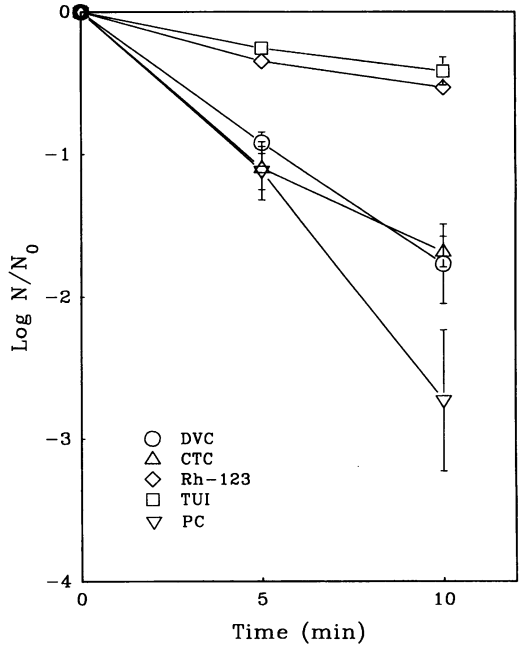

FIG. 2. Effect of monochloramine treatment (1 mg/liter) (pH 9.0) on *K. pneumoniae* KP1 biofilms. Bacterial activity was assessed by five different methods (n = 3). Bars represent the standard errors of the means.

Biofilm chloramination. Results of a similar experiment in which monochloramine (1 ppm) was used to treat Kp1 biofilms are shown in Fig. 2. The initial mean density of respiring cells on coupons was (1.67 ± 0.27) × 10^4 cells per mm^2, and the cell population stained with Rh 123 was (1.57 ± 0.29) × 10^4 cells per mm^2. Following 10 min of chloramination, a 1.59 log decrease was observed with the fraction of biofilm bacteria that reacted in the CTC reduction assay (Fig. 2). Higher counts were observed with the Rh 123 staining method (0.53 log reduction) after monochloramine treatment for 10 min. The DVC method showed a 1.76 log reduction, which is comparable to data from the CTC reduction assay. Statistical analyses showed that there was no significant difference between results obtained by CTC reduction and DVC ($P > 0.05$), but the results of the Rh 123 method were significantly different than those of the other three methods ($P < 0.05$). The results by the PC method again showed the largest decline (2.71 ± 0.50 logs) in bacterial culturability and an increase in confidence interval after a 10-min exposure to monochloramine. However, it should be noted that the log N/N_0 values obtained by the PC method with chlorine and monochloramine, respectively, after 10 min of exposure (Fig. 1 and 2) were not significantly different ($P > 0.05$).

TUI in biofilms after disinfection. TUI was used to estimate the RNA turnover rate of attached cells. Incorporation of [5,6-^{3}H]uridine by Kp1 biofilms after treatment with biocides is shown in Table 1. In order to compare the RNA turnover rate with the other physiological activities measured in this study, uptake of tritiated uridine by biofilm bacteria was also expressed as log N/N_0 (the log ratio of the counts per minute of surviving bacteria to the initial counts per minute of bacteria) at 5 and 10 min (Fig. 1 and 2). TUI by attached bacteria was significantly higher after 5 and 10 min ($P < 0.05$) of disinfection with monochloramine than with chlorine.

DISCUSSION

Results indicated a large variation in the responses of different physiological activities examined within Kp1 biofilms after disinfection. Control data obtained without exposure to biocides demonstrated that DVC, CTC, and Rh 123 yielded comparable results as described previously (37, 38). It should also be noted that these results were all in relatively close agreement with microscopic (acridine orange direct count) enumerations to detect the total bacterial population. However, differences among the various physiological processes that were examined became apparent after exposure to disinfectants, as illustrated in Fig. 1 and 2. Among all the methods studied (DVC, CTC, Rh 123, and TUI), chlorine always caused a greater reduction in the physiological activities assayed, although PC yielded comparable levels of bacterial culturability. In addition, the fraction of the active cell population detected by colony formation (PC) was always at least 1 log lower than data obtained by the other methods. These results agree with earlier reports (18, 38) suggesting that reliance on PC overestimates the efficacy of disinfection treatment.

The results reported here provide a comparison of the relative effects of biocides on different physiological processes

TABLE 1. Incorporation of [5,6-^{3}H]uridine by *K. pneumoniae* biofilms after chlorine (0.25 mg/liter) (pH 7.2) and monochloramine (1 mg/liter) (pH 9.0) treatment (n = 3)

Treatment time	[5,6-^{3}H]uridine incorporation (cpm) after treatment with:	
	Chlorine (% of control)	Monochloramine (% of control)
Control	103,784 ± 4,194 (100)	78,573 ± 4,050 (100)
5 min	37,030 ± 2,118 (35.7)	44,566 ± 6,220 (56.7)
10 min	23,749 ± 3,507 (22.9)	32,167 ± 7,630 (40.9)

within *K. pneumoniae* biofilms while the PC results suggest that comparable efficiencies were achieved with chlorine and monochloramine. With chlorine, the effect on culturability and viability (PC and DVC) was greater than its impact on respiratory activity (CTC), which was greater than its influence on transmembrane potential (Rh 123) and RNA turnover rate (TUI). DVC and CTC yielded comparable measures of the action of monochloramine. Rh 123 and TUI also produced comparable results. Monochloramine exposure caused greater decreases in culturability, respiratory activity, and viability than in transmembrane potential and RNA turnover rate. Although monochloramine appeared to cause slightly less damage to the cellular processes studied, the overall patterns of physiological consequences for both disinfectants are similar and some processes displayed significant differences in susceptibility. By way of a possible explanation, it is reasonable that tests, such as DVC and PC, which depend on the comprehensive integrity of cellular physiology would be more susceptible to biocides while discrete physiological processes would be more likely to display variable and reduced susceptibility. However, both disinfectants are considered effective in the control of biofilms, although monochloramine penetrates biofilms more effectively (14) and removes biofilm bacteria better (38).

The experimental system described here used a pure bacterial culture of an environmental isolate grown as thin biofilms (approx. 1 to 2 μm) under controlled laboratory conditions. It should be noted that thicker and more complex biofilm communities might respond differently to disinfection because of variations in cellular physiology (7–9) and greater production of extracellular polymers (15). However, the physiological approach reported here to assess the efficacy of biocides might be used in the study of mixed culture systems and thicker biofilms.

One noteworthy drawback associated with the DVC method is the difficulty in differentiating the elongation of individual cells within biofilms or aggregates, although image analysis technology can be useful (31). By contrast, utilization of CTC and Rh 123 allowed clear resolution of individual cells by epifluorescence microscopy. This can be facilitated through different combinations of excitation and barrier filters to allow the colors of stains and counterstains to be differentiated without causing confusion when enumerating bacteria. As an additional advantage, staining with either CTC or Rh 123 can be completed in 2 h, although additional time might be needed for data analysis. This approach provides more timely and useful information for the formulation of effective responses directed toward the control of biofilms.

CTC and Rh 123 have also been used recently with flow cytometry to determine respiratory activity (11) and dormancy (12) in bacteria as well as respiring autochthonous bacteria in drinking water and biofilms (29). There is a large number of other fluorogenic reagents utilized in biological and physiological applications with eukaryotic cells (10, 17). However, there are only a few microbiological uses of this analytical approach (5, 21, 33), yet there is great potential for expanded application of fluorogenic compounds to determine bacterial physiological and metabolic activities at the cellular level. This development is further facilitated by recent advances in scanning confocal laser microscopy, digital image analysis systems, and high-resolution cameras (5).

The use of fluorogenic indicators of metabolic activities in microscopy provides information describing the physiological status of individual cells and an assessment of specific cellular processes in situ within attached microbial communities. This approach is more accurate and goes beyond the conventional strategy, which is dependent on culturability. This study indicated that the use of fluorescent probes can reveal different physiological insights within bacterial biofilms that may be applied in the evaluation of disinfection efficacy.

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