Application of Flow Cytometry and Fluorescent In Situ Hybridization for Assessment of Exposures to Airborne Bacteria

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Current limitations in the methodology for enumeration and identification of airborne bacteria compromise the precision and accuracy of bioaerosol exposure assessment. In this study, flow cytometry and fluorescent in situ hybridization (FISH) were evaluated for the assessment of exposures to airborne bacteria. Laboratory-generated two-component bioaerosols in exposure chambers and complex native bioaerosols in swine barns were sampled with two types of liquid impingers (all-glass impinger-30 and May 3-stage impinger). Aliquots of collection media were processed and enumerated by a standard culture technique, microscopy, or flow cytometry after nucleic acid staining with 4',6-diamidino-2-phenylindole (DAPI) and identified taxonomically by FISH. DAPI-labeled impinger samples yielded comparable estimates of bioaerosol concentrations when enumerated by microscopy or flow cytometry. The standard culture method underestimated bioaerosol concentrations by 2 orders of magnitude when compared to microscopy or flow cytometry. In the FISH method, aliquots of collection media were incubated with a probe universally complementary to eubacteria, a probe specific for several *Pseudomonas* species, and a probe complementary to eubacteria for detection of nonspecific binding. With these probes, FISH allowed quantitative identification of *Pseudomonas aeruginosa* and *Escherichia coli* bioaerosols in the exposure chamber without measurable nonspecific binding. Impinger samples from the swine barn demonstrated the efficacy of the FISH method for the identification of eubacteria in a complex organic dust. This work demonstrates the potential of emerging molecular techniques to complement traditional methods of bioaerosol exposure assessment.

Agricultural environments have high concentrations of airborne bacteria (21, 22). These microorganisms can be causative agents of respiratory disease through infectious, allergic, or inflammatory mechanisms (19). Exposure-response relationships for airborne bacteria and respiratory disease have not been thoroughly identified, in part because of the use of exposure assessment methods that lack sensitivity, specificity, and reliability. Currently, the predominant analytical methods used for evaluating exposures in agriculture are culture or microscopic techniques (15, 16). For culture methods, the collected airborne bacteria are grown on media so that visible colonies form, which can be subsequently identified and enumerated. However, variables in this method may include (i) the inability of general nutrient media to meet the specific growth requirements of all species, (ii) large bacterial aggregations that mask single colonies, and (iii) physicochemical trauma to organisms during collection that makes them nonviable (21). Microscopic methods are based on concentrating the bacterial cells in an air sample onto a membrane filter that is subsequently stained with a nucleotide-specific fluorochrome to facilitate the identification of cells with the aid of an epifluorescence microscope. Microscopy is slow and tedious for the enumeration of bacterial cells, and a general lack of morphological distinction between bacterial species limits their taxonomic identification.

Fluorescent in situ hybridization (FISH) involves the use of fluorochrome-labeled nucleic acid probes to target rRNA within morphologically intact cells. Single bacterial cells with fluorochrome-labeled probes hybridized to their rRNA are identifiable within mixed populations by the use of fluores-

cence microscopy or flow cytometry (1). With selective targeting of regions of rRNA, which consist of evolutionarily conserved and variable nucleotide regions, any taxonomic level ranging from kingdom to species should be able to be specifically identified. However, adequate sensitivity has been suggested to be limited to cells in an active growth phase, since the number of rRNA copies within a cell to target with a probe is proportional to the rate of growth (6). Therefore, quiescent cells, which are commonly found in environmental samples may not hybridize a sufficient quantity of a fluorochrome-labeled probe to be detectable.

Flow cytometry (FCM) is useful for the rapid identification and enumeration of bacteria from aquatic environments (14). Individual cells can be objectively identified on the basis of light scattering and fluorescence emitted from fluorochromes bound to cells. Direct quantitation of the bacterial concentration in a sample is not possible, since most flow cytometers cannot directly measure the volume of an analyzed sample. However, the sample volume can be determined indirectly with the addition of a known concentration of fluorescent polystyrene beads to the bacterial sample. The number of beads enumerated in such a sample will be proportional to sample volume, thereby allowing bacterial concentration to be calculated (20).

Here we describe the adaptation of techniques developed for aquatic microbiology, FISH and FCM, for the identification and enumeration of airborne bacteria. The techniques are demonstrated with laboratory-generated aerosols and field samples, and the sensitivity, specificity, and reliability of these techniques are evaluated. As a field sample, bioaerosols were collected from within a swine confinement building. This environment was chosen because of this laboratory's long-standing interest in the respiratory health of swine producers and the knowledge that swine barns contain many nutrients to

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FIG. 1. Contour plot showing gated data acquisition from an unstained suspension of *P. aeruginosa* in post-log-phase growth and fluorescent polystyrene beads. Data from the region of background was not subsequently collected.

support bacterial growth; these include stored feed, pig epithelium, and excrement in open manure pits just meters beneath slatted floors. It was suspected that bacteria may contain sufficient rRNA to make FISH feasible for bioaerosol identification in this environment.

MATERIALS AND METHODS

Microorganisms. *Pseudomonas aeruginosa* (ATCC 10145; American Type Culture Collection, Rockville, Md.), *Escherichia coli* (ATCC 11775), and *Micrococcus luteus* (ATCC 4698) were grown at 37°C in nutrient broth (Difco Laboratories, Detroit, Mich.). Growth was monitored spectrophotometrically at 600 nm.

Aerosol samples. A PITT no. 1 nebulizer was used to produce laboratory-generated aerosols of freshly cultured microorganisms in a 200-liter chamber, as described previously (9). Field aerosols were collected from an eastern Iowa swine confinement building. An all-glass impinger-30 (AGI; ACE Glass Co., Vineland, N.J.) and a May multistage liquid impinger (May; Burkard Manufacturing, Rickmansworth, England) were used to sample all aerosols (4, 13). The liquid collection medium for both impingers consisted of 1% peptone (Difco) in filtered (pore size, 0.2 µm) reverse-osmosis-purified H₂O–0.01% Tween 80–0.005% Antifoam A (Sigma Chemical Co., St. Louis, Mo.) (22). The total sampling time was 30 min for each instrument, with a flow rate of 12.5 liters/min into 20 ml of medium for the AGI and 20 liters/min into 8 ml of medium at each stage for the May. Following sampling, aliquots of collection media were plated onto tryptic soy agar (Difco) containing 1% cycloheximide (Sigma) for the enumeration of CFU per cubic meter of air, as previously described (22). The collection media for the purposes of quantitative analyses were fixed with 1% formaldehyde and stored at 4°C until use. From aliquots of this media, bacteria were enumerated by the microscopic techniques described previously (23), with one modification: the filters were counted for 5 min instead of a specified number of microorganisms being counted on each filter. To compute the bacterial concentration in the liquid collection media by the microscopic technique, the following formula was used:

$$\text{number of bacteria per milliliter} = \frac{\text{area of filtration (201.1 mm}^2\text{)} \times \text{number of cells counted per filter}}{\text{area of reticule (0.0019 mm}^2\text{)} \times \text{number of reticules counted per filter} \times \text{filtered sample volume (milliliters)}} \quad (1)$$

The liquid collection media for purposes of qualitative analysis were washed in phosphate-buffered saline (PBS; pH 7.2); fixed overnight in cold, fresh 4% paraformaldehyde in PBS; and stored until use in equal parts of PBS and ethanol at –20°C (1).

FCM. The media used for quantitative analysis were incubated with 4',6-diamidino-2-phenylindole (DAPI; Sigma) overnight at 4°C. Monodisperse fluorescein-tagged 3.0-µm-diameter spherical polystyrene beads (Fluoresbrite; Polysciences, Inc., Warrington, Pa.) were added to each sample. The beads in suspension had a distribution that was 85% monodisperse, regardless of whether they were discriminated by microscopy or FCM. The stock concentration of monodisperse beads used was 3×10^5 beads ml^{–1} as determined by the microscopic technique mentioned above. The samples were vortexed prior to analysis. Analyses by FCM were completed with a EPICS 753 (Coulter Electronics, Hialeah, Fla.) equipped with lasers and optical filters for the measurement of forward

light scatter, 90° light scatter, and blue, red, and green fluorescence, all on logarithmic scales. Data file management, graphics, and subpopulation analysis were performed with Windows Multiple Document Interface, available as public domain software from the Scripps Research Institute (<http://facs.scripps.edu>). Contour plots used for the graphic presentation of data connect points for which events occur in equal frequency in steps of four. Subpopulations were identified within samples with two-dimensional plots and gated for the enumeration of events within that subpopulation. The following formula was used to compute the bacterial concentration of the liquid collection media by FCM:

$$\text{number of bacteria per milliliter} = \quad (2)$$

$$\frac{\text{cell count from FCM} \times \text{volume of beads added} \times \text{original bead concentration/milliliter}}{\text{bead count from FCM} \times \text{volume of cells added}}$$

To compute the concentration of airborne bacteria in a cubic meter of air, the following formula was used:

$$\text{number of bacteria per cubic meter of air} = \quad (3)$$

$$\frac{\text{number of bacteria per milliliter} \times \text{final liquid sample volume (milliliters)}}{\text{sampling flow rate (cubic meters/minute)} \times \text{sampling time (minutes)}}$$

FISH. Commercially synthesized probes (Bio-Synthesis, Inc., Lewisville, Tex.) were tagged at the 5' end with either Texas Red (TR) or fluorescein (fl). Probe sequences were (i) TR-EUB, universally complementary to eubacteria (target site, 16S rRNA 338 to 355; 5'-GCT-GCC-TCC-CGT-AGG-AGT-3') (1); (ii) fl-PSMg, previously shown to be specific for rRNA in *Pseudomonas aeruginosa*, *P. fluorescens*, *P. mendocina*, and *Comamonas acidovorans* (target site, 16S rRNA 440 to 454; 5'-CCT-TCC-TCC-CAA-CTT-3') (5); and (iii) TR-NotEUB, complementary to TR-EUB to control for nonspecific binding (5'-ACT-CCT-ACG-GGA-GGC-AGC-3') (25).

With the use of the collection media for qualitative analysis, the methodology for FISH was as previously reported (25). The hybridization conditions included a buffer (0.9 M NaCl, 20 mM Tris HCl [pH 7.2], 0.1% sodium dodecyl sulfate, 20% formamide), a probe concentration of 1 ng/µl, and incubation at 46°C for 2 h on a microscope slide. Following incubation, a washing buffer (180 mM NaCl, 20 mM Tris HCl [pH 7.2], 0.1% sodium dodecyl sulfate, 5 mM EDTA) was used for 20 min at 54°C to remove nonhybridized probe. The microscope slides were viewed with a laser scanning confocal microscope (MRC-600; Bio-Rad Laboratories, Hercules, Calif.) in the epifluorescence mode. The standard software delivered with the instrument completed the image processing. Hybridization conditions for FISH in suspension (FISHES) were similar to those for FISH. Approximately 10⁶ cells were suspended in 100 µl of hybridization buffer in a 1.6-ml microcentrifuge tube. Hybridization was stopped after 2 h by addition of 500 µl of cold PBS. FCM was used to measure the probe-conferred fluorescence of individual cells.

FIG. 2. Relationship between DAPI fluorescence and the DAPI concentration used for DNA staining of bacterial populations measured by FCM. The populations studied over a range of concentrations were *P. aeruginosa* in log-phase growth (open squares) (at 0.01 and 0.1 µg of DAPI per ml, there were two subpopulations exhibiting different fluorescence emission), *P. aeruginosa* in post-log-phase growth (solid squares), bacterial cells in a field sample (solid circles), the debris in the same field sample (open circles), and *M. luteus* in log-phase growth (open triangle). All samples had approximately 10⁶ bacterial cells ml^{–1}, and the median DAPI fluorescence of the beads was 180.

FIG. 3. Contour plot of a swine barn bioaerosol collected in the AGI, stained with DAPI ($10 \mu\text{g ml}^{-1}$), and mixed with fluorescent polystyrene beads. The ellipsoid encloses the region of events counted as bacterial cells.

The sensitivity and specificity of the previously published probes were confirmed by using suspensions of *P. aeruginosa* and *E. coli*, both in log-phase growth. By using laser scanning confocal microscopy, the incubation of the suspensions with TR-EUB rendered both species visible by fluorescence whereas incubation with TR-NotEUB did not make either species visible. Therefore, binding of the TR-EUB to bacterial cells was due to hybridization of the probe with the targeted rRNA. Similarly, the fl-PSMg probe was demonstrated as specific for *P. aeruginosa* by fluorescence (data not shown).

RESULTS

Detection of airborne bacteria by FCM. FCM was evaluated for its capability of distinguishing bacteria from background events of instrument noise and debris collected in field samples. A contour plot of a microbial suspension was used to identify the region of background events (Fig. 1). The coordinates of this region were confirmed with a blank sample, for which all events were within the demarcated background region (data not shown). This region of background events was gated from further data acquisition. The volume of a bacterial cell is highly correlated with the parameter of forward light scatter (18). With the proximity of the *P. aeruginosa* bacterial cells to the gate rejecting events for data acquisition, the volume of these organisms was approximately the lower size limit of detection for these FCM experiments.

DAPI is a fluorochrome that internucleates with DNA in situ to allow the detection and subsequent enumeration of bacterial cells. The concentration of DAPI that is optimal for distinguishing bacterial cells from debris collected in a field sample is shown in Fig. 2. For the *P. aeruginosa* sample at

DAPI concentrations of 0.01 and $0.1 \mu\text{g ml}^{-1}$, the suspension of bacteria did not stain uniformly. FCM identified two distinct populations of approximately equal proportions but with different fluorescence emissions. The *P. aeruginosa* sample at a DAPI concentration of $10 \mu\text{g ml}^{-1}$ was optimal for discriminating bacterial cells from nonstaining debris. The difference between fluorescence emission of cells and debris was approximately 2 orders of magnitude. At a concentration of $100 \mu\text{g}$ of DAPI ml^{-1} , the magnitude of the difference between the fluorescence emission of bacterial cells and debris is lower than it was at $10 \mu\text{g}$ of DAPI ml^{-1} .

Quantitative analysis of airborne bacteria by FCM. By using the optimal DAPI concentration to stain the collection medium of a swine barn aerosol and the parameter of 90° light scatter, the population of bacterial cells could be clearly discriminated from debris and beads, as shown in Fig. 3. This region of bacterial cells was gated for enumeration of microbial suspensions and field samples. Individual microbial suspensions were studied at five dilutions to evaluate the reliability of the quantitative analysis of bacteria over a wide concentration range. Quantitative analysis by the microscopy technique was done in parallel for comparison (Table 1). By analysis with FCM, the difference in the measured concentration of bacteria from dilution to dilution was correlated ($r = 0.99$) with that expected under the assumption of perfect dilution; i.e., a 10-fold serial dilution yielded a 10-fold reduction in measured concentration. In addition, the coefficient of variation (CV) was less than 15% for each dilution of bacterial cells. This is in stark contrast to the microscopy technique, for which the CV was 170% at the 1/1,000 dilution. Subjectively, the measured bacterial concentrations were comparable between the two methods of analysis.

The culture method, microscopy technique, and FCM were compared for their usefulness in estimating the airborne bacterial concentration within a swine barn (Fig. 4). Most striking is that the culture method underestimated the microorganism concentration by approximately 2 orders of magnitude in relation to the two nonculture methods. The concentrations of bioaerosols measured by FCM and quantitative microscopy were similar. Based on the collection stages of the May for all three methods of analysis, the percentages of airborne microorganisms of different diameters were as follows: less than $4 \mu\text{m}$, 5 to 10%; 4 to $10 \mu\text{m}$, 10 to 25%; greater than $10 \mu\text{m}$, 65 to 85%.

Qualitative analysis of a laboratory-generated bioaerosol by FISH. An aerosol mixture of *E. coli* and *P. aeruginosa* was generated and collected in an exposure chamber. The liquid medium from the AGI used to collect microorganisms was incubated with the TR-EUB and fl-PSMg probes. Both bacterial species were expected to hybridize the EUB probe, but

TABLE 1. Quantitative analysis of bacterial suspensions of *P. aeruginosa* by FCM and microscopy

Dilution ^a	FCM				Microscopy			
	Mean no. of cells counted	Mean no. of beads counted	Mean no. of cells ml^{-1b}	CV (%) ^c	Mean no. of cells counted/filter	No. of reticules/filter	Mean no. of cells ml^{-1d}	CV (%)
1	4,065	60	1.1×10^7	6.3	452	10	9.6×10^6	1.6
1/10	3,049	500	9.7×10^5	3.6	116	25	9.8×10^5	6.8
1/100	711	1,200	9.4×10^4	1.8	20	50	8.5×10^4	7.2
1/1,000	92	1,400	1.0×10^4	9.4	0.5	50	2.1×10^3	170
1/10,000	21	1,400	2.4×10^3	13				

^a For each method at each dilution, $n = 4$.

^b The mean number of cells per milliliter was calculated from equation 2 with a bead volume of $166 \mu\text{l}$ and a cell volume of $314 \mu\text{l}$.

^c CV, coefficient of variation for the estimate of the mean.

^d The mean number of cells per milliliter was calculated from equation 1 with a filtered sample volume of 0.5 ml .

FIG. 4. Comparison of three analytical methods for quantitative analysis of airborne bacteria collected in a swine barn. Data shown for May Total are summed from the three individual stages of the May sampler. Both concentrations estimated by microscopy and FCM were obtained from equation 3. The final sample volumes were 18, 6.3, 6.0, and 6.6 ml for AGI, May > 10 µm, May = 4 to 10 µm, and May < 4 µm, respectively.

only *P. aeruginosa* should hybridize the PSMg probe. As shown in Fig. 5, the probes could be used effectively in conjunction with laser scanning confocal microscopy to distinguish the two gram-negative bacterial species of similar morphologies within a laboratory-generated bioaerosol.

Variables of qualitative analysis by FISH. The use of specific nucleic acid probes was shown to be a feasible method for the identification of airborne bacteria, but important constraints were identified. Table 2 presents the limits of this method when the targeted rRNA is within quiescent cells or gram-positive bacteria. The probe-conferred fluorescence of *P. aeruginosa* cells in log-phase growth incubated with TR-EUB was clearly discernible from background fluorescence emission of the same cell types incubated with TR-NotEUB. Incubation with the TR-EUB probe did not permit cells of *P. aeruginosa* isolated from a 7-day-old culture plate or cells in post-log-phase growth to be distinguished from background fluorescence emission. Like *P. aeruginosa*, the *E. coli* cells were distinguishable from background fluorescence only during log-phase growth. In contrast, the gram-positive *M. luteus* cells were not discernible from background fluorescence emission even during log-phase growth.

A second variable of using FISH for identification of airborne bacteria is the changing characteristics of the sample following the multistep fixation of the sample in preparation for incubation with probes. The forward light scatter of events following a one-step fixation protocol (Fig. 6A) is significantly different from the forward light scatter of events following the multistep fixation protocol (Fig. 6B).

Qualitative analysis of a swine barn bioaerosol by FISHES. Air samples from a swine barn were incubated with TR-EUB and TR-NotEUB probes to evaluate the sensitivity of FISHES for bioaerosols in this environment. Figure 7 shows the dot plot of the AGI sample. In comparison to the negative control probe (Fig. 7A), the EUB probe (Fig. 7B) had a greater proportion of events above the line demarcating fluorescence emission. However, the separation of specific and nonspecific

FIG. 5. A laser scanning confocal obtained micrograph after FISH with the AGI-collected medium of a laboratory-generated aerosol of *P. aeruginosa* and *E. coli*, both in log-phase growth. The probes used were TR-EUB and fl-PSMg. All cells with actual green and red fluorescence emission are colored green, and those with only red fluorescence are shown as red.

fluorescent events was suboptimal. For all three samples analyzed, there was a statistically significant difference ($P < 0.01$) in the number of positive events between the two probes, as shown in Table 3.

DISCUSSION

The ability to identify exposure-response relationships between airborne bacteria and respiratory diseases would be facilitated by using an analysis method that can enumerate all the bacteria in an air sample by taxonomic class and aerodynamic diameter. FCM and FISH have not been previously applied and evaluated for the enumeration and identification of airborne bacteria in agricultural environments.

TABLE 2. Fluorescence emission of gram-positive and gram-negative bacteria at different phases of growth after FISHES

Probe	Median red fluorescence of:				
	<i>P. aeruginosa</i> log growth	<i>P. aeruginosa</i> post-log growth	<i>P. aeruginosa</i> 7-day old culture plate	<i>E. coli</i> log growth	<i>M. luteus</i> log growth
TR-EUB	31	2	2	19	2
TR-NotEUB	2	2	2	2	2

FIG. 6. Distribution of DAPI fluorescence and forward light scatter for events from the same collection medium but fixed by different methods. (A) The medium was fixed by addition of 1% formaldehyde. (B) The medium was washed in PBS, fixed overnight in 4% paraformaldehyde in PBS, and washed twice more in PBS.

Bacterial detection and quantitation. Agricultural dusts are a complex mixture of inorganic and organic components (7). Major constituents of the organic dusts are plant fragments and pollens, fungal hyphal fragments and spores, animal epithelium, and bacteria. DAPI is a fluorochrome that binds in a highly specific fashion to DNA and is useful for discriminating aquatic bacteria from inorganic particles (17). Similarly, in this study, bacteria labeled with DAPI were discernible from non-staining particles, which probably represented inorganic debris (Fig. 2 and 3). While the particles detected in the analysis by FCM that had high DAPI fluorescence emission were not verified to be bacteria, there are several points to support the assumption. First, the *x-y* coordinates for the region containing the suspension of *P. aeruginosa* were within the gated region of the events in the field sample. Second, fungal spores typically make up less than 1% of total microorganism counts in agricultural buildings (21, 22), so that their contribution to the total counts should be insignificant. Third, and most important, the agreement of the bacterium counts between microscopy and FCM indicated that the same events were probably enumerated.

For analysis of samples by microscopy, approximately 5×10^4 bacteria had to be collected on each 25-mm filter before

FIG. 7. Distribution of red fluorescence and forward light scatter for events with the same collection medium, but incubated with different probes. Both dot plots contain 5,000 events, and the demarcation line, which was arbitrarily placed, is at a consistent *y* coordinate.

the bacteria could be enumerated with confidence. The error in microscopy for analysis of small numbers of microorganisms could be reduced by counting more fields per filter, but error from investigator fatigue would probably increase and quenching of the fluorochromes following extended UV illumination would decrease the sensitivity of the method (10). A second approach to increase the sensitivity of the microscopy method is to filter a larger volume of collection medium to obtain more bacteria on a filter. However, this can lead to loss of counts due to obscuring of bacteria by inorganic and nonstaining organic debris.

TABLE 3. Comparison between probes of the positive events after FISHES of a swine barn bioaerosol

Impinger	No. of positive events with ^a :	
	NotEUB probe	EUB probe
AGI	258	788
May (>10 μm)	440	893
May (4–10 μm)	393	798
May (<4 μm)	— ^b	—

^a A positive event was defined as an event occurring above the line in Fig. 7.

^b —, not completed.

Potential use of FCM for other environments. A foreseeable constraint of using the FCM method for environments other than agricultural ones is the physical collection of bacterial concentrations sufficient for reliable analysis. Any filtration or centrifugation of collection media to concentrate a microorganism suspension is likely to have highly variable levels of recovery (3). For the suspensions of *P. aeruginosa* that were serially diluted in this study, the lowest concentration at 1/10,000 ($\approx 10^3$ cells ml^{-1}) in Table 1 deviated furthest from a 10-fold serial dilution, yielding a 10-fold reduction in relation to concentrations of other dilutions. In aquatic environments, limits of quantitation at cell concentrations of 10^2 bacterial cells ml^{-1} are achievable (8) provided that there is good separation between background events and bacteria events and that increased time for FCM analysis of the additional volume is available.

There are many air-sampling devices that use air filtration, impaction, or liquid impingement. The liquid impingement instruments, e.g., AGI or May, with their liquid collection media are the sampling instruments most amenable to quantitative analysis by FCM. Drawbacks of using the impingers are the limited sampling time of 30 min to avoid excessive evaporative loss of media, a lower temperature of operation of about 5°C, and a fixed operational flow rate. Hence, the concentration of microorganisms that can be collected in a liquid collection medium is essentially dependent on the environment. The concentration of bacteria in the collection media in the agricultural environment in this study ranged from 10^5 to 10^6 cells ml^{-1} . Less contaminated environments, such as indoor air of homes, at 10^2 CFU/ m^3 (11), may be below the limits of detection by FCM at the current sensitivity of the detectors.

FISH. The artificial bioaerosol generated in the exposure chamber was a simple mixture of *P. aeruginosa* and *E. coli*. As expected, FISH could be used for the taxonomic identification of single cells from this bioaerosol. In contrast to this simple bioaerosol, the field sample collected in the swine barn was a complex dust. In addition, it is doubtful that all the gram-negative bacteria in the field sample contained a large amount of rRNA like the artificial bioaerosol. Table 3 indicated an increase in the number of fluorescent events for the sample incubated with EUB compared to the parallel sample incubated with Not-EUB. Hence, gram-negative bacteria that contained a sufficient quantity of rRNA for adequate analytical sensitivity were collected in this air sample. Alternatively, the aggregation of cells caused by the fixation protocol for FISHERS may have played a role. The clumped cells, which would be detected as a single event by the flow cytometer, might, when combined, have hybridized with enough probe to increase fluorescence over background.

A present constraint for the widespread application of FISH in air microbiology is the inadequate sensitivity of the method. The sensitivity of FISHERS was shown in Table 2 to be clearly dependent on stage of microorganism growth and the species of microorganism. The stage of microorganism growth is an important variable, presumably since the rate of growth is proportional to the ribosome content (6). Therefore, quiescent cells have an insufficient concentration of rRNA for probes to target. Hence, application of this technique in field studies is currently limited to environments that contain ample nutrients to support continual growth, like activated sewage sludge (24). To increase sensitivity, approaches that have achieved limited success include the use of multiple fluorochrome molecules per probe, multiple probes per rRNA, and indirect signal amplification with antibodies to hapten-labeled probes (2). Even though the *M. luteus* cells were in log-phase growth, the fluorescence of these cells hybridized with the universal probe was

indistinguishable from background. The difference in the composition of cell wall between the gram-positive *M. luteus* and gram-negative *P. aeruginosa* and *E. coli* was likely responsible for the difference in the success of FISHERS. Successful permeabilization of gram-positive bacteria to allow the entry of probes has been shown, but the treatment protocol may have to be individually tailored for each species (12).

Summary. Adequate descriptions of the relationships of bioaerosol exposures to health outcomes have often proved elusive. The development of reliable methods for the enumeration and identification of bioaerosols will strengthen the power of epidemiological investigations, assist the establishment of a criterion for the existence of a health risk, and provide direction for preventive efforts. This work supported the use of FCM for the quantitation of bioaerosols in agriculture environments. General advantages of such FCM analysis are (i) enumeration of microorganisms regardless of viability, (ii) automation to avoid operator tedium, and (iii) objective discrimination of bioaerosols from inorganic debris by multiparameter analysis which could be standardized among laboratories. A general drawback of FCM analysis is the initial cost or accessibility to a flow cytometer. FISH proved sensitive and specific for the identification of microorganisms in log-phase growth, whether in suspension or in laboratory-generated aerosols. In addition, the data for FISHERS of the field sample suggested that gram-negative bacteria could be identified in the air of a swine barn. Further work should strive to validate FISHERS for the identification of bioaerosols in agriculture. Three goals of further work are (i) enhanced separation of microorganisms from inorganic debris in FCM dot plots, (ii) signal amplification of targeted quiescent cells to increase sensitivity, and (iii) cell wall permeabilization of gram-positive bacteria to make all bacteria reliable targets of probes.

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