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Changes in intrinsic fluorescence during the production of viable but nonculturable *Escherichia coli*

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The potential of intrinsic fluorescence spectroscopy to detect and differentiate viable but nonculturable bacteria in the presence of culturable bacteria was explored. *Escherichia coli* cells, starved for 210 days in nutrient-free normal saline, show new fluorescence emissions near 400 and 440 nm, and reduced emission near 340 nm.

Keywords: intrinsic fluorescence spectroscopy; viable but nonculturable bacteria; VBNC bacteria; *Escherichia coli*

Introduction

The existence of Gram-negative pathogenic bacteria that are viable but nonculturable (VBNC) poses a health concern since such microbes are unrecoverable by conventional procedures (ie, colony formation on nutrient agar) yet have been shown to be pathogenic when placed in host organisms [3]. The VBNC state is suggested to result from nutrient deprivation, which bacteria experience in most natural environments, and which correlates with the expression of starvation-specific genes [5]. Other characteristics of nutrient-deprived bacteria include changes in molecular composition (ie, decreased ATP levels and degradation of RNA), changes in shape, decreased size, and increased resistance to stress [5].

Since these physiological changes are associated with the inability of standard techniques, such as PCR, to detect certain VBNC bacteria [1], new detection methods are warranted. Intrinsic fluorescence spectroscopy is a prime candidate since it can provide real time, high speed analyses with minimal sample preparation. Also, it does not require the design and use of specific probes, oligomers, or antibodies. This report describes observations linking physical changes in starved *Escherichia coli* with changes in the intrinsic fluorescence.

Materials and methods

Organism, culture medium, and culture conditions

E. coli strain JM101 was maintained [7] on glucose M9 minimal media agar (Sigma Chemical Co, St Louis, MO, USA) enriched with thiamine (Fisher Scientific, Pittsburgh, PA, USA).

Preparation of VBNC bacteria, direct counts, and viability assays

One-milliliter aliquots from overnight cultures were transferred to sterile 1.5-ml polypropylene microcentrifuge tubes and the cells were gently pelleted by centrifugation at 1300 $\times$ *g* for 5 min. The supernatant fluids were removed and the pellets were gently suspended in 1 ml of sterile 0.9% NaCl. This washing process was repeated a total of three times to insure removal of all nutrient media. The samples were then aged at room temperature for up to 210 days.

Characterization of the aged cells was begun by centrifugation for 5 min at 1300 $\times$ *g* to remove larger (viable and culturable) bacteria. The supernatant fluid was isolated and the pellets were suspended in 0.9% NaCl. The supernatant fluid was then centrifuged again for 15 min at 16 000 $\times$ *g* to pellet the smaller bacteria. Again the supernatant fluid was isolated, and the pellet was suspended in 0.9% NaCl.

This last supernatant fluid was further processed using a Centricon 100 concentrator which allows the removal of soluble components (less than 100 000 MW) from the media by elution through a membrane and into a lower collection chamber while retaining and concentrating the bacteria in an upper chamber. One-half milliliter of the supernatant fluid was placed in the upper chamber of the Centricon and centrifuged according to manufacturer's protocol; the 0.40 ml of liquid effluent which passed through to the lower chamber was removed and saved for analysis by fluorescence spectroscopy. The 0.10 ml of liquid which remained in the upper chamber contained the bacteria and was diluted to 2.0 ml by the addition of sterile normal saline. The sample was centrifuged until approximately 0.25 ml of liquid remained in the upper chamber. The effluent was discarded, the bacteria were diluted to 2.0 ml by the addition of normal saline and centrifuged again. This process was performed a total of three times. An aliquot of the Centricon-treated supernatant fluid was passed through a two-stage 0.8/0.2-μm syringe filter (Gelman Sciences, Ann Arbor, MI, USA) to remove all bacteria which were greater than 0.2 μm in diameter.

These separation procedures yielded five samples (1st pellet of larger bacteria, 2nd pellet of smaller bacteria, one supernatant fluid, one filtered supernatant fluid, and the effluent from the Centricon concentrator). As a control, the entire centrifugation, pelleting, and filtration procedure was performed on an overnight culture. In this case, cells were only found in the first pellet. Excitation/emission spec-

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troscopy was also done on the aged sample prior to separation of the various bacterial fractions.

The number of colony forming units (CFU) contained in each sample was determined by serial dilution titers and plating (done in triplicate) onto LB agar. Direct counts (DC) were made using Acridine Orange stain, the final value is the average of 10 counts from different areas of the same slide [6]. Direct viable counts (DVC) were done by incubating bacterial samples in 0.25 g L^{-1} YT media and 20 μg ml^{-1} naladixic acid for 24 h [4]. The naladixic acid inhibits cell division but not cell growth. The incubated bacteria were stained with Acridine Orange, filtered onto Millipore filters (Millipore Corp, Bedford, MA, USA) and visually examined for growth using epifluorescence microscopy (1000× magnification and oil immersion). Direct viable counts were determined by counting the bacteria which had grown beyond 2 μm in length. Again the final value is the average of 10 counts from different areas of the same slide. Control studies were done on bacteria aged 24 h and all bacteria displayed growth beyond 2 μm.

Fluorescence spectroscopic methods

Intrinsic fluorescence was monitored by excitation/emission spectroscopy which records the entire fluorescence spectrum generated from a selected excitation wavelength. This acquisition procedure was repeated over a range of excitation wavelengths. The resulting fluorescence intensities for each pair of excitation and emission wavelengths were displayed as contour plots. This method is particularly appropriate because bacteria contain multiple chromophores and excitation/emission spectroscopy allows the interrogation of each one. Spectroscopy was performed on a SLM/AMINCO 8100A Fluorimeter (SLM Instruments, Urbana, IL, USA) employing the following conditions. Slit widths: 8 nm excitation, 16 nm emission; scan rate: 1 nm s^{-1}; integration time: 0.95 s; excitation range: 250–400 nm, 5-nm increments; emission range: from excitation +15 or 20 nm to twice the excitation wavelength or 600 nm in 2-nm increments. Fluorescence measurements were done in solution using a 3-ml quartz cuvette (with a 1.0-cm path length). Bacterial samples were prepared in 0.9% NaCl with $OD_{600\ nm}$ less than 0.1 [8].

Results and discussion

The results of the culturability, direct count, and direct viable count assays are summarized in Table 1. The 1st pellet at 1300 × *g* and the second pellet at 16000 × *g* had CFU, DC, and DVC values that agreed within a factor of 1 to 4; however, the supernatant fluid had a CFU value which was only 10% of its DC. The filtered supernatant fluid had approximately 7×10^5 cells ml^{-1} which were able to grow in the DVC test but were unable to form colonies on solid media. This contrasts with the behavior of pellets which had CFU and DVC values which were in close agreement and indicates that properties of VBNC bacteria were being acquired by the small cells in the supernatant and filtered supernatant fluid samples.

Table 1 Culturability (colony forming units), direct counts, and viability (direct viable count) of *E. coli* after 210 days of starvation

Sample	CFU ml^{-1}	DC (cells ml^{-1})	DVC (cells ml^{-1})
1st pellet at 1300 × *g*	$2.0 \pm 0.1 \times 10^7$	$8.0 \pm 0.2 \times 10^7$	$2.8 \pm 0.6 \times 10^7$
2nd pellet at 16000 × *g*	$1.1 \pm 0.1 \times 10^6$	$8.3 \pm 0.9 \times 10^6$	$1.9 \pm 0.2 \times 10^6$
Supernatant	$2.5 \pm 0.5 \times 10^5$	$2.4 \pm 0.4 \times 10^6$	$9.9 \pm 2.0 \times 10^5$
Filtered supernatant	<10	$1.8 \pm 0.7 \times 10^6$	$6.9 \pm 3.1 \times 10^5$

The spectroscopic results (Figure 1), show a common feature from water Raman scattering which appears as a prominent diagonal line at all excitation wavelengths above 305 nm. The level of Raman scattering is linearly proportional to the excitation intensity. Thus, Raman scattering varies with output intensity from the excitation source (note that the Raman intensity for 325 nm excitation is nearly constant in all plots). Figure 1a shows the excitation/emission spectra of starved samples aged 24 h. This spectrum shows a single fluorescence peak near 345 nm for all excitation wavelengths below 305 nm which we attribute to tryptophan fluorescence [2]. Figure 1b shows the spectrum from a sample aged 210 days. This spectrum shows the tryptophan peak in addition to two new fluorescence bands which include a shoulder extending the tryptophan fluorescence from 400 to 450 nm (following 300 nm excitation) and a peak centered near 435 nm for excitations greater than 330 nm.

The excitation/emission spectra of the 1300 × *g* pellet (not shown) closely resembles that for the 24-h sample except that a weak shoulder appears on the tryptophan band. This was also observed for the 16000 × *g* pellet but to a lesser extent (not shown). Figures 1c and 1d relate the two new peaks in the excitation/emission spectra to particular fractions of the aged bacteria. The spectra from the supernatant fluid (Figure 1c) shows only a trace of the tryptophan fluorescence and an emission peak near 400 nm (300–325 excitation). This is the band that appeared as a shoulder on the tryptophan emission in the mixed sample. The filtered supernatant fluid (not shown) shows only a hint of the 400-nm band and no other features. Finally, Figure 1d shows that the strong peak near 450 nm for 330–380 nm excitation comes from a soluble component and is presumably a low molecular weight metabolic byproduct. Efforts to identify the chromophores responsible for the 400-nm fluorescence band in the shrunken cells and the 440-nm band in the soluble component are ongoing.

These results demonstrate that VBNC-like bacterial isolates do not show significant fluorescence from tryptophan but have a new fluorescence band that absorbs near 300 nm and fluoresces near 400 nm. A soluble byproduct is also produced, as *Escherichia coli* are starved, that absorbs near 350 nm and emits near 440 nm.

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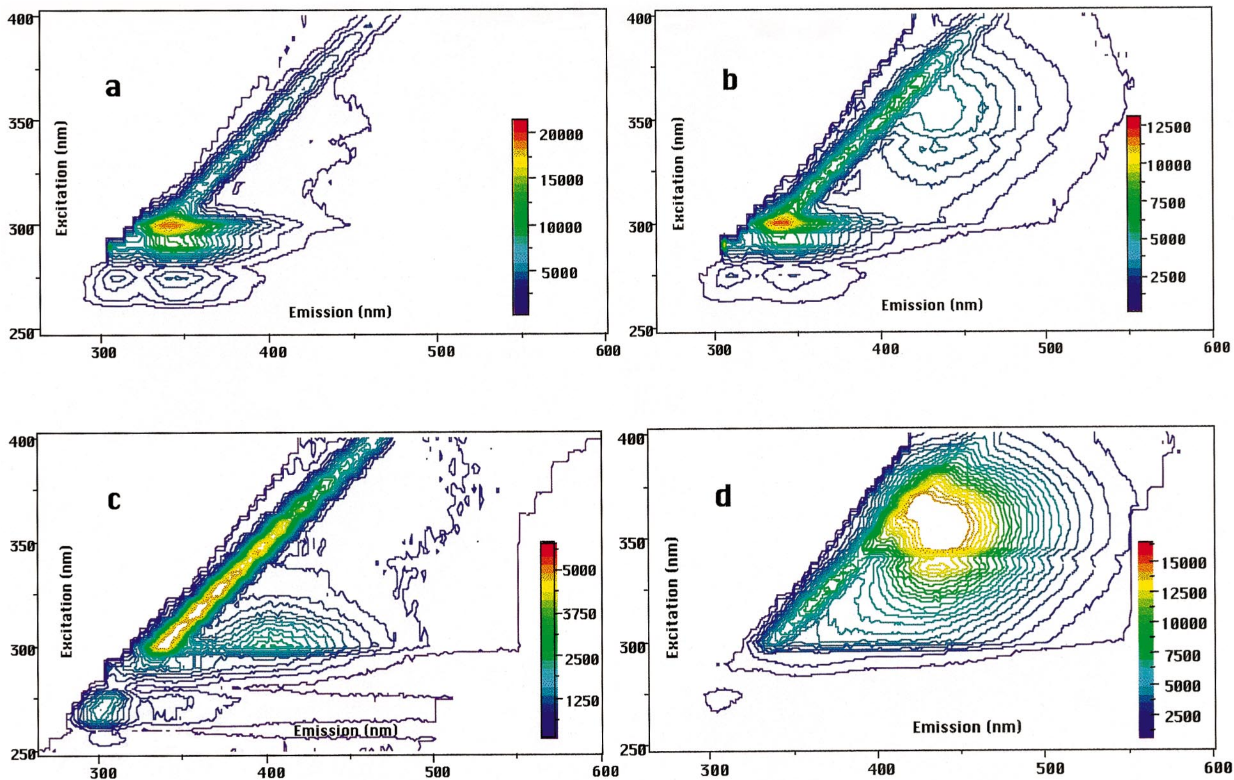

Figure 1 (a) Contour plot of excitation/emission spectra for *E. coli* cells starved for 24 h. Color legend indicates absolute fluorescence intensity. (b) Colour plot of excitation/emission spectra for *E. coli* cells starved for 210 days. This spectrum shows the tryptophan peak and two new fluorescence bands which include a shoulder extending the tryptophan fluorescence from 400 to 450 nm (following 300 nm excitation) and an emission centered near 435 nm for excitations greater than 330 nm. (c) Contour plot of excitation/emission spectra for *E. coli* cells starved for 210 days (Table 1). Spectra pertain to unpelletted bacteria in the supernatant fluid which were washed in a Centricon concentrator but not syringe filtered. The plot shows the new emission near 440 nm at 350 nm excitation. (d) Contour plot of excitation/emission spectra for low molecular soluble components (less than 100 000 MW) of the Centricon concentrator effluent. This fraction accounts for the strong fluorescence near 440 nm following 350 nm excitation.

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