Steady-State and Time-Resolved Spectroscopy of F420 Extracted from Methanogen Cells and Its Utility as a Marker for Fecal Contamination

Kyle D. Ashby,[†] Thomas A. Casey,*[,‡] Mark A. Rasmussen,*[,‡] and Jacob W. Petrich*[,†]

Department of Chemistry, Iowa State University, Ames, Iowa 50011-3111, and National Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, Ames, Iowa 50010

Methanogenic bacteria, which are common inhabitants of the animal digestive tract, contain the fluorescent compound F420 (coenzyme 420), a 7,8-didemethyl-8-hydroxy-5-deazariboflavin chromophore. F420 was characterized as an initial step in determining if this compound would be useful as a fluorescent marker for the detection of fecal and ingesta contamination. Using a single anion exchange chromatographic process, F420 was separated from other cell components of a *Methanobrevibacter* sp. cell culture. The extent of separation was determined spectroscopically. To aid in the development of possible techniques for the detection of fecal contamination using F420 as a marker, further spectroscopic investigation of F420 was conducted using steady-state and time-resolved fluorescence methods. The fluorescence lifetime of F420 in an elution buffer of pH 7.5 was found to be 4.2 ns. At higher pH values, the fluorescence decay, $F(t)$, was best described by a sum of two exponentials: at pH 13, $F(t) = 0.31 \exp(-t/4.20\ \text{ns}) + 0.69 \exp(-t/1.79\ \text{ns})$. Further investigation using front-faced fluorescence techniques has shown that emission from F420 can be collected efficiently from samples of methanogen cell cultures as well as from fecal material.

Keywords: *Fluorescence spectroscopy; fluorescent marker; F420; methanogen; methanogenic bacteria; meat contamination*

INTRODUCTION

Food safety is an important and continuing concern for consumers and the food industry. Human diseases caused by *Salmonella*, *Escherichia coli* O157:H7, *Listeria* and other pathogens are often traced to consumption of contaminated food. Human foodborne pathogens may be present in feces of livestock, and carcasses from these animals can be contaminated with feces during slaughter. Currently, to prevent fecal contamination, the Food Safety Inspection Service (FSIS) mandates "zero tolerance" for visually apparent fecal contamination on carcasses. FSIS also requires bacteriological culture on random carcasses as an indication of fecal contamination. These current methods for assessing "zero tolerance" are insensitive, subject to worker fatigue, time-consuming, and labor intensive. Alternative markers for fecal contamination, which are not subject to these limitations, would be of benefit to the meat processing industry.

A study was initiated to identify fluorescent markers useful for the detection of fecal and ingesta contamination. Others have observed that fecal matter commonly contains material that is fluorescent in regions of the visible spectrum (*1*). In their work, Anderson et al. (*1*) investigated the possibility of using fluorescence to identify and differentiate dietary components. Our work investigated the phenomena of fecal fluoresence from a food safety perspective because fecal contamination can carry pathogenic bacteria onto animal carcasses and meat products during processing. Subsequent consumption of this contaminated food can result in illness and, with certain foodborne pathogens, death.

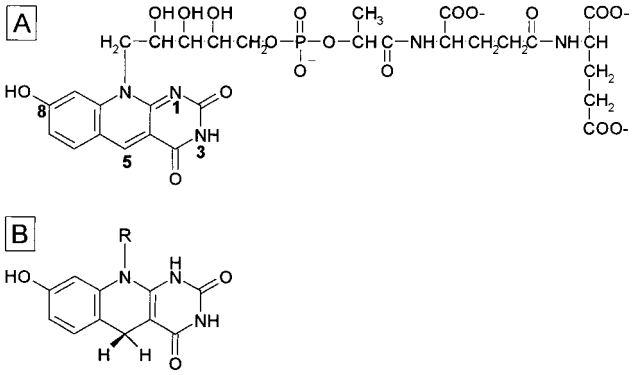

Figure 1. Structure of the methanogen electron carrier, F420; (A) is the oxidized, fluorescent form of F420, and (B) is the reduced, nonfluorescent form.

We conducted a survey of fluorescent compounds known to occur in bacteria that are common inhabitants of the anaerobic environment of the gut. Methanogens, which have a characteristic blue-green fluorescence, are one group of bacteria commonly found in the gut of animals. This characteristic is due to the presence of a fluorescent coenzyme, F420 (Figure 1), in the methanogen cells. Coenzyme F420 is ubiquitous among methanogens and serves metabolically as an electron donor for the reduction of carbon dioxide to methane (*2*−*4*). Its trivial name is derived from the 420 nm absorbance maximum observed for the oxidized form of the coenzyme (*5*). This form of the molecule (Figure 1A)

* Authors to whom correspondence should be addressed (e-mail tcasey@nadc.ars.usda.gov, mrasmuss@nadc.ars.usda.gov, or jwp@iastate.edu).

[†] Iowa State University [fax (515) 294-0105].

[‡] U.S. Department of Agriculture [fax (515) 663-7669].

10.1021/jf000689r CCC: $20.00
Published on Web 01/26/2001

exhibits a blue-green fluorescence at 470 nm with a quantum yield of fluorescence, ϕ_f, of 0.17−0.18, which we and others have measured using tryptophan as a reference (*6*). This fluorescent property has also been used by microbiologists to identify methanogenic bacteria by fluorescence microscopy (*7*, *8*).

The spectral features of oxidized F420 are pH dependent (*2*, *9*, *10*), and in acidic media, the absorbance maximum at 420 nm shifts to 380 nm (*7*, *11 12*). There is an isosbestic point at 401 nm with an extinction coefficient of 2.59×10^4 M^{-1} cm^{-1} (*5*, *7*). However, the extinction coefficient at 420 nm is 4.14×10^4 M^{-1} cm^{-1} (pH 7.5, 25 °C) (*11*, *13*). This coefficient changes as a function of temperature due to effects upon the pK_a of the 8-OH group (pK_a = 6.3) (*2*, *9*, *13*). The pK_a for the nitrogen at the 3-position is 12.3 (*2*). The reduced form of F420 (Figure 1B) loses its absorbance at 420 nm and becomes nonfluorescent (*7*, *9*, *12*). In vivo, the coenzyme is reduced by F420-reducing hydrogenases or by NADP reductases. The coenzyme is subject to oxidative and photodecomposition (*11*, *12*) reactions.

In our study, we isolated F420 from methanogen cells and examined its steady-state and time-resolved fluorescent properties. We relate these findings to the fluorescent properties of fecal material and suggest the use of F420 as a fluorescent marker for the detection of fecal or ingesta contamination on the carcasses of food animals.

MATERIALS AND METHODS

Animal Care and Sample Collection. Experimental procedures for animals were covered by protocols approved by the National Animal Disease Center's Animal Care and Use Committee. Ruminal and cecal ingesta samples were collected from cattle through surgically implanted cannula. Fresh fecal samples were obtained directly from the rectum of subject animals. All samples were transported to the laboratory in sterile, light-tight containers. To assess normal variation, animal samples were collected at random times, and these samples were frozen until analyses were conducted.

Isolation of Methanogen Cells. A methanogen was isolated from the ruminal contents of a cow using basal salts, rumen fluid, and bicarbonate-buffered, anaerobic culture broth (*14*). A headspace atmosphere of H_2/CO_2 (80%/20%, v/v) was used with hydrogen serving as the primary substrate for growth. Pressurized (200 kPa) culture tubes were incubated at 39 °C with agitation to facilitate gas transfer. For single-colony isolation, the culture medium was supplemented with 1.5% w/v agar. Tenfold dilutions from the primary culture were inoculated into molten agar medium (55 °C), and solidified agar roll tubes were prepared (*14*). Agar roll tubes were incubated at 39 °C under an atmosphere of hydrogen and carbon dioxide. Roll tubes were flushed with a fresh gas mixture daily during incubation. Well-isolated colonies were picked from the agar roll tubes under anaerobic conditions and transferred to the liquid culture medium. An isolated methanogen was identified and chosen for experimental use. A cell paste (0.73 g of wet wt) was prepared from liquid culture (825 mL of culture medium) by centrifugation (17000*g*) under anaerobic conditions. Exposure to light and oxygen was minimized, and the cell pastes were kept frozen (−70 °C) until use.

Extraction of F420 from Methanogen Cells. The method for extraction and isolation of the F420 was essentially that reported by Schonheit et al. (*11*). Briefly, acetone was precooled to −15 °C. Two milliliters of the precooled acetone was added to a vial containing 0.73 g (wet wt) of frozen methanogen cell paste. This mixture was protected from light during extraction with stirring at 4 °C for 30 min. Following extraction, the suspension was centrifuged and the supernate fluid was collected. A second 2-mL volume of chilled 50% acetone/water was added to the pellet, stirred for 30 min, and clarified by centrifugation. The supernates from both extractions were combined and diluted 1:2 with a 0.3 M NaCl/50 mM Tris-HCl buffer (pH 7.5). The resulting solution was loaded onto a Sephadex QAE A-25 ion exchange column that had a diameter of 1 cm and a length of 4.5 cm. The ion-exchange resin was pre-equilibrated in the dilution buffer, and the column was initially washed with 15 mL of this buffer. After sample loading, the column was eluted with 45 mL of 0.3 M NaCl/50 mM Tris-HCl buffer. Under these conditions, the F420 was retained on the column, as indicated by the yellow band observed, while all other components were eluted and discarded. To elute the F420, 15 mL of a 1.0 M NaCl/50 mM Tris-HCl buffer (pH 7.5) was applied to the column and 3 mL fractions were collected. Fractions containing the F420 were combined and used for the steady-state and time-resolved measurements.

Steady-State Fluorescence Measurements. The absorbance spectrum of the F420 in the eluting buffer was obtained using a Perkin-Elmer Lambda 18 UV−visible spectrophotometer. Excitation and emission spectra were obtained using a SPEX FluoroMax using an emission wavelength of 470 nm and excitation wavelengths of 295 and 420 nm.

For solid or opaque samples the front-faced fluorescence technique was utilized in both steady-state and time-resolved measurements, collecting emission from the surface of a 45° cuvette at right angles to the excitation. Samples were prepared as described above by adding acetone to frozen methanogen cell paste and stirring for ∼30 min. The resulting lysate was very opaque, so the emission spectrum was collected using the front-faced technique using an excitation wavelength of 420 nm.

Emission spectra were obtained from rumen, cecum, and fecal samples of two different cattle and fecal samples of one pig. Small volumes (10 μL) of an initial dilution (50 mg of wet weight in 3 mL of distilled water) of each sample were placed in 1 cm × 1 cm cuvettes containing 3 mL of 50 mM Tris buffer (pH 7.5). Small sample volumes were used to reduce the opacity of the samples as these spectra were using right-angle signal detection rather than the front-faced technique. The excitation wavelength was 430 nm. A Tris buffer blank spectrum was obtained so that contributions to each sample spectrum from water Raman scattering could be subtracted.

Time-Resolved Fluorescence Measurements. Time-correlated single-photon counting measurements were performed to determine the fluorescence lifetime of F420 in the elution buffer at pH 7.5 and 13. A Coherent 701 rhodamine 6G dye laser was synchronously pumped with 1−2 W of 532 nm output from an Antares 76s CW mode locked Nd:YAG laser. The 701 dye laser was cavity-dumped at 3.8 MHz. The dye laser pulses had an autocorrelation of ∼7 ps full-width at half-maximum (fwhm). Excitation of F420 at 295 nm was effected by focusing the dye laser pulses with a 5 cm lens into a crystal of $LiIO_3$ or KDP. Fluorescence was collected at right angles through a polarizer mounted at 54.7° to the excitation polarization and then passed through cutoff filters. A Hamamatsu 2809u microchannel plate, amplified by a B&H Electronics type AC3020 3.15 GHz preamplifier, provided the start signal while the sync out from the cavity dumper driver provided the stop signal. Constant-fraction discrimination of these signals was performed by a Tennelec TC 455 discriminator and time-to-amplitude conversion by an ORTEC 457 TAC. Data were stored in a Norland 5500 multichannel analyzer before transfer to and analysis with a personal computer. The time scale for the time-resolved experiments was 12.5 ns. The instrument response function of this system has a fwhm of 70−100 ps. Time-resolved fluorescence data were fit to a single exponential or a sum of exponentials

$$F(t) = \sum_{n=1}^{n} a_n \exp(-t/\tau_n) \quad (1)$$

by iteratively convoluting trial decay curves with the instrument response function and employing a least-squares fitting procedure to determine the lifetimes, τ_n, and preexponential

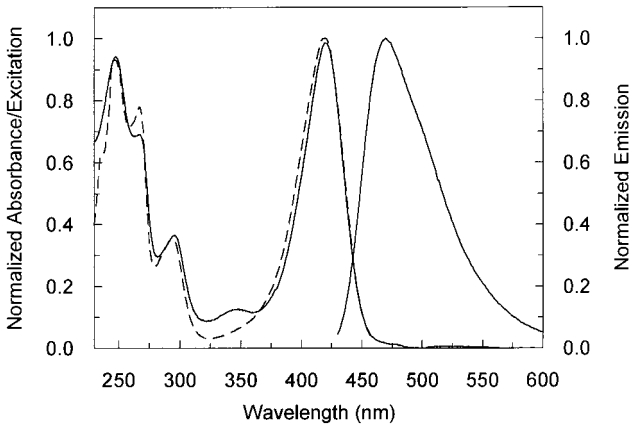

Figure 2. Absorbance (—, left tracing), excitation (- -, left tracing), and emission (—, right tracing) spectra of F420 in the elution buffer. The excitation wavelength for the emission spectrum was 295 nm. The emission wavelength for the excitation spectrum was 470 nm.

factors, a_n. A good fit is determined largely by the χ^2 criterion (*15*, *16*): $0.8 \leq \chi^2 \leq 1.2$.

The time-resolved fluorescence decay of bovine fecal matter was obtained using the front-faced technique with a 45° cuvette. The solid fecal matter was diluted (50 mg in 3 mL) with Nanopure water, which allowed for easier placement of the sample into the cuvette. The experiment was then performed in an identical manner as previously described for the F420 lifetime measurements with greater care given to filtering out the scattered excitation laser light. A large amount of excitation light was, nevertheless, reflected and scattered toward the detector from the front surface of the 45° cuvette. Despite our precautions, a considerable amount of this scattered light was detected in our measurements. We have ignored its contribution in analyzing the data.

RESULTS

The isolated methanogenic bacterium was determined to be *Methanobrevibacter* sp. Identification was based upon isolation from the rumen environment where this genus is the predominant methanogen, cell fluorescence, cell morphology, growth on hydrogen, production of methane, and lack of growth on carbohydrates.

Our success in purifying F420 from other components in the methanogen extract is demonstrated by the absorbance spectrum shown in Figure 2. This spectrum is very similar to spectra previously published for F420 (*9*, *11*). There were no observed differences, other than magnitude of intensity, in the emission spectrum (Figure 2) when the F420 sample was excited at 420 nm as compared to 295 nm, the excitation wavelength used for the time-resolved measurements.

Extracted F420, once separated and diluted in the elution buffer, was used in time-correlated single-photon counting to determine its lifetime. Figure 3 presents the fluorescence decays of F420 obtained at pH 7.5 and 13. These measurements yielded the same 4.2 ns lifetime component. Lifetimes were not measured at pH values below 7 due to pH-induced changes in the absorption spectrum. At low pH, the sample could not be sufficiently excited for the fluorescence decay to be determined.

The utility of F420 as a marker for fecal contamination was demonstrated when methanogen cells, feces, and other ingesta samples were examined. Figure 4 shows the emission spectrum of an acetone extract from methanogen cells. This emission spectrum was nearly identical to that of the purified F420 chromophore in

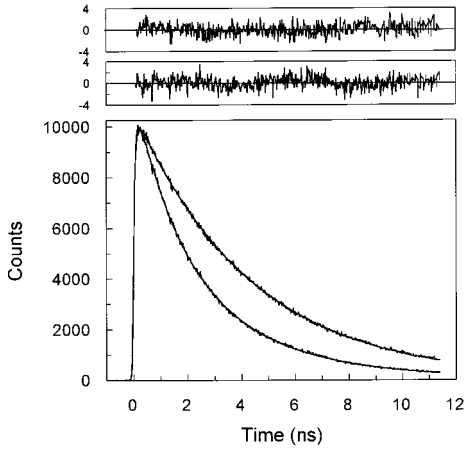

Figure 3. Lifetimes of the eluted F420 preparation at pH 7.5 and 13. At pH 7.5 (upper decay and residuals) $F(t) = 1.0 \exp(-t/4.25\ \text{ns} \pm 0.02)$ with a $\chi^2 = 1.15 \pm 0.1$. At pH 13 (lower decay and residuals) $F(t) = 0.31 \exp(-t/4.20\ \text{ns}) + 0.69 \exp(-t/1.79\ \text{ns})$ with a $\chi^2 = 1.39$.

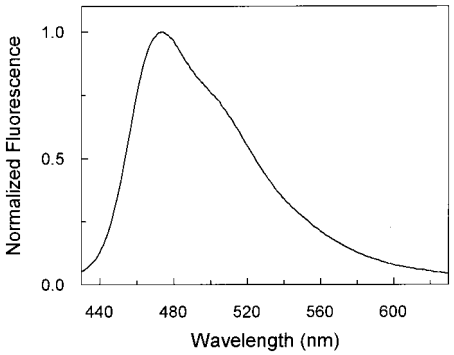

Figure 4. Emission spectrum of an acetone extract of methanogen cells. Excitation wavelength was 420 nm. Front-faced technique was utilized.

Figure 2. Front-faced analysis provided a satisfactory means by which to collect the emission from surfaces of these opaque samples. Similarly, and perhaps most importantly, we also observed an "F420-like" emission from various animal samples including feces and ruminal and cecal digesta (Figure 5). These materials are the predominant contaminants in a meat-processing plant.

Purified F420 had a fluorescence lifetime of 4.2 ns, but to use time-resolved methods of detecting F420 within fecal samples, this cofactor's lifetime must be resolved from scattered light and fluorescence from other emitting species. Figure 6 shows the lifetime decay of feces. A 45° cuvette was used given the opacity of the sample. This analysis revealed a 4.2 ns component with an amplitude of 0.24.

DISCUSSION

Methanogenic bacteria are very common if not ubiquitous inhabitants in the digestive tract of animals (*17*) including ruminants (*18*) and swine (*19*). They can also be found in animal feces (*20*). These bacteria contain the F420 chromophore. We have determined the fluorescence emission spectrum of this bacterium (Figure. 4). We have isolated F420 from a bacterium, *Methanobrevibacter* sp., which was obtained from bovine rumen contents, and we have determined the absorption, excitation, and emission spectra for purified F420 samples. The particular absorbance spectrum in Figure

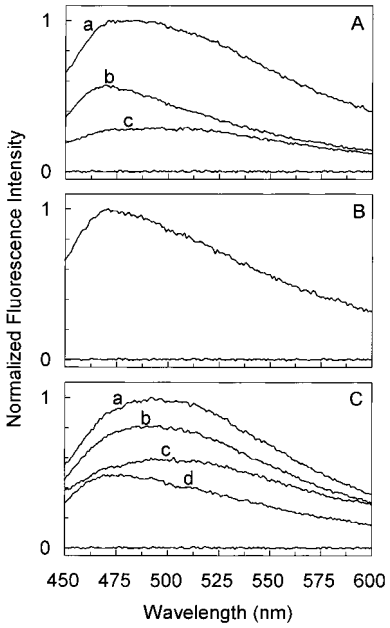

Figure 5. Fluorescence spectra of (A) rumen, (B) cecum, and (C) feces samples from two different cows, labeled 1 and 2, and a fecal sample from a pig. The excitation wavelength was 430 nm. The baseline in each panel at zero intensity is the emission from the 50 mM Tris dilution buffer after correction for Raman scattering from water. Each emission spectrum was corrected for Raman scattering by subtracting the contribution of Raman scattering from the Tris buffer. In (A) spectra a and b are rumen samples from cow 1 and spectrum c is from cow 2. (B) is a cecum sample from cow 2. In (C), spectrum a is a feces sample from cow 2, spectra b and d are from cow 1, and spectrum c is a pig feces sample. The spectrum within each set of samples with highest intensity was normalized to 1 with the other spectra normalized relative to it to preserve the relative fluorescence intensities.

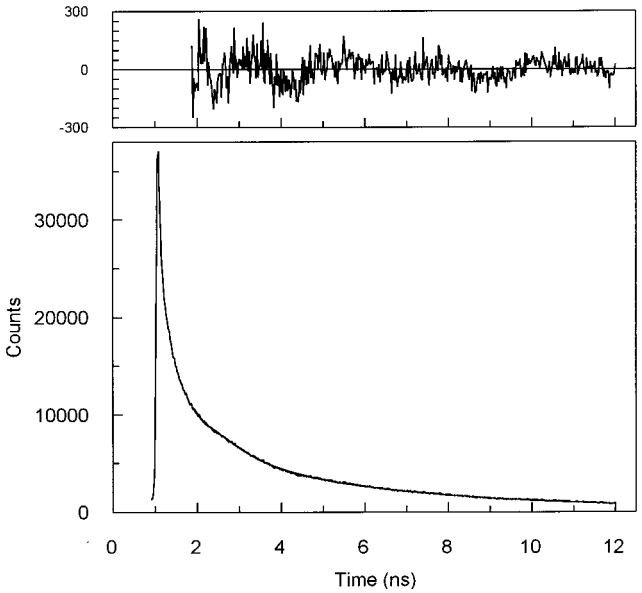

Figure 6. Fluorescence decay of bovine fecal material using the front-faced technique. $F(t) = 0.38 \exp(-t/0.140\ \text{ns}) + 0.38 (-t/0.682\ \text{ns}) + 0.24 (-t/4.20\ \text{ns})$; $\chi^2 = 1.55$. The fit was started at ~30% of the decay so as not to fit contributions to the decay from scattered or reflected excitation light. The residuals show some RF oscillations for which there was no correction.

2 was included to illustrate the effect of impurities in the eluted F420 sample. Our preparation did show a minor anomalous absorption band near 350 nm, but this band was not observed in the excitation spectrum, and it did not affect our fluorescence measurements. Additional preparations did not show any anomalous absorption bands. Thus, the separation technique was satisfactory in providing us with the F420 compound that was free of other possible fluorescent impurities.

F420 has a relatively high fluorescence quantum yield (0.17) and a long fluorescence lifetime. The fluorescence decays of F420 at pH 7.5 and 13 yielded the same 4.2 ns lifetime component. However, as the pH was increased through the pK_a of the nitrogen at the 3-position, a second component of fluorescence decay was observed. We interpret this to be due to the ionization of this nitrogen group.

The spectra of various animal samples including feces and digesta all displayed fluorescence emission with a peak near 470 nm. This signal corresponds to the emission observed with methanogenic bacterial cells and purified F420 preparations. Anderson et al. (*1*) also observed fluorescence emissions from sheep feces in the 470 nm region using laser-induced fluorescence. These observations illustrate that one can detect the fluorescent contributions of F420 in fecal and ingesta samples at wavelengths around 470 nm. It also suggests the possibility of detecting these contaminants on meat surfaces using F420 as one fluorescent marker.

Despite problems with scattered and reflected excitation light during our lifetime analysis, we observed a 4.2 ns lifetime component in the fluorescence detected from fecal samples. Even though care was taken to filter out the scattered and reflected light, it can be seen that the decay does contain significant extraneous contributions at short times. To avoid these contributions in our analysis, the data were fit by ignoring the early part of the decay curve and by beginning the fit of data at longer times. If this is not done, the artifactual short-time components dominate the fit and make it more difficult to resolve the contributions of actual fluorescent components. The fit revealed a 4.2 ns component with an amplitude of 0.24, illustrating the ability to observe emission from F420 even in a time-resolved measurement of a fecal sample. The amount of the emission arising from F420 was most likely larger given that where we chose to begin the fitting procedure is somewhat arbitrary and that we have ignored all scattered light contributions.

We have concluded that these bacteria may be good optical probes for detecting fecal and ingesta contamination of animal carcasses during processing. We have demonstrated that F420 fluorescence can be collected from methanogenic bacteria. Finally, and most importantly, for applications to problems of food safety, we have demonstrated that solid samples of fecal material contain optical signatures of F420. It appears that there are intrinsic optical markers in the gut of animals that can be used to monitor fecal and ingesta contamination of meat products.

LITERATURE CITED

(1) Anderson, D. M.; Nachman, P.; Estell, R. E.; Ruekgauer, T.; Havstad, K. M.; Fredrickson, E. L.; Murray, L. W. The potential of laser-induced fluorescence (LIF) spectra of sheep feces to determine diet botanical composition. *Small Ruminant Res.* **1996**, *21*, 1−10.

(2) Daniels, L. Biochemistry of methanogenesis. In *The Biochemistry of Archaea*; Kates, M., Kushner, D., Matheson, A. T., Eds.; Elsevier: Amsterdam, The Netherlands, 1993; pp 41−100.

(3) Ashton, W. T.; Brown, R. D.; Jacobson, F.; Walsh, C. Synthesis of 7,8-didemethyl-8-hydroxy-5-deazariboflavin and confirmation of its identity with the deazaisoalloxazine chromophore of *Methanobacterium* redox coenzyme F420. *J. Am. Chem. Soc.* **1979**, *101*, 4419−4420.
(4) Gorris, L. G. M.; van der Drift, C. Cofactor contents of methanogenic bacteria reviewed. *Biofactors* **1994**, *4*, 139−145.
(5) Wolfe, R. S. Biochemistry of methanogenesis. In *The Archaebacteria: Biochemistry and Biotechnology*; Danson, D., Hough, W., Lunt, G. G., Eds.; Portland Press: London, U.K., 1992; pp 41−49.
(6) Avouris, P.; Yang, L. L.; El-Bayoumi, M. A. Excited-state interactions of 7-azaindole with alcohol and water. *Photochem. Photobiol.* **1976**, *24*, 211−216.
(7) DiMarco, A. A.; Bobik, T. A.; Wolfe, R. S. Unusual coenzymes of methanogenesis. *Annu. Rev. Biochem.* **1990**, *59*, 355−394.
(8) Doddema, H. J.; Vogels, G. D. Improved identification of methanogenic bacteria by fluorescence microscopy. *Appl. Environ. Microbiol.* **1978**, *36*, 752−754.
(9) Eirich, L. D.; Vogels, G. D.; Wolfe, R. S. Proposed structure for coenzyme F420 from *Methanobacterium*. *Biochemistry* **1978**, *17*, 4583−4593.
(10) Eirich, L. D.; Vogels, G. D.; Wolfe, R. S. Distribution of coenzyme F420 and properties of its hydrolytic fragments. *J. Bacteriol.* **1979**, *140*, 20−27.
(11) Schonheit, P.; Keweloh, H.; Thauer, R. K. Factor F420 degradation in *Methanobacterium thermoautotrophicum* during exposure to oxygen. *FEMS Microbiol. Lett.* **1981**, *12*, 347−349.
(12) Cheeseman, P.; Toms-Wood, A.; Wolfe, R. S. Isolation and properties of a fluorescent compound, factor 420, from *Methanobacterium* strain M.o.H *J. Bacteriol.* **1972**, *112*, 527−531.
(13) Purwantini, E.; Mukhopadhyay, B.; Spencer, R. W.; Daniels, L. Effect of temperature on the spectral properties of coenzyme F420 and related compounds. *Anal. Biochem.* **1992**, *205*, 342−350.
(14) Ogimoto, K.; Imai, S. *Atlas of Rumen Microbiology*; Japan Scientific Societies Press: Tokyo, Japan, 1981; pp 171−173, 185.
(15) Chang, M. C.; Courtney, S. H.; Cross, A. J.; Gulotty, R. J.; Petrich, J. W.; Fleming, G. R. Time correlated single photon counting with microchannel plate detectors. *Anal. Instrum.* **1985**, *14*, 433−464.
(16) Cross, A. J.; Fleming, G. R. Analysis of time-resolved fluorescence anisotrophy decays. *Biophys. J.* **1984**, *46*, 45−56.
(17) Miller, T. L.; Wolin, M. J. Methanogens in human and animal intestinal tracts. *Syst. Appl. Microbiol.* **1986**, *7*, 223−229.
(18) Murray, R. M.; Bryant, A. M.; Leng, R. A. Methane production in the rumen and lower gut of sheep given lucerne chaff: Effect of level of intake. *Br. J. Nutr.* **1978**, *39*, 337−345.
(19) Kirchgessner, M.; Kreuzer, M.; Muller, H. L.; Windisch, W. Release of methane and of carbon dioxide by the pig. *Agribiol. Res.* **1991**, *44*, 103−113.
(20) Miller, T. L.; Wolin, M. J.; Kusel, E. A. Isolation and characterization of methanogens from animal feces. *Syst. Appl. Microbiol.* **1986**, *8*, 234−238.

Received for review June 5, 2000. Revised manuscript received November 29, 2000. Accepted December 20, 2000. This work was funded by a grant from the Food Safety Consortium and by Specific Cooperative Agreement 58-3625-7-113 with the ARS/USDA.

JF000689R