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MONITORING BIOFILM-INDUCED PERSISTENCE OF *MYCOBACTERIUM* IN DRINKING WATER SYSTEMS USING GFP FLUORESCENCE

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Introduction

Biofilms are ubiquitous in drinking water distribution systems. They are relatively impervious to mitigation treatments targeted for suspended cells, and have been implicated in blooms of coliforms and in harboring pathogens in otherwise properly maintained systems (1-6). To investigate the impact of biofilms on pathogen persistence in potable water, the attachment and retention of a *gfp*-transformed *Mycobacterium smegmatis* (MS) strain was monitored in laminar flowcells exposed to different concentrations of chlorination. On-line GFP fluorescence was used to measure non-destructively MS biomass levels in monoculture and mixed-culture biofilms.

Materials and Methods

Bacterial Strains and Growth Media: The *Mycobacterium smegmatis* strain was kindly provided by Dr. Vojo Deretic, University of Texas Health Sciences Center (7). The triculture mixed-species biofilm was composed of three bacterial species isolated from corroded copper drinking-water pipes. The bacteria were identified by fatty acid profiles as *Acidovorax* sp., *Bacillus* sp., and *Pseudomonas* sp. (data not shown).

M. smegmatis was maintained on enriched Middlebrook 7H9 media supplemented with 25 ug/mL kanamycin. The three drinking water isolates were maintained on tryptic soy agar. All media reagents were purchased from Difco Laboratories (Detroit, MI, USA).

Test System: A 1:1000 dilution of tryptic soy broth (TSB) in distilled deionized water was pumped through laminar flowcells in a once-through design. Each flowcell contained five stainless steel coupons inserted flush with the bottom of the chamber. Three hollow screws with a quartz disk attached at the end were positioned above each coupon and provided viewports to measure biofilm fluorescence (8). Fifty mL of a 48 h MS culture (~8.7 x 10^7 cells ml^{-1}) were injected into each flowcell and flow was stopped for 2 h to allow for cell attachment. The triculture was maintained in a chemostat using diluted TSB. A flow line from the chemostat was used to introduce the triculture cells into the flowcells for 4 h. Subsequently, the inoculation line was clamped off and sterile media flow was resumed at a rate of 10 mL min^{-1}. All experiments were performed in triplicate.

Biofilm fluorescence was measured using a fluorometer equipped with a fiber-optic attachment (Spex Industries Inc., Edison, NJ, USA). Tryptophan (ex. 295 nm; em. 340 nm) and GFP (ex. 395 nm; em. 509 nm) fluorescence readings were used to

determine the total biofilm and MS biomass respectively. Background fluorescence levels were subtracted from all subsequent readings (8,9).

Cell Enumerations: Viable MS cells were determined by plating suspensions of biofilm material onto Middlebrook 7H9 agar plates and measuring colony forming units. The MS colonies were morphologically distinct from the other bacterial components of the biofilms.

Total cell counts were determined by staining biofilm material with acridine orange as described by Arrage et al. (8) and examined under epifluorescent illumination.

Disinfection: MS biofilms and mixed-species biofilms were exposed to 0, 1, and 5 ppm total chlorine. Media amended with chlorine was pumped through the flowcells beginning 1 h before MS inoculation and continuing through the remainder of the experiment. For experiments involving mixed-species biofilms, the triculture cells were inoculated into the flowcells and allowed to form biofilms for 96 h prior to chlorine and MS addition.

Results and Discussion

The addition of MS into sterile flowcells resulted in the detection of GFP fluorescence at 3 h post-inoculation (1 h after the resumption of media flow), which decreased to background levels after 12 h (Fig. 1). This result was presumably due to the wash-out of unattached cells and occurred at all chlorine treatments by 24 h. Upon termination of the experiment, microscopic examination of the substratum revealed cell densities of approximately 400 cells cm^{-2} which did not vary significantly with chlorine concentration.

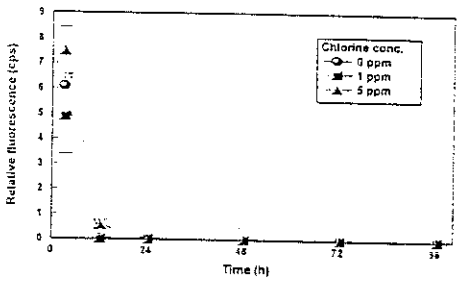

Fig. 1. On-line GFP fluorscence from MS cells inoculated into sterile flowcells.

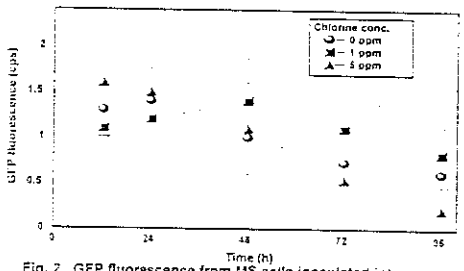

Fig. 2. GFP fluorescence from MS cells inoculated into flowcells containing a mixed-species biofilm.

When MS was inoculated into flowcells containing a 96 h triculture biofilm, GFP fluorescence was an order of magnitude greater than that recorded from the sterile environment (Fig. 2). The release of MS cells was more gradual in the presence of an established biofilm with GFP fluorescence detected up to 96 h post-MS inoculation. At this timepoint, MS fluorescence at the 0 and 1 ppm chlorine levels was 3-fold greater than that measured at 5 ppm chlorine. The on-line data was

supported by epifluorescent microscopic counts of GFP-expressing MS cells recovered from biofilms at the end of the experiment (Table 1). Although there was an inverse relationship between chlorine and the total number of MS cells, the proportion of those cells that were viable was independent of the chlorine concentration (Table 1).

Chlorine conc.	Microscopic cell counts (10^5 cells cm^{-2})	Viable cell counts (10^5 cells cm^{-2})	Viability Index (viable/total)
0 ppm	43 ± 8.5	7.8 ± 1.2	0.18
1 ppm	9.1 ± 6.3	1.3 ± 0.53	0.14
5 ppm	2.8 ± 1.9	0.55 ± 0.11	0.20

Table 1. Numbers of *M. smegmatis* cells recovered from mixed-species biofilms after 96 h exposure to chlorine.

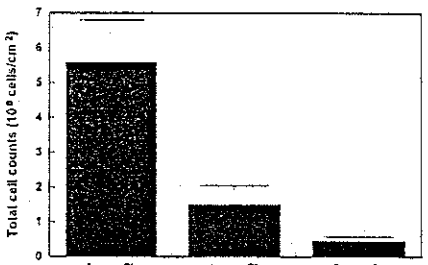

Fig. 3. Microscopic counts of acridine orange-stained biofilm cells after 96 h exposure to chlorine.

The results of this study suggest that MS attachment and retention was dependent on the amount of biofilm biomass present on the substratum, rather than on the chlorine concentration. There was a marked decrease in biofilm development when triculture cells were exposed to chlorine (Fig. 3). This may have resulted in a less adherent surface for planktonic MS cells. It has been shown that initial colonizing bacterial species can modify surfaces and enhance the subsequent attachment of succeeding microorganisms (10).

This study has demonstrated the utility of using a fluorescent tag to selectively identify a specific cell population within a multi-species biofilm. It has been widely reported that biofilms can mitigate the effectiveness of biocides and antibiotics on target organisms (1,4). Using an organism which has been transformed to express the tag allows for the ability to track its response to perturbations in its environment (i.e. chlorine) in real time which is not always possible when using lethal fluorescent or colorimetric stains.

Acknowledgments

We thank R. Kirkegaard and A. Cardwell for excellent technical assistance. This study was funded by the National Water Research Institute project #HRA 699-510-94.

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