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Cell Sorting of Formalin-Treated Pathogenic *Mycobacterium paratuberculosis* Expressing GFP

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ABSTRACT

GFP is widely used as a molecular tool for the study of microbial pathogens. However, the manipulation of these pathogenic microorganisms poses a health threat to the laboratory worker, requiring biosafety level II or III containment. Although the GFP fluorophore is tolerant to formalin, a thorough analysis of this treatment on fluorescent output in prokaryotic systems has not been described. In addition, the analysis of microorganisms expressing GFP often depends on specialized equipment, which may not be housed in biosafety level II or III laboratories. Therefore, we sought to develop a safe and effective method for manipulating the GFP-expressing pathogenic bacterium *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*) utilizing a formalin treatment that would permit the analysis of GFP fluorescence without requiring stringent biosafety containment. We demonstrate that formalin-treated *M. paratuberculosis* expresses 50% less fluorescence than viable cells, but this reduction is still compatible with spectrofluorometry and cell sorting. Furthermore, plasmid DNA that expresses GFP can be recovered efficiently from nonviable, sorted fluorescent cells. This approach is flexible, provides an additional margin of safety for laboratory personnel, and can be easily applied to other pathogenic microorganisms expressing GFP.

INTRODUCTION

The GFP from the jellyfish *Aequorea victoria* has been extremely valuable as a marker for the study of host-pathogen interactions. Applications for this technology are wide ranging and include the analysis of biofilm formation in *E. coli* and *Pseudomonas aeruginosa* (9), monitoring the behavior

of the enteropathogenic *E. coli* O157:H7 in ground beef (1) and determining the induction of specific genes during the course of infection in *Salmonella typhimurium* (22). Two unique advantages of the GFP reporter system are that it does not require exogenous substrates or co-factors and it is stable in prokaryotic systems (6). In mycobacteria, the expression of GFP has been documented for several species (2,7,11,17,19,24). It has been used to analyze various aspects of mycobacterial pathogenicity, including tissue localization during infections (17,18), monitoring intracellular trafficking pathways (24) and identifying specific genes with enhanced activity in macrophages (2).

The purified GFP from *A. victoria* requires an intact secondary and tertiary structure for fluorescence (4). The purified protein is stable over a wide temperature and pH range (26) but can be denatured by several different chemicals under conditions of prolonged exposure, high chemical concentrations, or temperatures of 40°C or higher (25). Surprisingly, very little information is available regarding the effect of formalin or glutaraldehyde fixation on the fluorescent properties of GFP. The pioneering study in which GFP was first expressed in both *E. coli* and *Caenorhabditis elegans* (6) also indicated that GFP was tolerant to both formalin or glutaraldehyde solutions. However, no subsequent work has quantified the effects of these chemicals on GFP fluorescence.

Experiments designed to evaluate bacteria expressing the GFP often rely on a fluorescence-activated cell sorting (FACS®) system, laser confocal microscopy, or spectrofluorometry. To minimize exposure to pathogenic microorganisms, it would be desirable to perform these analyses on nonviable organisms wherever protocols do not require live bacteria for downstream applications. Therefore, a successful strategy for preparing inactivated GFP-expressing pathogenic bacteria was developed that allows techniques based on GFP fluorescence to be performed. We illustrate this method with *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*), a bacterial pathogen of veterinary importance. These bacteria cause a severe gastroenteritis known as Johne's disease in cat-

tle and other ruminants (15). It has also been implicated as a potential cause of Crohn's disease in humans (16), although a causal link between Crohn's disease and *M. paratuberculosis* has not yet been established (23). The use of formalin treatment may be applied to any pathogenic microorganism expressing GFP to reduce the risks of accidental exposure.

MATERIALS AND METHODS

Bacterial Strains, Growth Conditions, and Plasmids

M. paratuberculosis strains K-10 and K-10(pWES4) were grown at 37°C in M²OADC²TW broth as described previously (12). Where required, 15 g/L Bacto agar (Difco Laboratories, Detroit, MI, USA) was added to solidify the medium. Kanamycin (Sigma-Aldrich, St. Louis, MO, USA) was added to the media at 50 µg/mL for growing K-10(pWES4). For the spectrofluorometry and FACS analyses, all bacterial cultures were grown to an A₆₀₀ of 0.5–0.9, which corresponds to approximately 1.5 to 3 × 10⁸ cfu/mL. The construction of the *E. coli*-*Mycobacterium* spp. shuttle plasmid pWES4 has been described previously (19). This plasmid contains the wild-type GFP cDNA from *A. victoria* downstream from the *M. bovis* BCG *hsp60* promoter (P_{hsp60}) and a kanamycin resistance gene as a selectable marker.

DNA Manipulations and Southern Blotting

M. paratuberculosis K-10 was transformed with the plasmid pWES4 using previously described methods (12). For plasmid recovery experiments, total DNA was prepared from formalin-treated *M. paratuberculosis* strain K-10(pWES4) by the method of Belisle and Sonnenberg (3), and transformed into *E. coli* DH5α ultracompetent cells (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. Plasmid DNA was prepared from *E. coli* strains either by the alkaline lysis miniprep method (20) or with the Wizard® Miniprep kit (Promega, Madison, WI, USA). For Southern analysis, chromosomal and plasmid DNA was digest-

ed with *EcoRI*, which cuts only once in the plasmid pWES4. Southern hybridization and detection were done as described previously (20). A 635-bp DNA fragment from pWES4 was PCR-amplified using the primers GFPfor (5'-CTTGTGAATTAGATGGTGAT-3') and GFPrev (5'-CTGTTACAACTC-AAGAAGGACC-3'). Thermal cycling conditions consisted of one cycle of 94°C for 4 min, 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and one cycle of 72°C for 4 min. This probe was labeled with α -[³²P]dCTP (3000 Ci/mmol) using the *Rediprime*® DNA Labeling System (Amersham Biosciences, Piscataway, NJ, USA) as described by the manufacturer.

Spectrofluorometry Assays

Recombinant *M. paratuberculosis* strain K-10(pWES4) was grown to mid-log phase, split into two aliquots, and harvested by centrifugation. One aliquot was resuspended in 10% formalin containing 0.05% Tween® 80 for 3 h, while the remaining aliquot was immediately washed in PBS-Tween. After formalin treatment, bacteria were washed in PBS-Tween as described above. To eliminate cell clumping, bacteria were sonicated and dispersed through a 26-g

needle as described previously (27). For the estimation of the lower limits of fluorescence, bacterial cells were adjusted to an A_{600} of 0.5 (approximately 1.5×10^8 cfu/mL) and diluted serially from 1.5×10^8 to 1.5×10^0 . For subsequent spectrofluorometry assays, bacteria were adjusted to an A_{600} of 1.67 (approximately 5×10^8 cfu/mL). Dilutions were made ranging from 5×10^8 to 5×10^6 cfu/mL in 1/10 logarithmic unit increments (e.g., 5×10^8 , 4×10^8 , 3×10^8 cfu/mL, etc.). For all assays, triplicate samples consisting of 200 μ L each dilution were aliquoted into black flat-bottom 96-well microplates (Dynex, Chantilly, VA, USA). The intensity of fluorescence was measured in the bottom reading mode using a Dynex MFx® spectrofluorometer with an excitation wavelength of 485 nm and emission at 538 nm. The mean for triplicate wells containing PBS-Tween was used as a background subtraction for all test wells, and results were expressed in fluorescence intensity units.

Flow Cytometry and Cell Sorting

The wild-type and recombinant *M. paratuberculosis* strains were grown to mid-log phase and harvested as described above. After processing to ob-

tain single cell suspensions, each culture was adjusted to a final OD_{600} of 0.5. Non-fluorescent and fluorescent bacteria were mixed in ratios of approximately 10:1, 1:1, and 1:10, followed by analysis in a FACSvantage® cell sorter (Becton Dickinson, San Jose, CA, USA) equipped with an argon-ion laser emitting at 488 nm and a 520/20 nm bandpass filter for collection of GFP fluorescence. Bacteria were detected by side scatter, and fluorescence and side scatter data were collected with logarithmic amplifiers.

RESULTS AND DISCUSSION

Expression of GFP in Formalin-Treated *M. paratuberculosis*

The fluorescent nature of the GFP protein is such that only one chromophore is associated with each protein molecule (10,26). Thus, the ability to detect its fluorescence is tightly linked to the levels of GFP expression in the heterologous system. In our experiments, the recombinant *M. paratuberculosis* strain expressed GFP fluorescence levels between 3 and 30 times above background levels for bacterial concentrations from 5×10^6 to 5×10^7 cfu/mL (Figure 1). The level of fluorescence expression for the wild-type GFP protein reported by others for *E. coli*, *Pseudomonas putida* (5), and *M. bovis* BCG (17) are similar, ranging from 3 to 10 times above background levels. This suggests that variations in methodology and/or genetic background have minimal effects on the expression of this protein in different microorganisms. It is possible that the use of a red-shifted GFP mutant could significantly increase the sensitivity of this assay, as others have reported 20- to 35-fold increases in fluorescence intensity for viable bacteria that express these proteins (8). However, it is unknown if the expression of these altered proteins is toxic for *M. paratuberculosis*, which would result in fluorescence output that is considerably below the optimum sensitivity levels reported for other bacteria.

Our goal was to develop a method that would safely inactivate pathogenic microorganisms while retaining compatibility with the use of the GFP re-

Figure 1. GFP expression levels of formalin-treated and untreated K-10(pWES4). The fluorescence intensity of formalin-treated and untreated bacteria expressing the GFP was measured at 538 nm using a spectrofluorometer. Each data point is the average of triplicate measurements. No Tx = no formalin treatment; formalin = 3 h exposure to formalin.

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porter system. Therefore, we examined the capability of recombinant *M. paratuberculosis* to retain GFP fluorescence after formalin fixation. Although treatment with 10% buffered formalin did not visibly affect fluorescence when viewed at 100× magnification with a fluorescent microscope fitted with a FITC filter (data not shown), spectrofluorometry assays did show a decrease in fluorescence of approximately 50% in the formalin-treated

bacteria (Figure 1). For these assays, the lowest concentration with detectable fluorescence was 1.5×10^6 cfu/mL for both formalin-treated and untreated bacteria (data not shown). As expected, there was a linear correlation between fluorescent output and the number of bacteria from 5.0×10^6 to 5.0×10^8 cfu/mL for both formalin-treated and untreated *M. paratuberculosis* (Figure 1). It is possible that the decrease in fluorescent output observed

in formalin-treated cells is a result of either an alteration in the absorbance/excitation spectrum of the fluorescent molecule or that the protein itself is slowly denatured upon exposure to formaldehyde. Other studies have reported that high concentrations and/or prolonged exposure to organic solvents, detergents, and chaotropic agents cause a loss in native and mutant GFP fluorescence intensity that is most likely due to denaturation of the protein (14,25).

FACS Analysis of Formalin-Treated *M. paratuberculosis* Cells

The GFP reporter system is well suited for separating fluorescent and non-fluorescent bacteria by FACS analysis. Since the previous study indicated that formalin treatment could affect GFP fluorescence, we next examined the ability of GFP-expressing and wild-type *M. paratuberculosis* to be separated by FACS analysis. Both non-fluorescent and fluorescent *M. paratu-*

Figure 2. Flow cytometric analysis of mixed populations of fluorescent and non-fluorescent bacteria. The results are shown as the relative amounts of bacteria versus the log₁₀ of fluorescence. Mixed populations of non-fluorescent (K-10) and fluorescent (GFP) bacteria were analyzed at ratios of 10:1 (A), 1:1 (B), and 1:10 (C) for fluorescent output.

Figure 3. Southern analysis of plasmid DNA recovered from inactivated *M. paratuberculosis*. Formalin-killed *M. paratuberculosis* K-10 and K-10(pWES4) were mixed at a 1:1 ratio and sorted through a flow cytometer as described in the Materials and Methods. Lane 1, chromosomal DNA recovered from inactivated *M. paratuberculosis* and digested with *EcoRI*. Lane 2, pWES4 recovered by transforming *E. coli* with DNA from sorted, inactivated *M. paratuberculosis* and digested with *EcoRI*, which cuts once within the plasmid. Lane 3, undigested, recovered pWES4. Lane 4, control pWES4 digested with *EcoRI*. Lane 5, uncut control pWES4 plasmid DNA.

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berculosis strains were monitored individually for light output, and cutoff values from these results were used to analyze mixed populations of bacteria. Formalin-treated wild-type *M. paratuberculosis* was mixed with GFP-expressing bacteria at ratios of approximately 10:1, 1:1, and 1:10. Bacteria were then sorted and analyzed for fluorescent output (Figure 2, A–C). Analysis of 5×10^3 particles in replicate experiments consistently resulted in 3%–5% of the population gated to the fluorescent fraction for the 10:1 (wild-type:GFP-expressing) ratio (Figure 2A), 33%–35% in the fluorescent fraction for the 1:1 ratio (Figure 2B), and 68%–82% of the particles in the fluorescent fraction for the 1:10 ratio (Figure 2C). Since cutoff values were gated conservatively, it is expected that a portion of the bacteria actually expressing the GFP will be identified as non-fluorescent using these parameters. Our re-

sults demonstrate that despite a reduction in fluorescence caused by formalin treatment, accurate separation of fluorescent and non-fluorescent *M. paratuberculosis* is possible. Furthermore, these data are consistent with similar flow cytometry results for viable cultures of *M. smegmatis*, *M. bovis* BCG (11), and *Lactococcus plantarum* (13). Thus, formalin treatment of GFP-expressing microorganisms is compatible with this technology.

Recovery of Plasmid DNA from Formalin-Treated *M. paratuberculosis* Cells

Specific applications, such as the screening of a promoter library, require the analysis of plasmid DNA from bacterial populations differentially expressing the GFP. We determined the feasibility of rescuing the pWES4 plasmid from cell-sorted, inactivated bacte-

rial cells. Total DNA was prepared from the fluorescent fraction of formalin-inactivated and sorted *M. paratuberculosis* and transformed into *E. coli*. A random transformant harboring the pWES4 plasmid was chosen for further analysis by Southern blotting. As seen in Figure 3, lane 1, pWES4 was present in the total DNA preparation from the fluorescent fraction of the sorted, inactivated *M. paratuberculosis*. Furthermore, this plasmid was successfully transformed into and subsequently recovered from *E. coli* (Figure 3, lanes 2 and 3). A comparison of this plasmid DNA (recovered pWES4) to another preparation of pWES4 that had never been transformed into *M. paratuberculosis* (control pWES4) does not show any differences in either the size or the migration pattern for both uncut and *Eco*RI-digested DNA (Figure 3, lanes 2–5). Our data also indicate that plasmid DNA can be successfully recov-

ered from inactivated *M. paratuberculosis* in a form that is suitable for further manipulation. Although recovery of plasmid DNA from inactivated *E. coli* has been previously reported (21,28), these results demonstrate that it is possible to obtain sufficient DNA from formalin-treated cells that amplification in an alternate bacterial host may not be required.

In conclusion, we have developed a novel method for manipulating recombinant pathogenic mycobacteria expressing the GFP that eliminates the necessity for performing techniques utilizing viable bacteria under biosafety level II or III conditions. Thus, studies designed to analyze intracellular survival of mycobacteria could incorporate formalin treatment of host cells after infection with mycobacteria and before FACS analysis. Specifically, recombinant strains of mycobacteria carrying the GFP gene under the control of differentially expressed promoters could be utilized to infect either primary macrophages or macrophage cell lines under the appropriate biosafety level conditions. Following this, macrophages containing intracellular bacteria could be lysed, the bacteria collected and treated with formalin, sorted via flow cytometry, and DNA extracted from the fluorescent bacteria for further analysis. This strategy has broad applications for the analysis of other pathogenic microorganisms and will provide an additional level of safety for laboratory personnel.

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