

ADENOSINE TRIPHOSPHATE ENHANCES THE  
FLUORESCENCE OF 4',6-DIAMIDINO-2-PHENYLINDOLE  
(DAPI)-LABELED *ESCHERICHIA COLI* O157:H7<sup>1</sup>

006591

ABSTRACT

*Live cells of E. coli O157:H7 were labeled by 4',6-diamidino-2-phenylindole (DAPI) in buffers of different pH. The extent of labeling was relatively insensitive to pH in the range of 6.5 to 9.5. The fluorescence intensity of ~ 10<sup>4</sup> DAPI-labeled bacteria per mL in optical cuvettes could be detected by a luminescence spectrometer. With a fluorescence microplate reader attachment, less than 10<sup>3</sup> of labeled bacteria could be measured. DAPI-labeling inhibited the growth and respiratory activities of the bacteria. The addition of 0.5 to 6 mM concentrations of ATP induced a substantial increase in the fluorescence of labeled bacteria. Maximal enhancement by ATP was observed from bacteria still maintaining low levels of physiological activities. The enhancement favored more alkaline media with pH greater than 9. A replacement of ATP with ADP or AMP diminished the extent of enhancement. Other triphosphate nucleotides did not enhance fluorescence of DAPI-labeled bacteria. Comparable ATP enhancements were also observed with *Pseudomonas alcaligenes* and *Shewanella putrefaciens*. Solubilization/destruction of cell membranes of labeled bacteria by detergents essentially eliminated the ATP enhancement. Absorption and fluorescence spectroscopic measurements indicated that ATP could interact with free and bound DAPI. These results suggest that observed ATP enhancement in fluorescence*

<sup>1</sup>Mention of brand or firm names does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

## INTRODUCTION

The contamination of food by pathogenic microorganisms results in numerous poisoning cases in the United States. One heavily cited pathogen is the bacterium *Escherichia coli* O157:H7. Although the *E. coli* has not been implicated in nearly as many cases as other pathogenic bacteria such as *Salmonella*, *Campylobacter jejuni*, or *Listeria monocytogenes*, it has been associated with a high frequency of fatalities relative to the other pathogens. Deaths caused by *E. coli* O157:H7 can be attributed to the development of hemolytic uremic syndrome and subsequent kidney failure in those infected by the bacteria. To minimize the possibility of food poisoning incidence, development of sensitive bacterial pathogen detection for food safety is therefore desired.

Fluorochrome staining of nucleic acids may significantly increase the sensitivity of direct counting of bacteria in environmental samples by epifluorescence microscopy (Kepner and Pratt 1994). The two fluorochromes most often used are 3,6-bis[dimethylamino] acridinium chloride (acridine orange [AO]) and 4',6-diamidino-2-phenylindole (DAPI) with the latter rapidly replacing AO as the most applied bacteria stain. DAPI is believed to bind to DNA (Kapusinski and Szer 1979; Larsen *et al.* 1989; Manzini *et al.* 1983) preferentially at AT-rich regions (Portugal and Waring 1988) through interactions with the phosphate groups along the DNA strand (Matsuzawa and Yoshikawa 1994). Recent reports indicate that DAPI may also interact with RNA (Tanious *et al.* 1992), proteins (Mazzini *et al.* 1992) and phospholipids (Favilla *et al.* 1993). Interactions with nucleic acids and protein increase the quantum yield of DAPI fluorescence. The interaction with phospholipids is noted with changes in the wavelength of emission maximum of DAPI fluorescence.

DAPI-labeling has been applied for detecting and quantifying of bacteria in different types of environmental samples, e.g., from Antarctic soil (Bloem *et al.* 1986) to oyster tissue homogenates (Crosby and Peele 1987). In aqueous samples, the fluorescence intensity of DAPI-labeled bacteria is influenced by a number of environmental parameters such as UV irradiation (Baleux and Got 1996), presence of disinfectants (Paquin *et al.* 1994; Saby *et al.* 1997), and high salt content (Kepner and Pratt 1994). The specific fluorescence of bacteria also appears to be controlled by the availability of carbon nutrients (Ross *et al.* 1996). These complications must be taken into account for utilizing DAPI-labeling techniques for bacterial enumeration in environmental samples by automated instrumentation. On the other hand, it is desirable to search for biochemical conditions which can enhance the fluorescent signals of DAPI-labeled bacteria and thus, the detection

sensitivity. In this work, we present results to demonstrate that the sensitivity of bacterial detection by DAPI-labeling may be further enhanced by ATP.

## MATERIALS AND METHODS

### Culture Conditions

*E. coli* O157:H7 (B1409, Center for Disease Control and Prevention, Atlanta, GA), *Pseudomonas alcaligenes* (DCMS 25), and *Shewanella putrefaciens* (ATCC 8071) were cultured in BHI (brain-heart infusion) medium according to standard procedures. Aliquots of 25 mL of BHI solution in 50-mL flasks were inoculated from refrigerated slants of *E. coli* cultured from selective media. The flasks were placed in an incubator and shaken at 160 rpm for 18 h at 37C. The cell density was checked qualitatively by light-scattering measurement at 650 nm and quantitatively with plate count on BHI agar. Typically, *E. coli* O157:H7 cultures after 18 h of incubation had absorbance readings of about 0.2 at 650 nm after a 30-fold dilution and contained about  $2 \times 10^9$  CFU/mL. The same culture procedure was also used for other bacteria.

### Labeling of Bacteria

Aliquots of 1-mL cultures of *E. coli* O157:H7 at different stages of growth were centrifuged at  $10,285 \times g$  in a BHG Hermle (Gosheim, Germany) Z360K centrifuge for 10 min at 25C. The bacterial pellets were quickly suspended to  $10^7$  cells per mL in buffers consisting of 10 mM Tris and 150 mM NaCl (TBS buffer) with different pH values. Aliquots of DAPI solution in water (1 mg/mL) were added to the bacteria suspensions with gentle vortexing for 1 min. Excess DAPI was removed by centrifugation at  $10,285 \times g$  for 10 min and DAPI-labeled *E. coli* O157:H7 were resuspended in 1 mL of the TBS buffer. Serial dilution of labeled bacteria with proper media was performed to yield suspensions with desired cell concentrations. In this study, "DAPI-labeled bacteria" referred to bacterial cells collected by centrifugation after DAPI treatment and then suspended in DAPI-free media.

### Fluorescence Measurements

The extent of labeling and the kinetics of the labeling were followed at 22C using a Perkin-Elmer (Norwalk, CT) LS-50B luminescence spectrometer. The fluorescence spectra were obtained by illumination at 359 nm and recorded in the wavelength range of 400-650 nm. When only fluorescent intensity was determined, the excitation and emission wavelengths were then set at 359 and 461 nm, respectively. A 96-well fluorescent plate reader attachment to the spectrometer

was used to determine the fluorescent intensity of DAPI-labeled bacteria collected on the filter membranes (5-mm circles) of Fluoricon GF assay plates by Idexx Inc (Westbrook, ME).

### Measurements of Bacterial Respiration and Growth

After being treated with different dosages of DAPI, labeled cells of *E. coli* O157:H7 were washed by centrifugation with TBS buffer (pH 7.5) to remove excess DAPI. The oxygen consumption rate of  $\sim 2.0 \times 10^7$  bacteria in a thermostated Gilson Medical (Middleton, WI) respiration cell filled with 1.9 mL of TBS (pH 7.5) buffer containing 50 mM glucose was measured by a YSI (Yellow Springs, OH) 5331 oxygen electrode attached to a YSI 5300 biological oxygen monitor.

To determine the effects of DAPI treatment on bacterial growth, labeled cells of the *E. coli* were applied to BHI agar culture plates and incubated at 37C for 18 h. The CFU counts were then determined by the use of a Model 500A Spiral System Bacteria Colony Counter (Gaithersburg, MD).

### Chemicals

DAPI, calf thymus DNA (Type I) and *E. coli* DNA (from strain B) were purchased from Sigma Chemical Co (St Louis, MO). All other reagents used were of highest purity obtainable commercially and were used without further purification.

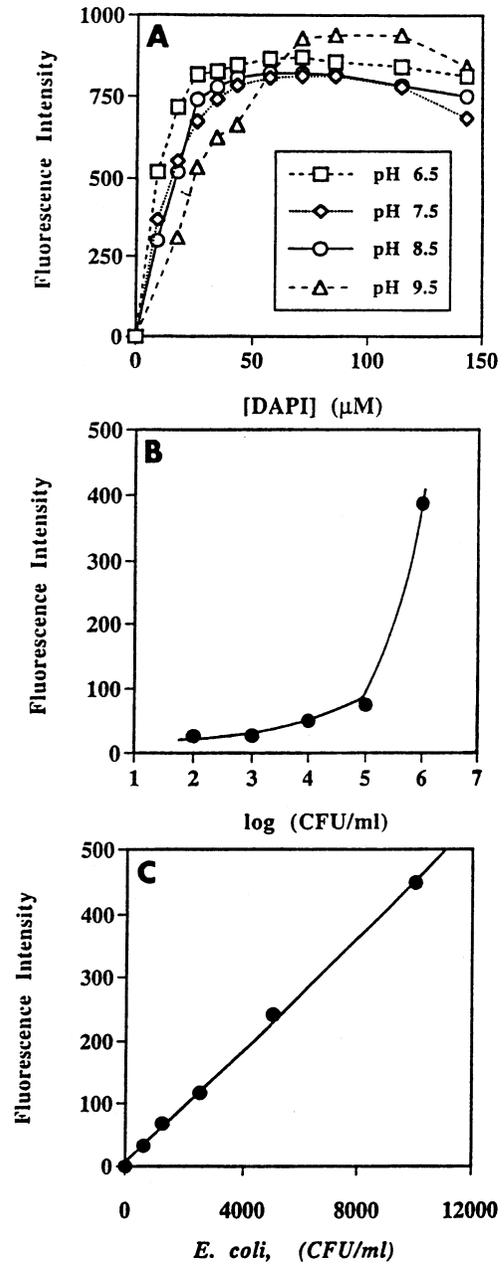
## RESULTS AND DISCUSSION

### Labeling of Bacteria

Live cells of *E. coli* O157:H7 harvested at stationary phase (CFU  $\geq 2.0 \times 10^9$  per mL) were diluted to  $10^7$  CFU/mL and treated with various concentrations of DAPI at different pHs to determine the concentration of DAPI needed to reach maximal labeling (Fig. 1a). As shown, the labeling appeared to reach a saturation level at 86  $\mu$ M of DAPI and showed no appreciable pH dependence. With serial dilutions, we determined that the fluorescence associated with labeled bacteria could be easily detected at a level of  $10^5$  cells per mL by standard spectroscopic measurements using 0.5-cm quartz cuvettes and  $\sim 10^3$  cells (total) when concentrated on Fluoricon filter membrane (Fig. 1b and c). It should be noted that the results do not imply a better sensitivity of microplate-reader measurement since the concentration of labeled bacteria on the filter was not determined.

A time course of DAPI-labeling is shown in Fig. 2a. The labeling of the bacteria essentially reached completion within 100 s. The fluorescent intensity of

DAPI LABELING OF *E. COLI* O157:H7



**FIG. 1. LABELING OF *E. COLI* O157:H7 BY DAPI AT DIFFERENT pH**  
 The fluorescence intensities of labeled bacteria, after free DAPI removed, are shown in 1a. The fluorescence intensities were measured directly (1b) or on filter membranes (1c). Data represent the average of measurements with an error range of  $\pm 20\%$ .

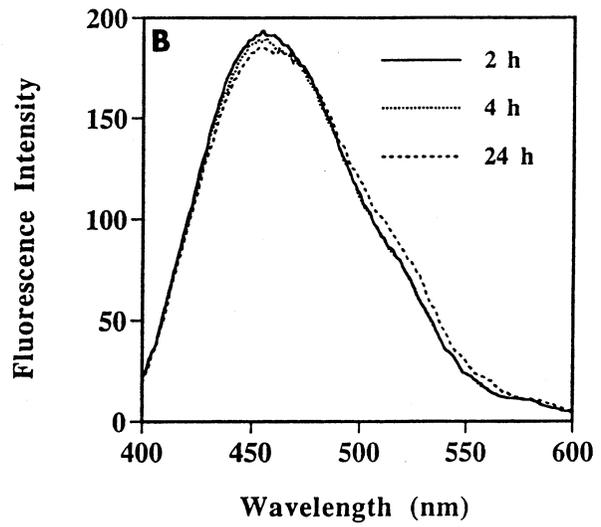
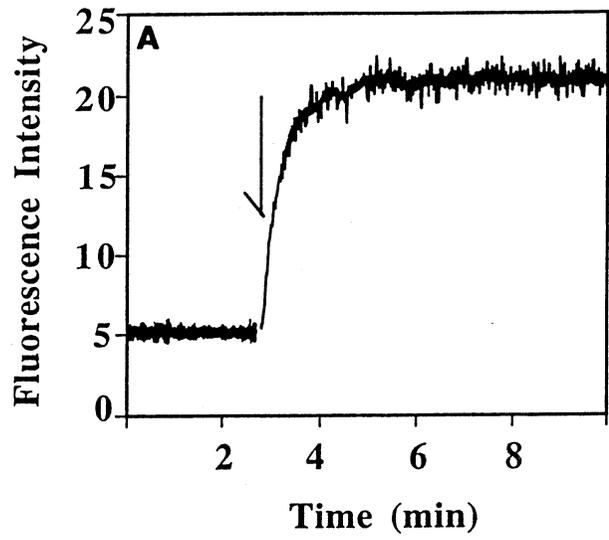
labeled bacteria remained nearly unchanged for at least 24 h at room temperature in the dark in TBS buffers (Fig 2b). As described, between pH 6.5 to 9.5, the reactivity of *E. coli* O157:H7 to DAPI, measured by the fluorescence intensity, was essentially independent of pH (Fig. 1a). These results suggest that DAPI-labeling may be used to enhance the detection of the bacteria by fluorescence measurements; however, it should be mentioned that DAPI-labeling by itself does not provide any information on the specificity of detected microorganisms.

#### **Effects of the Labeling on the Cell Viability**

It has been previously reported by Vaulot and Partensky (1992), that the extent of DAPI-labeling may be affected by DNA replication state of cell cycle as modulated by nutrient availability. Under laboratory culture conditions, *E. coli* O157:H7 did not show significant differences in reactivity to DAPI treatment at various stages of growth (Fig. 3a) as indicated by a constant specific fluorescent intensity along the growth curve. However, the labeling appeared to affect some cell functions. The bacterial cells were allowed to react with different concentrations of DAPI in buffer solutions. After the removal of DAPI, the activities of labeled *E. coli* O157:H7 were tested. As shown (Fig. 3b), DAPI-labeled *E. coli* O157:H7 exhibited minimal growth on BHI agar culture plates for at least 18 h. The DAPI treatment also decreased the respiratory activity of the bacteria (Fig. 3b). However, the sensitivity of respiration to the treatment is considerably less than that of cell growth. After treated with 25  $\mu$ M DAPI, the labeled bacterial cells, retained ~50% of respiration activity in buffer. With applied DAPI at 175  $\mu$ M, the labeled cells still exhibited more than 25% activity. The DAPI-labeling induced inhibition, mentioned in Fig. 3b, might be expected from the possible interactions with nucleic acids, proteins and/or phospholipids. Since cellular nucleic acids are not directly involved in respiratory activity, DAPI interactions with nucleic acids should not be responsible for observed inhibition in oxygen consumption. The exact molecular origins of described inhibition remain to be established.

#### **Effects of ATP on DAPI-labeling**

Since DAPI-labeling of *E. coli* O157:H7 strongly inhibited cell growth and respiration, it was possible to use its fluorescence to enumerate the bacteria without possible complications of cell division. One possible way to increase fluorescence intensity and improve detection sensitivity is to increase the uptake of DAPI by the bacteria. In biological systems, uptake or active transport of many molecules is known to link to ATP-consuming processes associated with cell membranes. We first tested the addition of ATP during the labeling stage of *E. coli* O157:H7 by DAPI. After centrifugation to remove excess DAPI, no difference in fluorescence intensity of labeled bacteria was noted (data not shown). Thus, ATP did not



**FIG. 2. KINETICS AND STABILITY OF DAPI-LABELING**  
 (a) To TBS buffer (pH 9.0) containing 30  $\mu\text{g}$  of DAPI,  $2.0 \times 10^6$  *E. coli* O157:H7 cells were added (indicated by arrow). (b) The same DAPI-labeled *E. coli* O157:H7 suspensions were measured at the end of labeling mentioned in (a) 2, 4, and 24 h after storage in the dark at 22C.

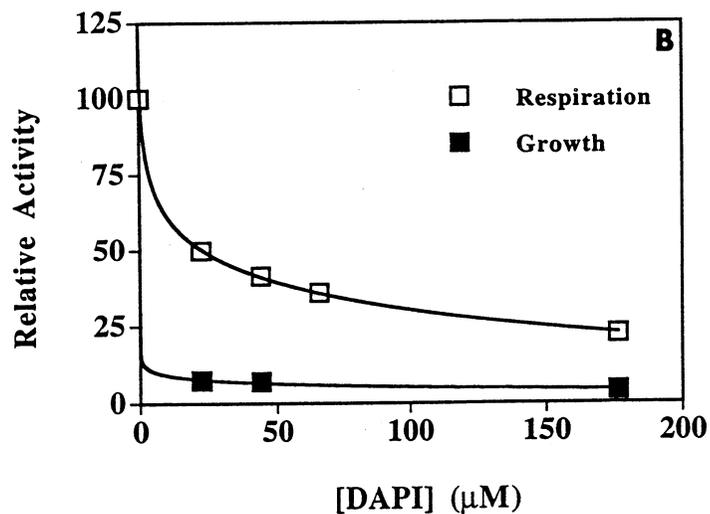
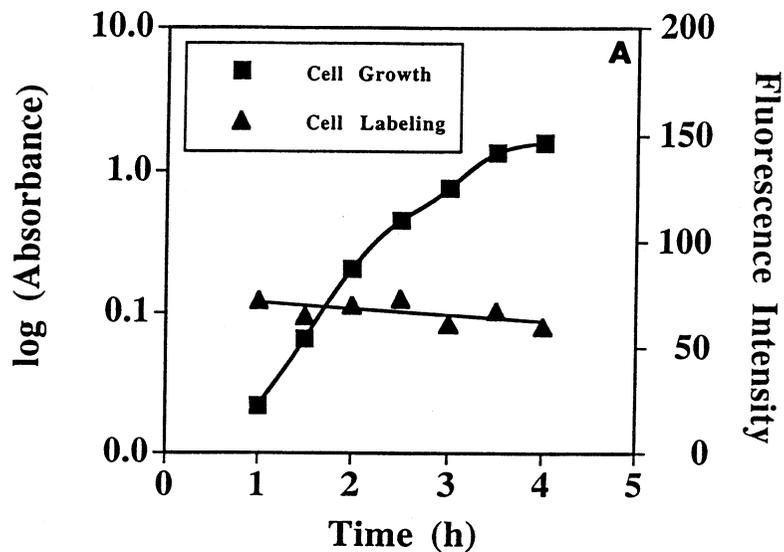


FIG. 3. PROPERTIES OF DAPI-LABELED *E. COLI* O157:H7

(a) DAPI reactivity of the *E. coli* at different stages of culture. Solid squares represent culture growth (absorbance, 650 nm). Solid triangles represent fluorescence intensities of bacteria treated with 30 μg/mL DAPI. (b) Plate counts (BHI agar) and oxygen consumption of *E. coli* labeled with different dosages of DAPI. Cell density was determined 18 h after inoculation (solid squares). Oxygen consumption rates were measured (open squares). Data represent averages of triplicated measurements with relative errors less than 10%.

facilitate the uptake of DAPI by the cells of *E. coli* O157:H7.

Yet, the addition of ATP to the suspensions of DAPI-labeled *E. coli* O157:H7 rapidly and significantly enhanced the fluorescence intensity in alkaline media. Keeping the labeled bacteria concentration constant, fluorescence enhancement increased as applied ATP concentration increased (Fig. 4a). This enhancement was affected by the pH of the medium. Under experimental conditions, maximal enhancement by ATP was observed in alkaline media with pH greater than 9.0. At pH 7.0, no significant enhancement was observed.

The extent of enhancement at pH 9.5 exhibited a dependence on the dosage of DAPI used for the labeling of bacteria. As shown in Fig. 4b, the relative enhancement by ATP first increased rapidly as DAPI concentration increased and then decreased to a slower rate upon further increase in DAPI concentration. Comparing the effects of DAPI-labeling on the growth and respiratory activities (Fig. 3b) it appears that most of the enhancement occurred with the labeled bacteria still retaining higher levels of respiration activity. Indeed, when heat-killed bacteria *E. coli* O157:H7 were used for DAPI-labeling, no appreciable ATP enhancement was observed (data not shown). However, the comparison also indicated that physiological viability does not appear as an absolute requirement for observed enhancement.

The described ATP enhancement is not unique to *E. coli* O157:H7. A similar enhancement was also observed with *Pseudomonas aeruginosa* and *Shewanella putrefaciens*. However, the levels of ATP stimulation were different. Experimentally, bacteria with the same cell density ( $\sim 10^7$  cells per mL) were treated with DAPI (30  $\mu\text{g}/\text{mL}$ ). After removing excess DAPI, the fluorescence intensities of labeled bacteria were measured with and without 5 mM of ATP in the TBS buffer, pH 9.5. The observed fluorescence enhancements with DAPI-labeled *E. coli* O157:H7, *Pseudomonas alcaligenes*, and *Shewanella putrefaciens* were 3.45, 2.75 and 2.15, respectively. The enhancement to the fluorescence of labeled *E. coli* O157:H7 appeared to be quite specific to ATP. When ATP was replaced by ADP or AMP or other nucleotide triphosphates (CTP, GTP, TTP, and UTP), no enhancement of the fluorescence was observed.

#### **Requirements for ATP Enhancement**

After recording enhanced fluorescence, labeled bacteria were further incubated in ATP-containing medium for 10 min at room temperature and then collected by centrifugation. Upon re-suspension in ATP-free medium, no enhancement of fluorescence was observed; however, the enhancement returned immediately when ATP was re-introduced. To have ATP outside the bacterial cell to enhance the fluorescence of intracellular DAPI suggested that certain membrane-related functions, e.g., uptake of ATP into the cells, may be required for the enhancement. Indeed, the addition of low concentrations of Triton X-100 or sodium dodecyl

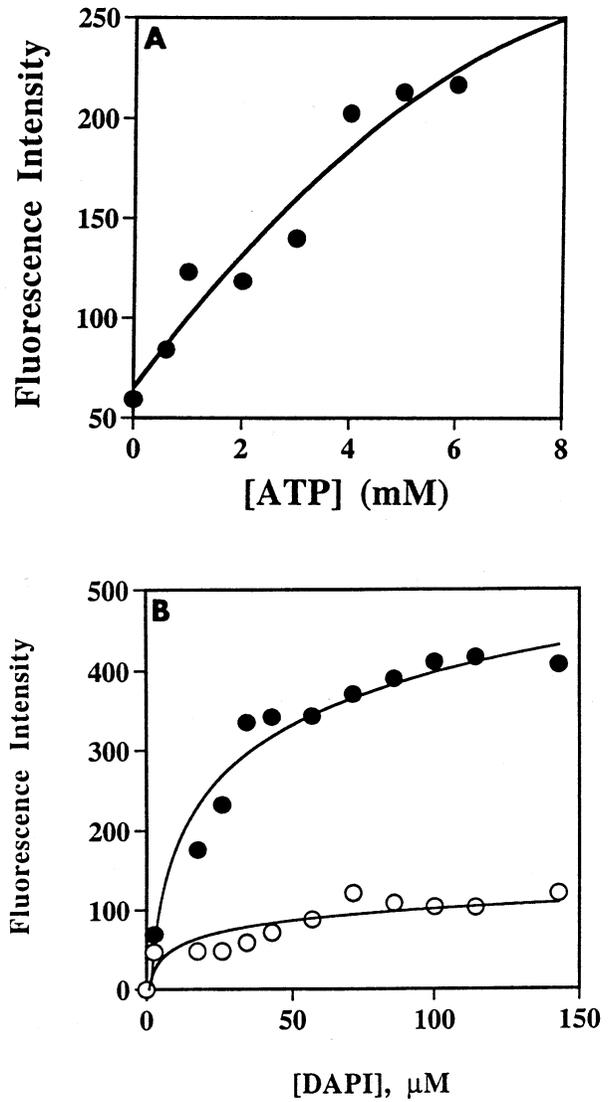


FIG. 4. EFFECTS OF ATP ON FLUORESCENCE INTENSITY OF DAPI-LABELED *E. COLI* O157:H7

(a) ATP Concentration Dependence. *E. coli* O157:H7 harvested at stationary phase were labeled with 30  $\mu$ g of DAPI per mL at pH 9.5 and suspended in TBS buffer pH 9.5 containing different concentrations of ATP. (b) DAPI Dosage Dependence. *E. coli* cells were labeled with different concentrations of DAPI. After removing the excess DAPI, the fluorescence of labeled cells was then measured in the presence of zero (open circle) and 5 mM ATP (filled circle) at pH 9.5. The data represent averages of triplicated measurements with relative errors less than 10%.

sulfate (SDS) to perturb the membrane structure essentially eliminated the ATP enhancement of DAPI-labeled *E. coli* O157:H7 (Table 1). The general increase of fluorescence caused by the detergents may be due to a re-partition of DAPI from cell components to detergent micelles. Under microscopic observation, detergents appeared to disrupt the integrity of the cell envelope of DAPI-labeled *E. coli* O157:H7 (data not shown).

TABLE 1.  
EFFECTS OF DETERGENTS ON THE FLUORESCENCE OF DAPI-LABELED  
*E. COLI* O157:H7<sup>1</sup>

Media Used	no ATP	+2 mM ATP
TBS buffer (pH 9.0)	1.0	2.7
TBS buffer (pH 9.0) + 0.2% Triton X-100	3.8	3.9
TBS buffer (pH 9.0) + 0.1% SDS	7.6	6.6

<sup>1</sup>Cells of *E. coli* O157:H7 harvested at stationary phase were treated with 30 µg of DAPI per mL. The fluorescence intensities of labeled bacteria were then determined in the described media with or without 2 mM of ATP. Data shown represented averages of triplicated experiments with relative errors as ± 10%, were normalized by assigning the intensity in TBS buffer (pH 9.0) as 1.0.

#### Interactions Between DAPI and ATP

The results of Table 1 suggest that the uptake of ATP into cells by intact membranes is necessary for enhancement. DAPI-labeling of the bacteria mainly involves noncovalent bindings with cellular components. The binding equilibria also predict the presence of certain levels of free DAPI inside the bacteria. Once inside labeled cells, ATP may interact with bound as well as free DAPI intracellularly leading to an enhancement of the fluorescence. Thus, possible ATP interactions with free and DNA-bound DAPI were investigated.

In buffered TBS solutions (pH 7.5 and 9.5), the presence of ATP caused a slight but noticeable red shift of the absorption maximum and a significant increase (more than 3-fold) in the fluorescence intensity, and thus, the relative quantum yield of DAPI labels (Table 2). Under the same experimental conditions, the absorption properties of DAPI bound to DNA did not show any noticeable changes by the presence of ATP. As expected, the binding of DAPI to DNA caused a substantial increase in fluorescence intensity (~ 40-fold), but the presence of ATP induced only small changes in the fluorescence intensity (less than 10%) of DNA bound-DAPI. These experimental results suggested that the biochemical components in cells may interact differently with ATP. Thus, described ATP enhancement in fluorescence intensity of DAPI-labeled *E. coli* O157:H7 was most likely due to interactions of ATP with free and/or bound DAPI in cells.

TABLE 2.  
CALCULATED RELATIVE QUANTUM YIELD OF DAPI UNDER DIFFERENT CONDITIONS

Conditions	Normalized Relative Quantum Yield	
	No ATP	+2mM ATP
<u>pH 7.5</u>		
TBS buffer	1.0	3.1
TBS buffer + DNA (Calf Thymus)	33.7	34.5
TBS buffer + DNA ( <i>E. coli</i> )	28.5	25.1
<u>pH 9.5</u>		
TBS buffer	1.0	3.4
TBS buffer + DNA (Calf Thymus)	38.3	43.0
TBS buffer + DNA ( <i>E. coli</i> )	39.3	38.0

Ratios,  $\{ \text{fluorescence intensity (F) at 461 nm} / [ \text{absorbance (A) at 359 nm} ] \}$  of DAPI under different conditions were first calculated. The relative quantum yield,  $\{ F/A \}_2 / \{ F/A \}_1$ , was then calculated where subscripts 1 and 2 refer to pH 7.5 TBS buffer only and with DNA additions, respectively. The relative quantum yields were then normalized by assigning the relative quantum yield of DAPI in pH 7.5 TBS buffer as 1.0. The data represented averages of triplicated experiments with relative errors as  $\pm 15\%$

## CONCLUSION

In this work, we demonstrated that DAPI-labeling could be a sensitive method to quantify *E. coli* O157:H7. The application of this method for a practical purpose requires that the target organism can be selectively isolated from others by suitable means, e.g., immuno-interactions. We discovered that the sensitivity of DAPI-labeling could be significantly enhanced by the presence of ATP. While the exact origin of this specific enhancement remains to be determined, preliminary characterization experiments suggested that the effect might relate to certain bacterial membrane processes linked to the uptake and/or utilization of ATP. Model system studies demonstrated that ATP could interact with free and bound DAPI to affect the fluorescence properties. Thus, the observed ATP enhancement in labeled *E. coli* O157:H7 can be attributed to interactions between ATP and DAPI inside intact cells.

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