Fluorescence Lifetime Characterization of Bacteria Using Total Lifetime Distribution Analysis with the Maximum Entropy Method

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Total lifetime distribution analysis was employed to obtain fluorescence lifetime profiles of the intrinsic fluorescence of *Pseudomonas fluorescens, Escherichia coli, Bacillus subtilis,* and *Staphylococcus epidermidis.* The lifetimes were measured using a multiharmonic Fourier transform phase-modulation fluorometer which can simultaneously measure the phase shift and demodulation at many modulation frequencies. The 364-nm line from an argon-ion laser and the 325- and 442-nm lines from a helium–cadmium laser were used for sample excitation. Broad emission windows were used to capture as much of the bacterial emission as possible for the lifetime measurements. The maximum entropy method was used to recover lifetime profiles from the multifrequency phase-modulation data. At all three excitation wavelengths, the bacteria exhibited three lifetime components, in the ranges of 0.5–1, 2–3, and 4–8 ns. Using 325-nm excitation, a fourth component, in the range of 9–14 ns, was recovered in all of the bacteria; using 364-nm excitation, the fourth component was resolved only in the two Gram-negative bacteria (*P. fluorescens* and *E. coli*). Excitation at 364 nm provided the most reproducible lifetime profiles and showed some differences among the four bacteria.

INTRODUCTION

The morphological similarities of bacteria make it impossible to identify most bacteria based upon the physical appearances of the cells. Traditional identification procedures employ biochemical tests that distinguish between the physiological differences of bacteria. The tests reveal the metabolic profiles and antibiotic immunities, among other things, of the isolated bacteria which are the basis for the taxonomic divisions. The time required to identify an isolated culture correctly can range from a few minutes to a few days, depending on the number and type of tests needed for a positive iden-

tification. Commercially available identification systems are available which simultaneously perform several tests and then provide a probabilistic identification to the user.[1–4] These systems require 4 to 24 h to identify correctly unknown isolates to the species, and sometimes subspecies, level. Newer techniques for identifying bacteria have been developed in recent years that involve the use of DNA and RNA probes.[5–10]

Previous work has been done in the endeavor to use fluorescence measurements as a means of bacterial identification. These include the use of fluorescent probes[11] and enzyme assays of fluorescent substrates or products,[12–14] as well as measurements of the spectral and lifetime characteristics of intrinsic fluorophores.[15–18] Fluorescence at short excitation wavelengths (250–300 nm) is dominated by protein fluorescence, particularly

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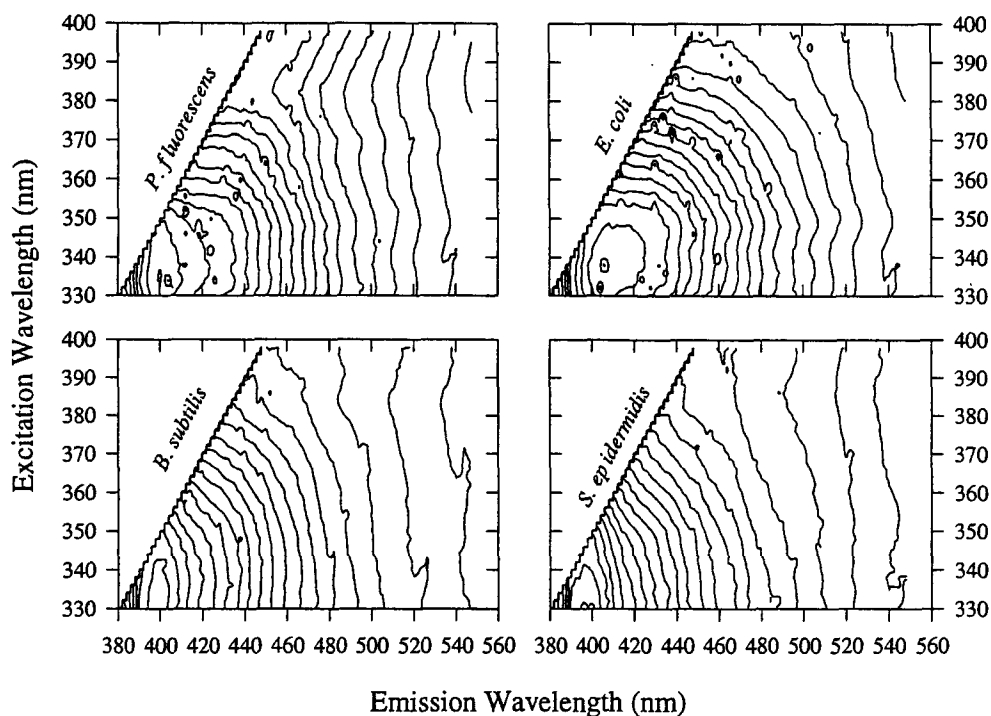[2] To whom correspondence should be addressed.

Fig. 1. Fluorescence excitation emission matrices (EEMs) of the four bacteria.

from tryptophan residues, and is not likely to provide much discrimination. Intrinsic fluorescence at longer wavelengths is due to a wider variety of compounds and may therefore show greater variation among different genera of bacteria. Even bacteria with similar spectral properties may show some differences in their decay profiles due to heterogeneity of fluorophores and the microenvironments experienced by those fluorophores in the bacteria.

In order to evaluate the possibility of lifetime discrimination, it is necessary to characterize fully the lifetime profiles of the complex fluorescence decay of bacteria. Previous studies[16-18] have used measurements at several excitation wavelengths and narrow bandpass filters in the emission channels, coupled with nonlinear least-squares data analysis. Such an approach uses only a fraction of the total emission in the lifetime measurement, which reduces the signal-to-noise ratio. The recovered lifetime profile of complex samples may also be oversimplified through reliance on conventional nonlinear least-squares data analysis, in which the decay data are fitted to models of a few discrete lifetime components.

Detailed characterization of the total fluorescence decay of complex samples can be performed using total lifetime distribution analysis (TLDA).[19] In TLDA, a commercially available, multiharmonic Fourier trans-

form phase-modulation fluorometer (MHF) provides simultaneous measurements of the phase and modulation of a fluorescence signal at multiple modulation frequencies,[20] from which the lifetime profile is recovered. Instead of measuring fluorescence lifetimes using a narrow emission window, measurements in TLDA are made over a broad window that is selected to include all or most of the sample emission. Using this approach, we obtain a complete lifetime profile which includes all of the different fluorophores which contribute to the signal. Moreover, inclusion of the entire sample emission in the lifetime measurement provides a greater intensity for an improved signal-to-noise ratio.

The frequency-domain lifetime data that are collected on the MHF are analyzed using the self-modeling maximum entropy method (MEM).[21,22] MEM finds a solution that satisfies the dual criteria of minimization of the χ^2 goodness-of-fit function and maximization of the Shannon-Jaynes statistical entropy function. It recovers a lifetime distribution in which the area under each lifetime peak represents the fractional intensity of that lifetime component. The advantages of MEM over conventional nonlinear least squares for fluorescence lifetime analysis are that it requires no prior knowledge or assumptions about the number or distribution of lifetime components and it recovers a single, unique solu-
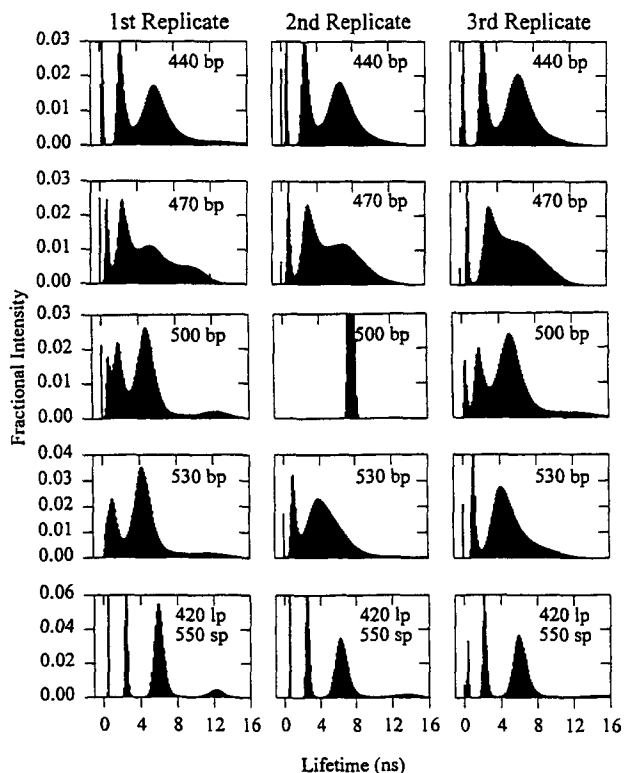
Fig. 2. MEM lifetime profiles for triplicate samples of *P. fluorescens* (excitation at 364 nm) using five emission windows. bp = bandpass, lp = longpass, sp = shortpass.

Fig. 3. Average lifetimes and fractional intensities of triplicate results in Fig. 2. Error bars indicate ±1 SD.

tion. This is important for complex samples, which generally are not well represented by simple models of a few discrete lifetime components.

In this paper, we describe the TLDA of four genera of bacteria. Comparison of the recovered profiles at three excitation wavelengths is used to determine the feasibility of identifying or classifying bacteria through their intrinsic fluorescence lifetimes. In a broader context, detailed analysis of the intrinsic fluorescence of bacteria is an important step toward the use of fluorescence, either intrinsic or extrinsic, as a tool for studying bacterial cultures.

EXPERIMENTAL

Gram-negative bacteria *Pseudomonas fluorescens* and *Escherichia coli* and Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus epidermidis* were obtained from Central Scientific. Stock plates of each of the four genera were grown on Trypticase-nutrient agar plates (Becton–Dickinson) and stored in a refrigerator at 5°C. Sample plates of Trypticase-nutrient agars were inoculated with cells from the stock plates, and the bacteria
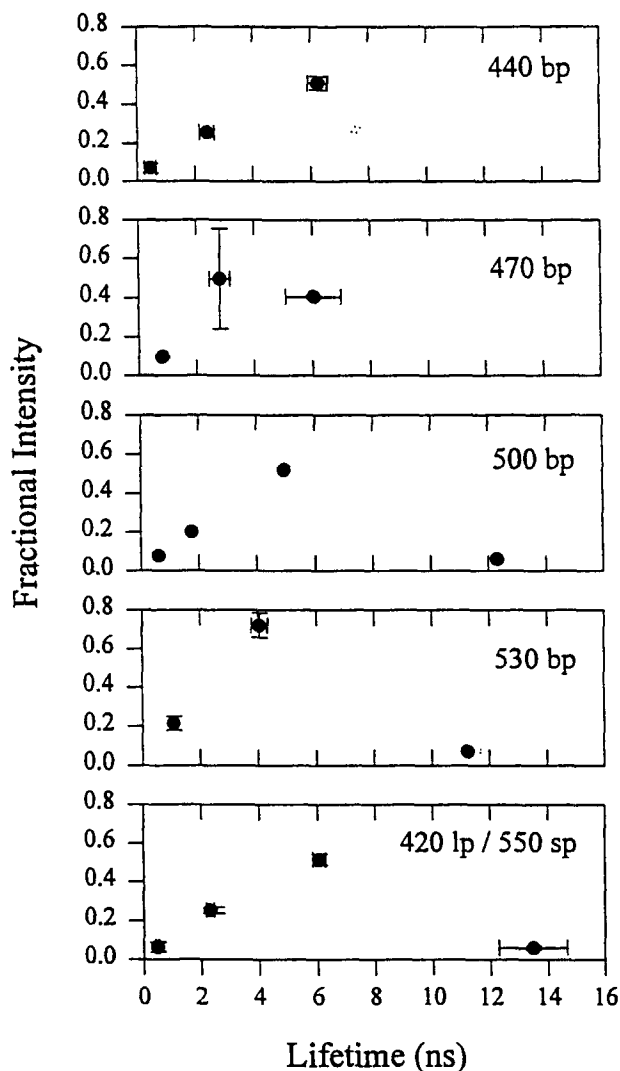
were grown for 24 h before use. Cells were scraped from the sample agars and washed three times in a phosphate-buffered saline solution (Sigma; 10 mM phosphate, 120 mM NaCl, 2.7 mM KCl, pH 7.4). After washing, the bacteria were suspended in 100% ethanol and sonicated for about 15 min. The suspensions were then set aside for approximately 15 min to allow the larger cell aggregates to settle. A portion of the supernatant was diluted with ethanol to lower the absorbance of the solution to 1–0.9 AU at 364 nm.

Fluorescence excitation–emission matrices (EEMs) were collected for each genus of bacteria using a SLM 48000S spectrofluorometer (Spectronic Instruments, Inc.) with a 450-W xenon arc lamp for sample excita-
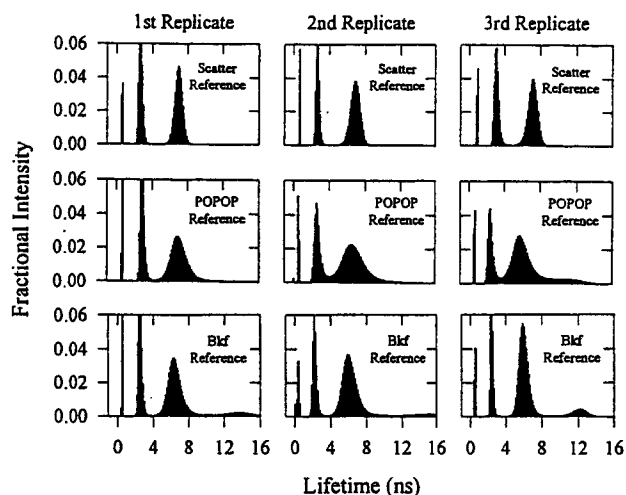
Fig. 4. MEM lifetime profiles for triplicate samples of *P. fluorescens* (excitation at 364 nm), measured vs three lifetime references.
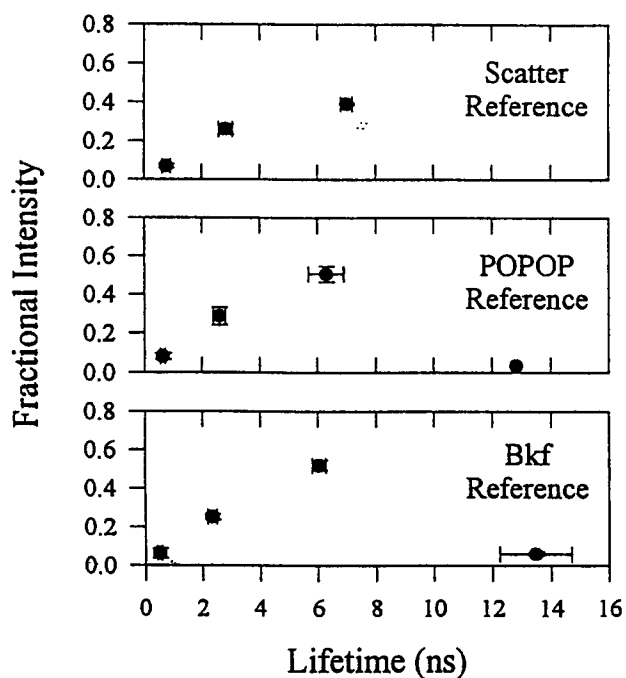

Fig. 5. Average lifetimes and fractional intensities of triplicate results in Fig. 4. Error bars indicate ± 1 SD.
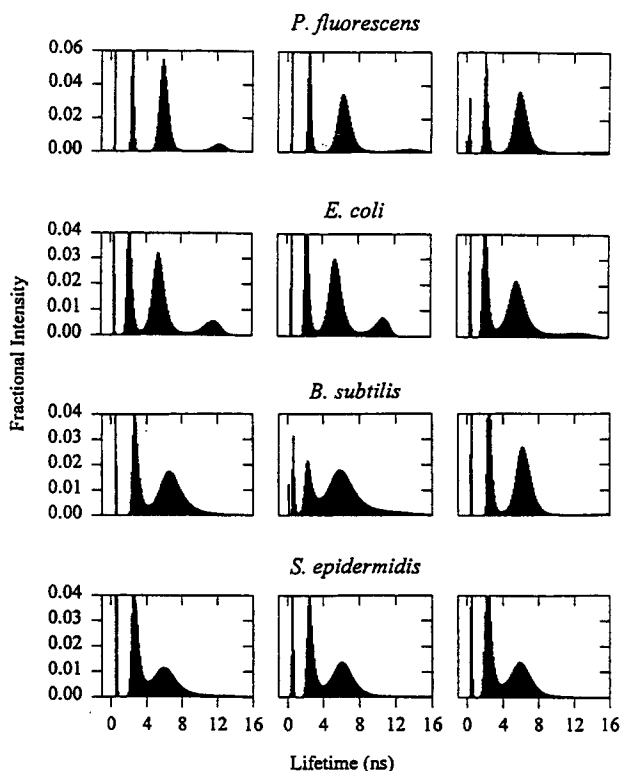

Fig. 6. MEM lifetime profiles for triplicate samples of the four bacteria using excitation at 364 nm, BkF as the lifetime reference, and the emission window defined by a 420-nm longpass/550-nm shortpass filter combination.

tion. Wavelengths ranged from 330 to 400 nm for excitation and 380 to 550 nm for emission. All monochromator slit widths were set to 4 nm. A solution of pure ethanol was measured after each sample for blank correction.

Lifetime measurements were performed using a multiharmonic Fourier transform phase-modulation flu-

orometer (Model 4850 MHF; Spectronic Instruments, Inc.). In the first series of experiments, a water-cooled argon-ion laser (Model Innova 307; Coherent) tuned to the 363.8-nm line (power ≥115 mW) was used for sample excitation. The lifetime profiles of *P. fluorescens* were measured versus a benzo(k)fluoranthene (BkF) lifetime reference ($\tau \approx$ 7.4–7.7 ns), using different filters to define the emission window. The filters included 440-, 470-, 500-, and 530-nm bandpass filters (10-nm bandwidth) and a broad window defined by the 420-nm longpass/550-nm shortpass filter combination. The lifetime profiles of *P. fluorescens* were also measured, using the broad emission window, versus scattered light (τ = 0 ns) and POPOP (τ = 1.3 ns) lifetime references. The lifetime profiles of all four bacteria were then measured versus the BkF reference using the 420-nm longpass/550-nm shortpass filter combination. Each of these sets of experiments was repeated on 3 days using freshly grown samples.

In the second series of experiments, the lifetimes of the bacteria were measured using the 325-nm (12-mW) or 442-nm (50-mW) laser line from a helium–cadmium
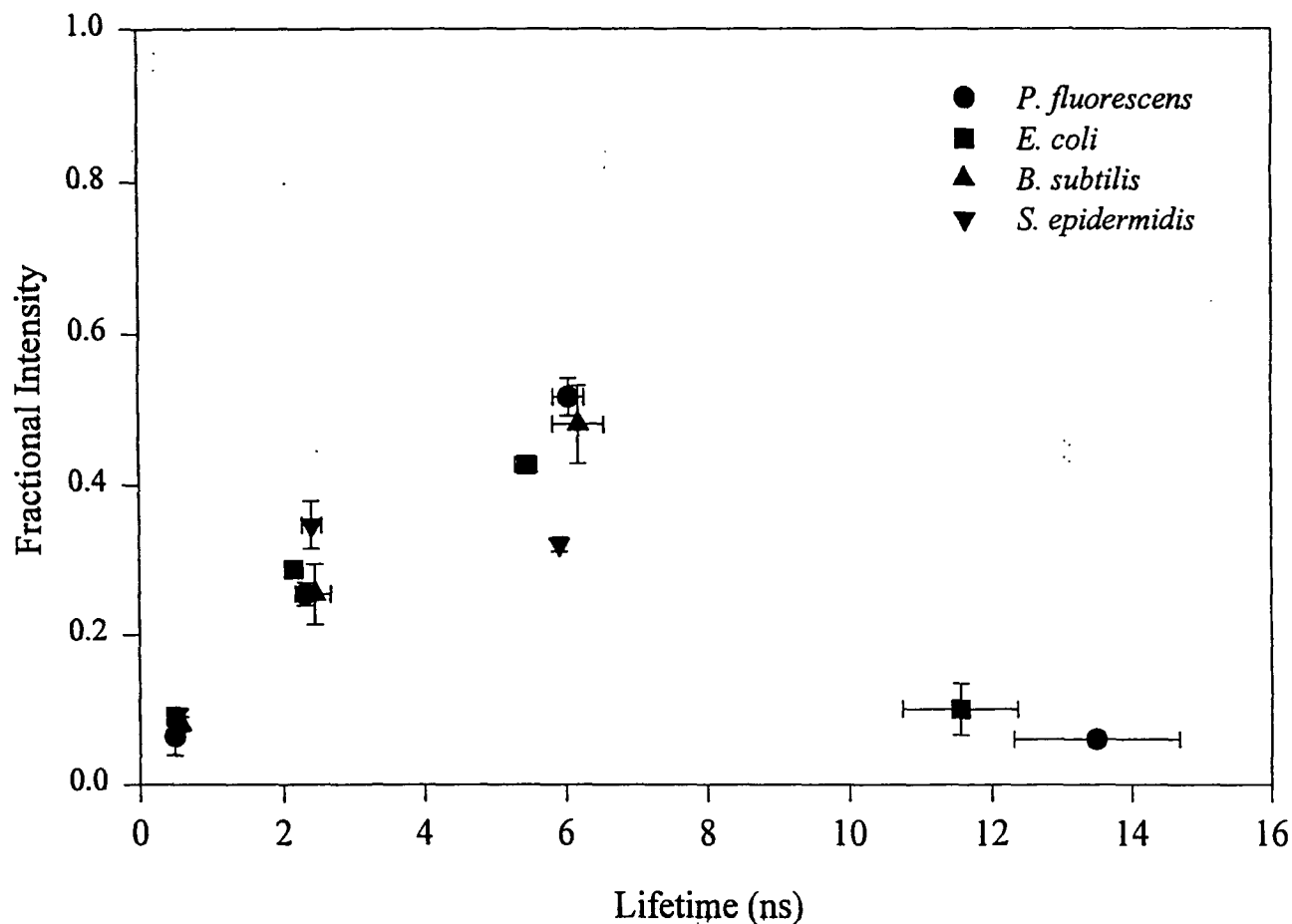
Fig. 7. Average lifetimes and fractional intensities of triplicate results in Fig. 6. Error bars indicate ± 1 SD.

laser (Model 4240NB; Liconix) for sample excitation. For experiments employing 325-nm excitation, BkF was used as the reference fluorophore and the emission window was defined by a 380-nm longpass/550-nm short-pass filter combination. For experiments involving 442-nm excitation, perylene (τ = 3.8 ns) was used as the reference fluorophore and the emission window was defined by a 520-nm longpass filter.

The lifetime profile of each sample was determined in triplicate, using the average of 10 pairs of sample-reference measurements for each determination. Each of the 10 measurements in a determination was the internal average of 100 signal samplings. The three data files for the triplicate determinations were linked, and the linked file was analyzed using maximum entropy method (MEM) software from Maximum Entropy Data Consultant Ltd. (Cambridge, UK). The lifetime window used in the MEM analysis ranged from 0.01 to 40 ns, divided into 300 evenly spaced cells. The wide-ranging lifetime window was used in order to provide the program with

a place to put the long and short noise. Only the range of 0.01 to 16 ns is shown in the figures since little information was contained above 16 ns. Lifetime peaks were integrated using the MEM software to obtain the mean lifetime and fractional intensity of each peak. Several of the MEM lifetime profiles also were analyzed using GRAMS/386 peak integration software, in which the peaks are approximated by Gaussian functions. However, there were no significant differences between the MEM integration and the GRAMS/386 integration, so only the MEM results are presented.

RESULTS

The fluorescence excitation–emission matrices (EEMs) of the four genera are shown in Fig. 1. The spectra are dominated by a single, broad peak around 320- to 350-nm excitation and 380- to 420-nm emission. The peak maximum of *S. epidermidis* is farthest to the
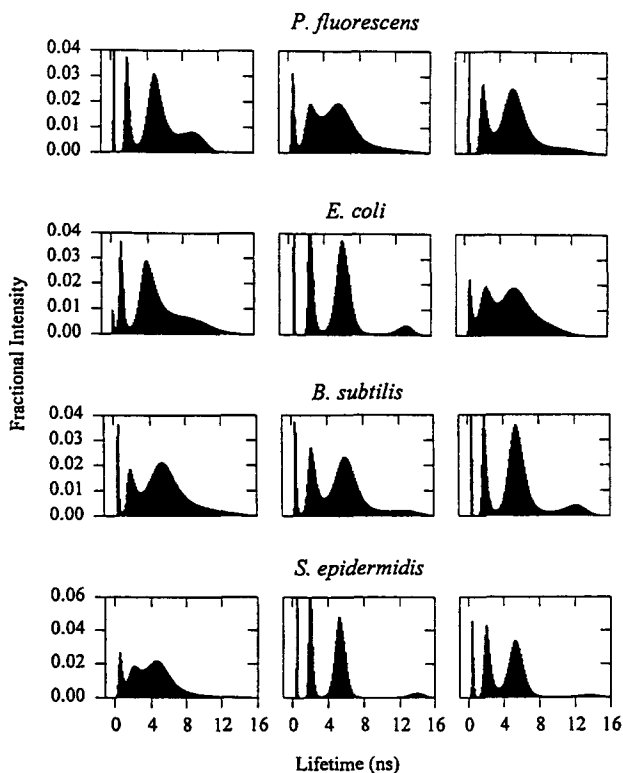
Fig. 8. MEM lifetime profiles for triplicate samples of the four bacteria using excitation at 325 nm, BkF as the lifetime reference, and the emission window defined by a 380-nm longpass/550-nm shortpass filter combination.

blue, and *P. fluorescens* and *E. coli* are farthest to the red. The only significant contribution from the blank was from scattered light. Dilution of the samples resulted in proportional decreases in sample fluorescence, indicating that the samples were in the linear fluorescence range despite their high absorbances.

Lifetime measurements were originally attempted with the cells suspended in an aqueous phosphate-buffered saline solution, but the fluorescence signal was too weak for lifetime measurements through the bandpass filters. Significant enhancement was achieved by suspending the cells in ethanol, which was therefore used for all spectral and lifetime measurements. The ethanol killed the bacteria, which may have disrupted some of the cells or separated fluorophores from enzymes or other binding environments that could quench the fluorescence. When the cells were filtered out of the suspension, the filtrate produced a small fluorescence signal. This indicated that the ethanol leached a few fluorophores out of the bacteria, but the majority remained within the cells.

Figure 2 shows the lifetime profiles of the three replicates of *P. fluorescens* that were recovered using

each of five emission windows and excitation at 364 nm. The replicates show good reproducibility for all of the lifetime windows except the second replicate for the 500-nm bandpass window. This anomalous profile is thought to be the result of entering the wrong reference lifetime into the computer software when the data were being collected. Figure 3 is a different representation of the results shown in Fig. 2. Each of the lifetime peaks recovered by MEM is represented by the lifetime at the peak maximum and the fractional intensity which is calculated from the peak area. This representation allows a more quantitative comparison among different profiles. The results shown in Fig. 3 are the averages of the peak lifetimes and fractional intensities for the three replicate samples for each emission window. Standard deviations are shown as well, except for the 500-nm bandpass window, since only the first and third replicates were used.

Several trends are observed in Figs. 2 and 3. The peaks are sharpest, indicating the best lifetime resolution, for the 420-nm longpass/550-nm shortpass window. There is a gradual decrease in the second and third lifetimes as the bandpass emission window is moved to longer wavelengths until, using the 530-nm bandpass, there are only two lifetimes below 10 ns. There is also a longer lifetime in the range of 9–14 ns that begins to appear in the 500- and 530-nm bandpass filters and is clearly visible in the 420-nm longpass/550-nm shortpass window. Interestingly, the 440-nm bandpass window and the 420-nm longpass/550-nm shortpass window resulted in very similar lifetime profiles with the exception of the appearance of the fourth, long-lifetime component in the latter.

The recovered lifetime profiles varied with the different bandpass filters because of the changing contributions to the spectrum as the emission window was redefined by successive filters. It is not surprising, therefore, that four peaks were recovered for the broad 420-nm longpass/550-nm shortpass window, which passes most of the total emission signal, while the bandpass windows generally revealed only three components. The failure to resolve four components when the bandpass filters were used is due in some cases to the spectral window and in others to the relatively poor signal-to-noise ratio, which broadens the peaks beyond complete resolution. Previous studies have shown that the widths of the peaks recovered by MEM increase as the signal-to-noise ratio decreases,[23] and this effect can be seen in the MEM plots in Fig. 3. Noise may also be seen in a single lifetime cell at 0 ns (short noise) or a small peak at 40 ns (long noise; not shown in the figures).

In order to determine the effects of lifetime reference on the recovered lifetime profiles in the TLDA ex-
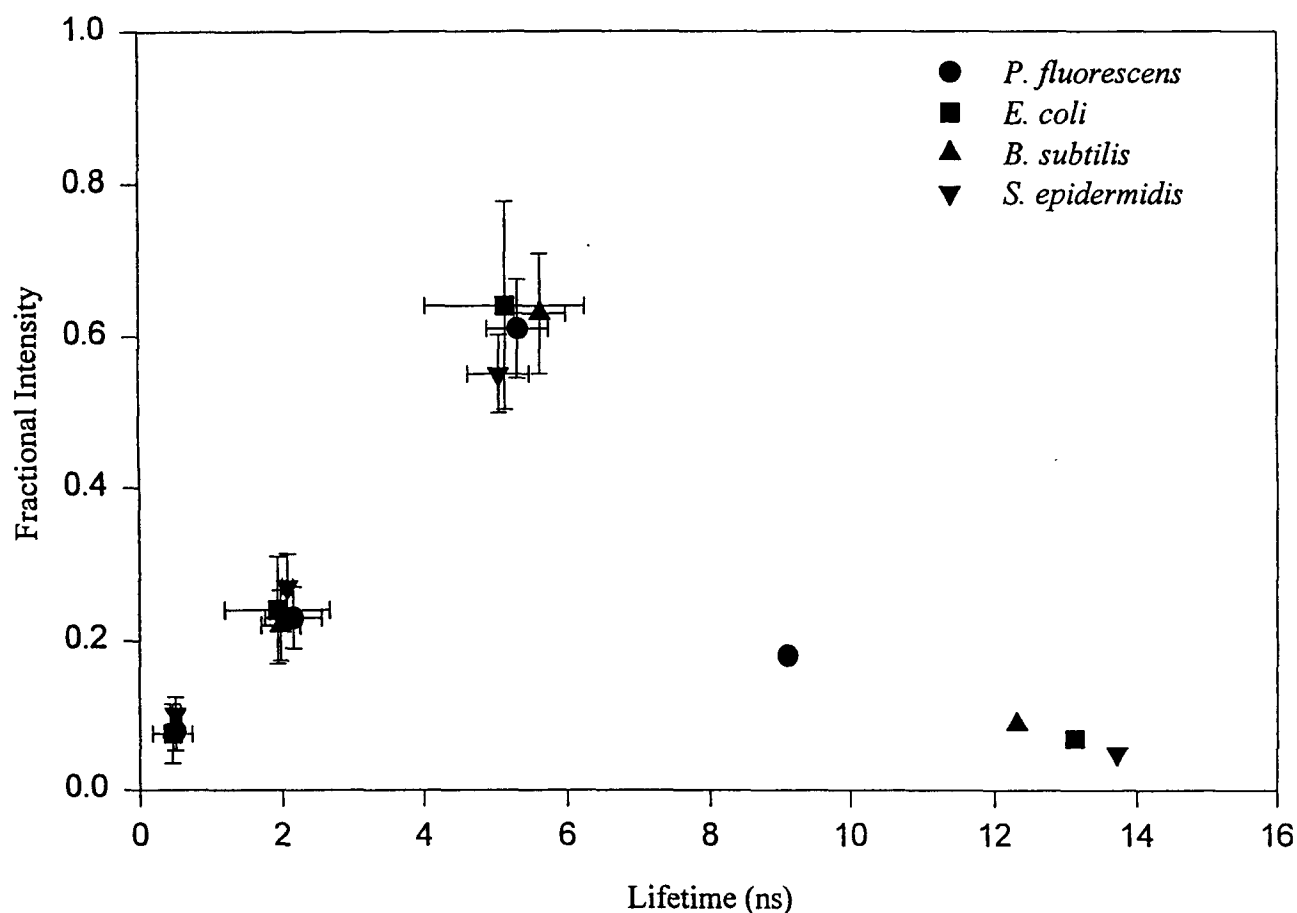
Fig. 9. Average lifetimes and fractional intensities of triplicate results in Fig. 8. Error bars indicate ± 1 SD.

periment, *P. fluorescens* was measured using three references: scattered light, POPOP, and BkF. As shown in Figs. 4 and 5, all three references resulted in three well-defined lifetime peaks. POPOP shows a possible fourth lifetime longer than 10 ns. The use of BkF as the lifetime reference allowed resolution of the fourth, long-lived component, thereby shortening the lifetimes of the other three components by removing the influence of the unresolved, long component. The dependence of the lifetime results on the lifetime reference is most likely due to the complexity of the bacterial decay. There is uncertainty associated with the multifrequency phase-modulation data of both the sample and the reference. This uncertainty affects the ability of MEM to resolve the various lifetime components. Errors in lifetime recovery due to the reference fluorophore are thought to be reduced by using references with lifetimes and spectral properties similar to those of the sample.[24,25] It follows that a longer-lifetime reference such as BkF would be better able to resolve the long-lifetime components in

the bacteria. It should be noted that spectral similarity between sample and reference, which is desirable in order to minimize the detector color effect, has been shown to be less of a problem when MEM is used instead of NLLS to analyze the data.[26]

The lifetime profiles of the four genera of bacteria excited at 364 nm are shown in Fig. 6. The lifetimes were measured versus BkF using the 420-nm long-pass/550-nm shortpass filter combination to define the emission window. For each genus, the MEM analyses of these data show excellent consistency between replicates. All of the genera have three primary lifetime peaks, and the Gram-negative *P. fluorescens* and *E. coli* have a fourth peak at approximately 13 ns. The third, longest-lifetime peaks in the MEM profiles of *B. subtilis* and *S. epidermidis* have long "tails," which suggest the possibility of a longer lifetime component, but a fourth component could not be reproducibly resolved. Figure 7 shows the average peak lifetimes and fractional intensities for each of the bacteria. The peak lifetimes of the
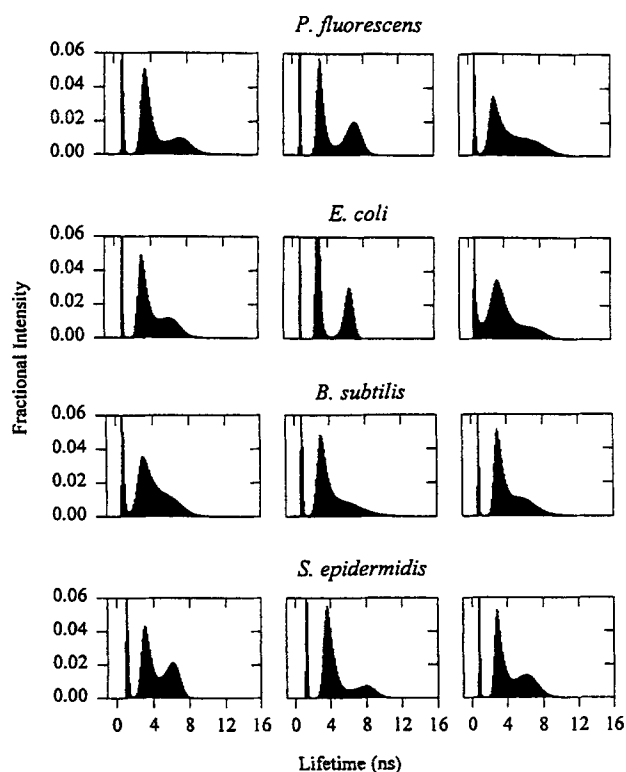
Fig. 10. MEM lifetime profiles for triplicate samples of the four bacteria using excitation at 442 nm, perylene as the lifetime reference, and the emission window defined by a 520-nm longpass filter.

three peaks common to the four genera are similar, but there are measurable differences among their fractional intensities, particularly of the third lifetime around 6 ns.

The lifetime profiles from 325-nm excitation were not as reproducible as those obtained from 364-nm excitation. Figure 8 shows the large variation in the recovered lifetime distributions, which resulted in large standard deviations for the lifetimes and fractional intensities (Fig. 9). Because of the large deviations, there are no statistical differences among the lifetimes or the fractional intensities of the four bacteria. For all four bacteria, the fourth lifetime component does not have standard deviation bars because it was resolved in only one or two of the three replicates. The large amount of noise in the data collected with excitation at 325 nm was probably due to the relatively low power of the laser line. The 325-nm line had only 12 mW of power, compared to 50 mW from the 442-nm line and at least 115 mW from the 364-nm line.

Excitation at 442 nm resulted in slightly better reproducibility relative to that at 325 nm (Figs. 10 and 11). For the Gram-negative *P. fluorescens* and *E. coli*, the third lifetime component does not have standard de-

viation bars in Fig. 11 because it was resolved in only two of the three replicates. This lack of resolution is the cause of the large error bars for the fractional intensities of the second lifetime for these two bacteria. The lifetime profiles for *B. subtilis* had large shoulders on the second lifetime peak that were never resolved into an actual third lifetime. This resulted in the small error bars for *B. subtilis* in Fig. 11.

DISCUSSION

Living bacteria may contain varying amounts of certain essential molecules such as NADH, FAD, FMN, pterin compounds, and folic acid that may fluoresce under favorable conditions in the spectral regions used in this work.[17] The fluorescence may vary not only among different genera, but also among different cultures of the same genus, due to environmental and nutritional influences and metabolic pathways. The results for the four bacteria in this work show that if the bacteria are grown under identical nutrient conditions, the four genera show some differences among their lifetime profiles, particularly when excited at 364 nm, where the best reproducibility was obtained. However, it should be noted that the lifetime profiles may be dependent not only upon nutrition, but also upon the incubation temperature and the growth stage of the culture.[27]

The lifetime profiles recovered by MEM analysis include broad peaks which may be comprised of contributions from several different fluorophores with similar lifetimes. The bacteria exhibit three such lifetime components, in the ranges of 0.5–1, 2–3, and 4–8 ns at all three excitation wavelengths. All of the bacteria also show a fourth, long component of 9–14 ns in one or more of the replicates when excited at 325 nm. The fourth component is recovered only for the Gram-negative when excited at 364 nm, and not at all when excited at 442 nm. This long-lifetime component is therefore associated with absorption at shorter wavelengths and is more prominent in the Gram-negative bacteria. The lifetimes of the three main components increase as the excitation wavelength increases. Aside from these differences, excitation at 325 and 364 nm resulted in similar lifetime profiles in which the fractional intensities were greatest for the third lifetime component. Excitation at 442 nm resulted in distinctly different lifetime profiles in which the second lifetime component provided the largest fractional contribution to the total intensity.

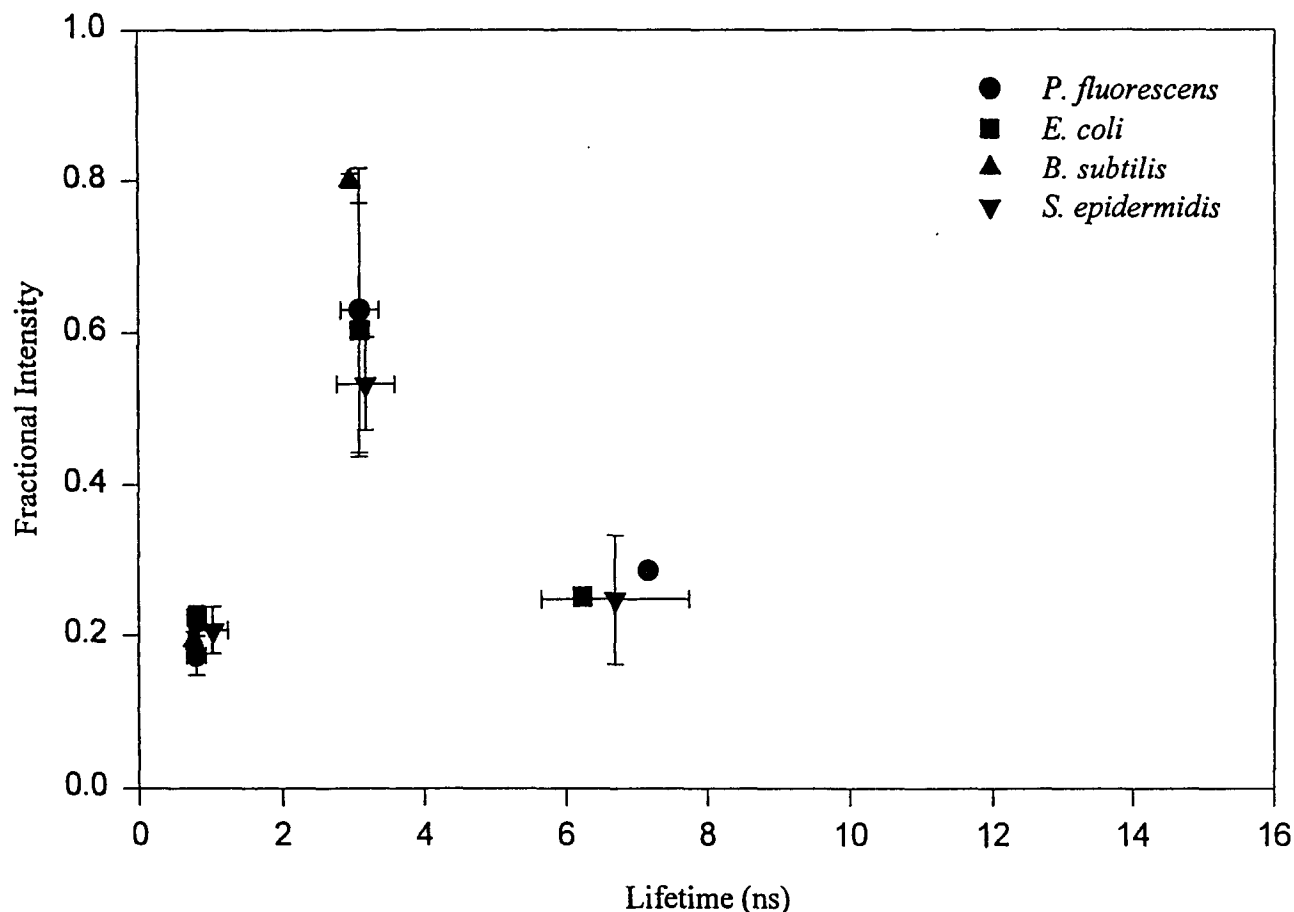The lifetime results of this work show some differences from the results previously reported for these bac-

Fig. 11. Average lifetimes and fractional intensities of triplicate results in Fig. 10. Error bars indicate ± 1 SD.

teria using similar excitation wavelengths.[17] Although the lifetimes fall into the same general ranges, the previous report often recovered only two components or, in the case of *P. fluorescens*, one component. These differences arise from the use of narrow bandpass filters and nonlinear least-squares data analysis with simple, discrete component models in the previous work, compared to broad emission windows and self-modeling MEM analysis in TLDA. The lifetime profiles from TLDA provide a more complete representation of the total, complex fluorescence decay at each excitation wavelength.

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REFERENCES

1. P. S. Amy, D. L. Haldeman, D. Ringelberg, D. H. Hall, and C. Russell (1992) *Appl. Environ. Microbiol.* **58**, 3367.
2. B. Holmes, M. Costas, M. Ganner, S. L. W. On, and M. Stevens (1994) *J. Clin. Microbiol.* **32**, 1970.
3. B. Holmes, M. Costas, T. Thaker, and M. Stevens (1994) *J. Clin. Microbiol.* **32**, 2221.
4. W. J. Looney, A. J. C. Gallusser, and H. K. Modde (1990) *J. Clin. Microbiol.* **28**, 1519.
5. R. Amann, W. Ludwig, and K.-H. Schleifer (1992) *FEMS Microbiol. Lett.* **100**, 45.
6. W. Manz, U. Szewzyk, P. Ericsson, R. Amann, K.-H. Schleifer, and T.-A. Stenström (1993) *Appl. Environ. Microbiol.* **59**, 2293.
7. R. I. Amann, L. Krumholz, and D. A. Stahl (1990) *J. Bacteriol.* **172**, 762.
8. E. F. DeLong, G. S. Wickham, and N. R. Pace (1989) *Science* **243**, 1360.
9. M. N. Widjojoatmodjo, A. C. Fluit, and J. Verhoef (1995) *J. Clin. Microbiol.* **33**, 2601.
10. R. I. Amann, W. Ludwig, and K.-H. Schleifer (1995) *Microbiol. Rev.* **59**, 143.
11. D. C. Shelly, I. M. Warner, and J. M. Quarles (1983) *Clin. Chem.* **29**, 290.
12. J. T. Coburn, F. E. Lytle, and D. M. Huber (1985) *Anal. Chem.* **57**, 1669.

13. C.-P. Pau, G. Patonay, C. W. Moss, G. M. Carlone, T. M. Rossi, and I. M. Warner (1986) *Clin. Chem.* **32**, 987.

14. C.-P. Pau, G. Patonay, C. W. Moss, D. Hollis, G. M. Carlone, B. D. Plikaytis, and I. M. Warner (1987) *Clin. Chem.* **33**, 337.

15. M. J. Sorrell, J. Tribble, L. Reinisch, J. A. Werkhaven, and R. H. Ossoff (1994) *Lasers Surg. Med.* **14**, 155.

16. S. K. Brahma, M. P. Baek, D. Gaskill, R. K. Force, W. H. Nelson, and J. Sperry (1985) *Appl. Spectrosc.* **39**, 869.

17. R. A. Dalterio, W. H. Nelson, D. Britt, J. F. Sperry, J. F. Tanguay, and S. L. Suib (1987) *Appl. Spectrosc.* **41**, 234.

18. R. A. Dalterio, W. H. Nelson, D. Britt, J. Sperry, D. Psaras, J. F. Tanguay, and S. L. Suib (1986) *Appl. Spectrosc.* **40**, 86.

19. L. B. McGown, S. L. Hemmingsen, J. M. Shaver, and L. Geng (1995) *Appl. Spectrosc.* **49**, 60.

20. G. Mitchell and K. Swift (1990) *SPIE Proc.* **1204**, 270.

21. J. C. Brochon, A. L. Livesey, J. Pouget, and B. Valeur (1990) *Chem. Phys. Lett.* **174**, 517.

22. A. L. Livesey and J. C. Brochon (1987) *Biophys. J.* **52**, 693.

23. J. M. Shaver and L. B. McGown (1996) *Anal. Chem.* **68**, 9.

24. J. R. Lakowicz, H. Cherek, and A. Balter (1981) *J. Biochem. Biophys. Methods* **5**, 131.

25. D. A. Barrow and B. R. Lentz (1983) *J. Biochem. Biophys. Methods* **7**, 217.

26. J. M. Shaver and L. B. McGown (1996) *Anal. Chem.* **68**, 611.

27. B. Bronk and L. Reinisch (1993) *Appl. Spectrosc.* **47**, 436.