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A Rapid, Direct Method for Enumerating Respiring Enterohemorrhagic *Escherichia coli* O157:H7 in Water

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Simple, rapid methods for the detection and enumeration of specific bacteria in water and wastewater are needed. We have combined incubation using cyanoditolyl tetrazolium chloride (CTC) to detect respiratory activity with a modified fluorescent-antibody (FA) technique, for the enumeration of specific viable bacteria. Bacteria in suspensions were captured by filtration on nonfluorescent polycarbonate membranes that were then incubated on absorbent pads saturated with CTC medium. A specific antibody conjugated with fluorescein isothiocyanate was reacted with the cells on the membrane filter. The membrane filters were mounted for examination by epifluorescence microscopy with optical filters designed to permit concurrent visualization of fluorescent red-orange CTC-formazan crystals in respiring cells which were also stained with the specific FA. Experiments with *Escherichia coli* O157:H7 indicated that both respiratory activity and specific FA staining could be detected in logarithmic- or stationary-phase cultures, as well as in cells suspended in M9 medium or reverse-osmosis water. Following incubation without added nutrients in M9 medium or unsterile reverse-osmosis water, the *E. coli* O157:H7 populations increased, although lower proportions of the organisms reduced CTC. Numbers of CTC-positive, FA-positive cells compared with R2A agar plate counts gave a strong linear regression ($R = 0.997$). Differences in injury did not appear to affect CTC reduction. The procedure, which can be completed within 3 to 4 h, has also been performed successfully with *Salmonella typhimurium* and *Klebsiella pneumoniae*.

Novel methods to detect bacteria in a range of environments which include drinking water and wastewater are needed. New approaches should be rapid, so that results can be obtained and appropriate action can be taken within an 8-h day. To offer an advantage over differential or selective culture methods, the innovative procedure should facilitate the detection and enumeration of specific viable bacteria. There has also been an increasing emphasis on the detection of specific organisms, particularly pathogens, rather than enumeration of indicator bacteria. Many existing techniques meet some, but not all, of these requirements.

Cultural methods have been used to detect and enumerate bacteria in environmental samples for over a century. However, for almost 50 years it has been known that these methods yield only a small percentage of the actual population in a sample (54). Early explanations for the discrepancies between culture and direct counting methods included aggregation of bacteria into clumps (18, 53) and failure to form colonies on plating media because of particular nutritional requirements (17). Other inadequacies of traditional plate counting methods, including cell injury and the viable but nonculturable state, have been discussed by several investigators (14, 16, 18, 25, 30, 31, 39). While membrane filter techniques permit concentration of bacteria and their separation from inhibitory materials in the sample, they suffer from the drawbacks inherent in plating methods because of their reliance on colony formation.

In the older most-probable-number (29) and more recent presence-absence (7) approaches, liquid culture media which may facilitate improved detection of some bacteria compared with plating methods are employed. These methods have, however, been criticized for their lack of precision and possible biases (1). In recent years, media have been developed for the simultaneous detection and confirmation of coliforms and *Escherichia coli* (1, 13, 26). While these systems offer advantages over conventional liquid media, especially in terms of the time required to obtain a confirmed result, there are persistent statistical concerns relating to the most-probable-number and presence-absence techniques on which they are based, such as their wide confidence intervals and their biases.

The direct viable count method was developed to detect individual viable bacteria in water (23). This relatively rapid method involves incubation of cells with nutrients and a quinolone antibiotic, usually nalidixic acid, which prevents cell division. As a result, nutrient-responsive cells elongate and may be enumerated microscopically. The operator must judge which cells have elongated, a procedure which can be difficult, especially when mixed populations are examined, although the use of image analysis may help to overcome this objection (41). Another rapid approach is the microcolony technique (52), in which cells are incubated for a few hours and allowed to form microcolonies which are then stained and enumerated microscopically.

Total direct microscopic techniques, such as the acridine orange direct count (9, 15) and the direct epifluorescent filter technique (32), permit enumeration of the total number of bacteria but do not distinguish between species, and assessment of their viability can be problematic (27). An advantage of these filter techniques is the concentration of bacteria, which increases sensitivity. Procedures which have been developed to detect metabolic activity of single cells or populations do not allow concurrent identification. Examples of these are procedures using tetrazolium salts, such as 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) (46) or 5-cyano-2,3-di-4-tolyl-tetrazolium chloride (CTC) (38), and other fluorochromes, including rhodamine 123 and fluorescein diacetate (28). Techniques which detect specific bacteria, including the fluorescent-antibody (FA) method (4) and molec-

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ular methods such as the PCR (3) and the use of nucleotide probes (51), give no indication of viability or physiological activity.

Enteropathogenic bacteria in water, including *E. coli* (11) and *Shigella* spp. (10), have been enumerated by a membrane filter FA technique, and the direct epifluorescent filter technique has been adapted for FA detection of *E. coli* O157 (47). In addition, both the direct viable count and microcolony methods have been used directly on membrane filters in combination with the FA technique to permit enumeration of specific viable salmonellae (12, 37). INT incubation to detect respiratory activity has been combined with FA staining to enumerate viable *Thiobacillus ferrooxidans* in natural water samples (2). In this procedure, cells which had been filtered, incubated with INT, and stained with FA were transferred to a microscope slide by a gelatin transfer technique and examined by epifluorescence microscopy.

We have developed a rapid, direct filtration-incubation procedure which facilitates an assessment of respiratory activity concurrently with the detection of specific bacteria, in this case enterohemorrhagic *E. coli*, serogroup O157:H7. In the first step bacteria concentrated on a membrane filter are incubated with CTC to detect respiratory activity (33). The cells on the membrane are then fixed and reacted with a specific FA prior to enumeration by epifluorescence microscopy. A patent application has been filed for this method (34). In this report, the procedure is described, including the effects of exposing the cells to liquids in the presence or absence of other species. Enterohemorrhagic *E. coli* O157:H7 was chosen to demonstrate the technique because of its increasing public health significance.

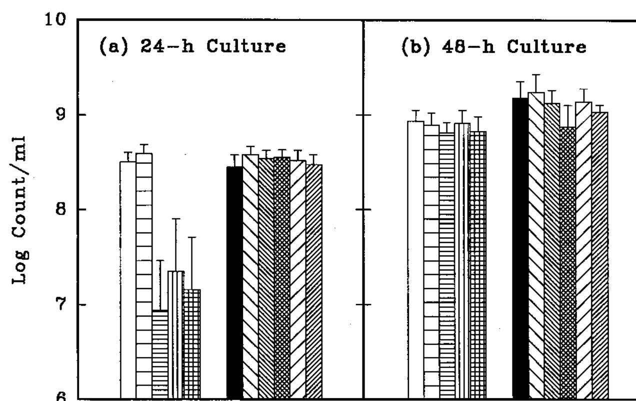

FIG. 1. *E. coli* O157:H7 cultures grown in CA-YE medium to logarithmic phase (24 h) (a) and stationary phase (48 h) (b). Cultures were diluted in PBS and enumerated by plate counts on R2A agar (▭), TLY agar (▭), TLYD agar (▤), MSA (▥), or MacConkey lactose agar (▦) and by direct microscopic counts as follows: DAPI-stained control (■), DAPI staining after CTC incubation (▧), CTC positive with DAPI (▧), FA control (▩), FA after CTC incubation (▨), and CTC positive with FA (▨). Bars indicate standard errors of the means.

MATERIALS AND METHODS

Bacterial strains and growth conditions. A culture of *E. coli* 932, serotype O157:H7, was provided by the U.S. Environmental Protection Agency, Cincinnati, Ohio. The culture was streaked on MacConkey sorbitol agar (MSA) and tryptone lactose yeast extract (TLY) agar (42) to determine purity, harvested from TLY agar into 20% glycerol–2% peptone, and frozen (−70°C) as stocks. Identity was confirmed by using API 20E test strips (bioMérieux, Hazelwood, Mo.). Unless specified otherwise, all media and solutions were prepared with reagent grade water (Milli-Q UV Plus; Millipore Corp.) and sterilized by autoclaving at 121°C for 20 min.

Frozen stock cultures were inoculated into 100-ml volumes of medium containing 0.3% Casamino Acids and 0.03% yeast extract (CA-YE) in sidearm flasks, at a ca. 10^5-CFU/ml starting inoculum, and incubated at room temperature (RT) with shaking at 100 rpm for 24 to 25 h to obtain cells in mid- to late logarithmic phase with continued incubation to stationary phase at 48 to 50 h. Optical density readings (Klett-Summerson Photometric Colorimeter) were made periodically throughout the incubation to confirm the phase of growth.

For starvation experiments, 5 ml of 48-h CA-YE culture was centrifuged with refrigeration at ca. 3,000 × *g* for 15 min and resuspended in 5 ml of sterile distilled water. These suspensions were diluted to obtain ca. 10^5 target cells per ml in 100 ml of either sterile M9 medium without nutrients (36) or Millipore reverse-osmosis (RO) water. Fresh RO water, which normally contains 10^2 to 10^3 CFU of heterotrophic bacteria per ml, was incubated at RT on a shaker at 100 rpm for 5 days to develop a stable bacterial population. Stabilized RO water usually contained ca. 10^5 cells per ml, and it was desirable to inoculate the water with about an equal number of *E. coli* O157 cells. Thus, the target number of 10^5 *E. coli* O157 cells per ml was adopted. The M9 and RO cell suspensions were incubated at RT on the shaker for an additional 48 h. The sterile M9 medium was used to determine the effects of starvation on the CTC and FA reactions, and the RO water was used to determine the effects of starvation and competition by indigenous heterotrophic microorganisms on FA specificity.

Cell suspension preparation. An aliquot of CA-YE culture was diluted to 10^{-1} or 10^{-2} in 0.22-μm-pore-size-filtered (Millipore type GS) autoclaved phosphate-buffered saline (PBS; pH 7.5) and vortexed at full speed for 1 min (Fisher Vortex Genie 2). A sample of this suspension was diluted in PBS (usually a further 10^{-2} or 10^{-3} dilution) to obtain ca. 10^5 cells per ml. This suspension was decimally diluted in sterile distilled water blanks for enumeration by a modified drop plate method (35) on R2A agar (1), TLY agar, TLYD agar (TLY with deoxycholate) (42), MSA, and MacConkey lactose agar. Cell injury was estimated as the percent difference between counts on the TLY and TLYD agar media. The same suspension was membrane filtered and examined by staining with 4,6-diamidino-2-phenylindole (DAPI), the CTC reduction assay, FA staining, and the combined CTC-FA procedures described below.

DAPI staining. The cell suspension (usually 5 or 10 ml) was filtered through a black polycarbonate membrane (25-mm diameter, 0.2-μm pore size; Nuclepore). The membrane was transferred to a 25-mm-diameter absorbent pad (Millipore) saturated with 0.6 ml of sterile water and 0.1 ml of 37% formaldehyde and incubated for at least 5 min at RT to fix the cells. The membrane was replaced on the filter apparatus, covered with 0.5 ml of a 10-μg/ml prefiltered solution of DAPI (Sigma), and incubated for 5 min before the stain was vacuumed off. This DAPI concentration and this staining time are well within the ranges used by others (22). The membrane was placed in a small petri dish on a 47-mm-diameter absorbent pad (Millipore) and allowed to dry. Dried membranes were kept under refrigeration for up to 7 days before examination. Immediately before microscopic examination, the membrane was placed on a drop of carbonate-buffered glycerol (pH 9; glycerol with 0.05 M carbonate buffer) on a microscope slide, and another drop of glycerol was placed on top, followed by a coverslip which was firmly pressed down to flatten the membrane filter. Filters were examined under oil immersion epifluorescence by using a Leitz Ortholux II microscope with Leitz filter block B2 (excitation, 350 to 410 nm; dichroic mirror, 455 nm; suppression filter, 470 nm).

Membrane filter CTC reduction assay. A membrane filter CTC reduction assay was performed as described elsewhere (33). Essentially, the cell suspension was filtered through a black polycarbonate membrane, and the membrane filter was transferred to an absorbent pad saturated with 0.85% physiological saline containing 5 mM CTC (Polysciences). Following incubation at RT for 1.5 h, the membrane filter was lifted, 0.1 ml of formaldehyde (37%) was dispensed onto the pad, and the membrane filter was replaced and incubated for at least 5 min. The membrane filter was stained with DAPI on a filter apparatus, dried on an absorbent pad, and mounted in buffered glycerol under a coverslip. The mounted filter was examined by epifluorescence microscopy (Leitz Ortholux II) by using light filters for DAPI (Leitz filter block B2) or CTC (Leitz filter block N2.1; excitation, 515 to 560 nm; dichroic mirror, 580 nm; suppression, 580 nm). Both the DAPI stain and CTC-formazan crystals could be observed together by using filter block H (excitation, 420 to 490 nm; dichroic mirror, 510 nm; suppression, 520 nm).

FA staining. The cell suspensions were filtered as described above, transferred to a pad saturated with physiological saline and formaldehyde, and incubated for at least 5 min. The membrane was transferred to a small petri dish which was placed on a slide warmer (Fisher) at ca. 65°C for 10 min and then was allowed to cool. A drop (50 μl) of 0.5% hydrolyzed gelatin (pH 7.2) was placed on the filter and spread over the surface; the filter was heated on a slide warmer at ca. 45°C for 5 to 10 min (until almost dry) and then allowed to cool. For *E. coli* O157:H7, a drop (50 μl) of fluorescein isothiocyanate-conjugated O157 antibody (Kirkegaard & Perry Laboratories) diluted 1:100 in PBS was placed on the membrane filter and spread with the top end of a 200-μl dispenser tip. The petri dish lid which contained the saturated pad was replaced to form a humidity chamber for the 20-min incubation. The membrane filter was gently removed from the dish, transferred to a filtration apparatus, and washed with three 0.5-ml volumes of PBS. The filter was either dried and stored as stated above or mounted in buffered glycerol under a coverslip for microscopic examination. Epifluorescence microscopy was performed as described above by using Leitz

TABLE 1. Percents injured and CTC-reducing cells[a]

Culture	% Injury[b]	% CTC+/ DAPI+[c]	% CTC+/ FA+[d]
CA-YE			
24 h	91.4 ± 6.1	90.8 ± 2.3	91.0 ± 1.5
48 h	16.1 ± 8.5	79.9 ± 13.0	69.7 ± 11.4
M9, before incubation	4.9 ± 5.2	85.5 ± 8.5	71.3 ± 10.5
RO, before incubation	6.0 ± 5.6	82.1 ± 7.4	80.3 ± 4.0
M9, after incubation	24.9 ± 12.0	79.8 ± 5.2	78.0 ± 2.5
RO, after incubation	17.2 ± 13.1	55.5 ± 6.2	76.2 ± 7.4

[a] Suspensions of *E. coli* O157:H7 cells were prepared with PBS after incubation in CA-YE medium (mid-logarithmic growth phase, 24-h culture; stationary phase, 48-h culture). Suspensions of cells from stationary-phase cultures were also examined before and after incubation in M9 medium or RO water for 48 h at RT. Results are means ± standard errors ($n = 4$).

[b] [(TLY CFU − TLYD CFU)/(TLY CFU)] × 100.

[c] (CTC-positive, DAPI-stained cells/DAPI-stained cells) × 100.

[d] (CTC-positive, FA-stained cells/FA-stained cells) × 100.

filter block H. FA method controls were prepared by substitution of PBS for the fluorescein isothiocyanate-conjugated FA solution, followed by staining with DAPI to determine if the FA procedure resulted in any changes in total cell numbers.

Combined CTC reduction and FA procedure. Cell suspensions were filtered and incubated with CTC for 1.5 h as described above and then transferred to a pad saturated with physiological saline and formaldehyde. After 5 min of incubation with formaldehyde, the filter was heat fixed, reacted with FA for 20 min, rinsed, and mounted for microscopic examination. Leitz filter block H was used to visualize both CTC-formazan crystals and FA-positive cells, and filter block N2.1 was utilized to confirm and enumerate the CTC-positive cells. The direct FA O157 procedure results were compared with the number of FA-positive cells for the combined CTC-FA technique to determine if the CTC incubation affected the FA results.

RESULTS AND DISCUSSION

Viable *E. coli* O157:H7 cells mounted on black polycarbonate filters and stained with DAPI following incubation with CTC appeared blue-green with bright red-orange spots of CTC-formazan when examined by epifluorescence microscopy using Leitz filter block H. Similar results were observed with CTC-FA-stained cells, although the cells appeared larger because of the FA attached to extracellular antigenic material.

Logarithmic- and stationary-phase cultures. Plate counts on R2A and TLY agar increased by less than 1 log between the 24- and 48-h incubation times (Fig. 1). Optical density readings showed that the CA-YE culture was in the mid- to late logarithmic phase of growth at around 24 h of incubation and that it had reached stationary phase before 48 h. The final cell concentrations in this relatively low-nutrient medium (0.3% Casamino Acids and 0.03% yeast extract) were ca. 10^9 CFU/ml. Growth on TLYD agar, MSA, and MacConkey lactose agar was severely restricted for the 24-h cultures but not for the 48-h samples (Fig. 1). There was over 90% injury in the actively growing 24-h cultures (Table 1), while less than 20% injury was detected for the stationary-phase 48-h cultures. This observation is consistent with chemostat data which show that faster-growing bacteria are more sensitive to antimicrobial agents (24). The MacConkey media also detected almost 100-fold fewer *E. coli* O157 cells in the 24-h cultures than the nonselective R2A and TLY media (Fig. 1). The MacConkey media, like TLYD agar, contain the bile salt surfactant deoxycholate, which is well known for its inhibitory effect on target bacteria (25). It is interesting that in this study, the actively growing cultures were most sensitive to the selective effects of these media.

Suspensions of CA-YE cultures diluted in PBS at 24 and 48 h of incubation stained only with DAPI, and those incubated with CTC before DAPI staining increased in total cell numbers by ca. one-half log from 24 to 48 h (Fig. 1). More than 90% of the cells in the logarithmic-phase (24-h) sample were CTC positive, and 70 to 80% of the stationary-phase (48-h) cells reduced CTC (Table 1).

DAPI staining of cells after CTC incubation yielded slightly higher counts compared with simple DAPI staining for both 24- and 48-h CA-YE cultures (Fig. 1; Table 2). The numbers of CTC-reducing cells enumerated by the CTC-FA technique were almost identical to those determined by the CTC-DAPI method for the growing cultures, and about 40% fewer cells were detected in the stationary phase (Table 2). Performing the FA procedure following CTC incubation did not appreciably affect the numbers of CTC-positive cells, except for the stationary-phase cultures.

M9 medium and RO water suspension. When stationary-phase cells were centrifuged, resuspended in M9 medium, and incubated on a shaker for 48 h at RT to facilitate starvation, there was a slight increase in viable plate counts (Fig. 2). Little injury (ca. 5%) was detected in the M9 and RO water suspensions before incubation, and about 20 to 25% of these popu-

TABLE 2. Ratios of *E. coli* O157:H7 cell counts determined by the CTC-DAPI, FA, and CTC-FA procedures to cell counts for DAPI-stained controls[a]

Culture	Ratio for:				
	CTC-inc, DAPI[b]	CTC+, DAPI[c]	FA control[d]	CTC-inc, FA+[e]	CTC+, FA+[f]
CA-YE					
24 h	1.35 ± 0.11	1.23 ± 0.10	1.28 ± 0.17	1.17 ± 0.10	1.07 ± 0.10
48 h	1.21 ± 0.23	0.88 ± 0.09	0.65 ± 0.18	0.95 ± 0.15	0.61 ± 0.12
M9, before incubation	1.28 ± 0.09	1.10 ± 0.02	1.39 ± 0.49	1.53 ± 0.40	1.06 ± 0.20
RO, before incubation	1.15 ± 0.15	0.94 ± 0.12	0.82 ± 0.13	0.93 ± 0.24	0.79 ± 0.20
M9, after incubation	1.00 ± 0.06	0.79 ± 0.05	0.91 ± 0.05	0.97 ± 0.10	0.84 ± 0.09
RO, after incubation	0.82 ± 0.17	0.48 ± 0.12	0.48 ± 0.16	0.41 ± 0.17	0.45 ± 0.27

[a] Cultures were prepared as described in Table 1. Results are means ± standard errors ($n = 4$). DAPI-stained controls contained no CTC or FA.

[b] Cells stained with DAPI after CTC incubation.

[c] CTC-positive, DAPI-stained cells.

[d] No CTC or DAPI.

[e] FA-stained cells after CTC incubation.

[f] CTC-positive, FA-stained cells.

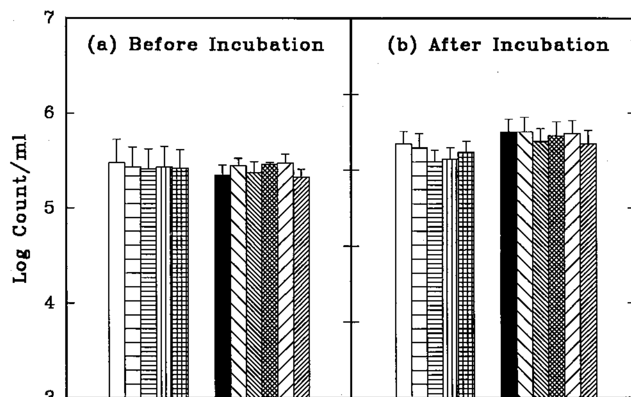

FIG. 2. Agar plate counts and direct microscopic counts of 48-h CA-YE cultures of *E. coli* O157:H7 which were centrifuged and resuspended in M9 medium without a carbon source (a) and incubated at RT for 48 h (b). Fill patterns and error bars are the same as for Fig. 1.

lations were injured after incubation (Table 1). The MSA counts of sorbitol-negative colonies in the RO water suspension samples confirmed that the R2A and TLY agar plate counts were composed almost totally of the introduced *E. coli* O157 cells, which outnumbered the natural bacterial population in RO water, according to plate count data (Fig. 3).

Total DAPI cell counts in M9 medium and, more obviously, in RO water after 48 h of incubation increased somewhat from those taken at the time of inoculation (Fig. 2 and 3). Interestingly, the plate counts on media including nonselective R2A and TLY did not indicate this increase (Fig. 3), even though the plates were incubated at RT for 5 to 7 days. The apparent increases in cell numbers in M9 medium and RO water might be attributed to the use of endogenous reserves in the cultured *E. coli* O157 cells, because the M9 medium was not supplemented with a carbon source. It is also possible that the increases observed with DAPI resulted from growth of RO water subpopulations which were not detected on R2A or TLY agar. In RO water, the larger increase in DAPI counts following incubation (Fig. 3) compared with the slight increase in FA counts suggested that heterotrophic bacteria which were natural contaminants in the RO water, rather than the introduced *E. coli* O157, grew during incubation. Almost 50% of the O157 cells were CTC negative after incubation in RO water (Table 2). The DAPI-stained cells included the natural population of RO water organisms in addition to the introduced *E. coli*. Immediately after the M9 cell suspension was prepared, the FA control counts and CTC-incubated, FA-stained counts were up to 1.5-fold greater than the DAPI control counts (Table 2). This suggested that DAPI staining did not detect all the *E. coli* O157 cells when they had been centrifuged and resuspended in M9 medium. After incubation in M9 medium, the DAPI control counts were only slightly greater than the FA counts (Table 2). We conclude that the FA counts are at least equivalent to, if not greater than, DAPI counts for similar populations.

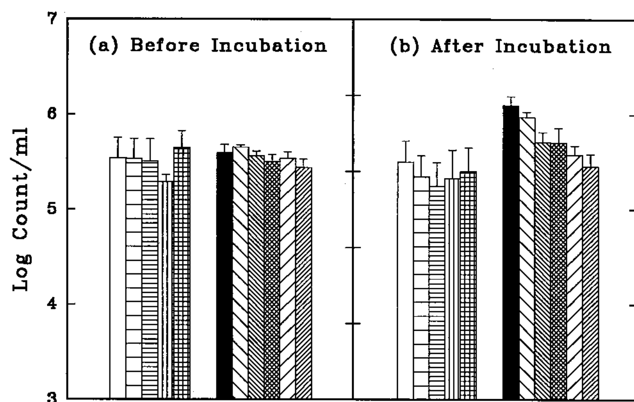

FIG. 3. Enumeration of 48-h CA-YE *E. coli* O157:H7 cultures which were centrifuged, resuspended in RO water (a), and incubated for 48 h at RT (b). Fill patterns and error bars are the same as for Fig. 1.

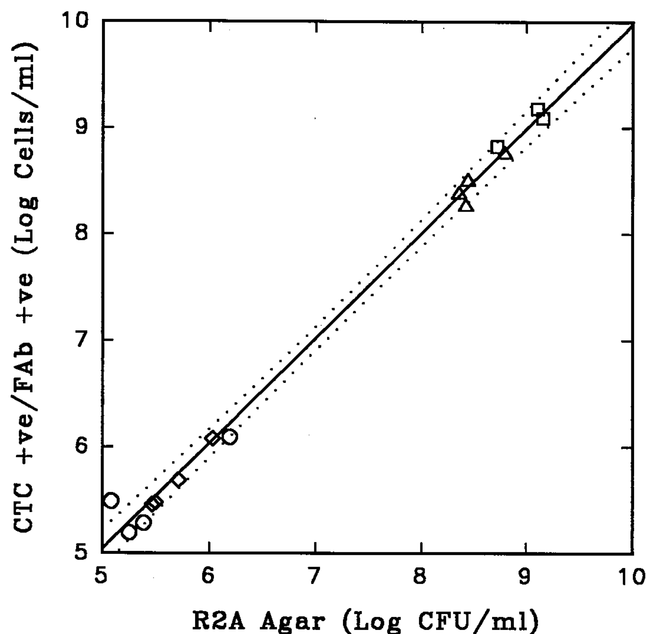

FIG. 4. Regression of counts of CTC-positive *E. coli* O157:H7 cells counterstained with FA against R2A plate counts. △, CA-YE logarithmic-phase (24-h) cultures; □, CA-YE stationary-phase (48-h) cultures; ○, M9 suspensions before incubation; ◇, M9 suspensions after incubation for 48 h at RT. ——, regression line; · · ·, 99% confidence interval. $R = 0.997$.

During incubation in RO water, the increased numbers of DAPI-stained cells indicated that the indigenous bacterial population multiplied (Fig. 3). The proportions of cells staining either with DAPI following CTC incubation or by FA methods decreased (Table 2). In addition, less than half of the RO water-incubated cells detected by DAPI or FA staining after CTC incubation showed respiratory activity by reducing CTC.

Regression of numbers of respiring *E. coli* O157:H7 cells enumerated by the CTC-FA technique with R2A agar plate counts showed that the methods gave comparable results over a wide range of cell concentrations (Fig. 4). Most values fell within the 99% confidence interval, and the regression coefficient was 0.997. This analysis included logarithmic- and stationary-phase cells diluted in PBS and stationary-phase cells which had been washed and resuspended in M9 medium, as well as the same populations after incubation for 48 h in M9. Cell injury, which was detected by enumeration on TLY and TLYD agar plates, varied significantly between these populations (Table 1), while the CTC counts were generally in agreement with R2A agar plate counts (Fig. 4). These results suggest that cell injury did not significantly affect CTC reduction.

The FA technique is an established method for detecting and identifying species which have been cultured and for which

antibody preparations are available. Danielsson et al. (10, 11) adapted the FA procedure for use with membrane filters, and others have successfully applied this approach in combination with the direct viable count (12) and microcolony (37) methods to enumerate viable pathogenic bacteria. The FA method has also been used in combination with INT staining to detect specific respiring bacteria (2).

Incubation with CTC to detect respiratory activity was originally proposed for Erlich's ascites tumor cells (45) and has since been used to enumerate respiring bacteria in water (8, 38, 40). Respiratory activity in populations of enteric bacteria in a polar marine environment (43) and in native bacteria in soil (48) has also been examined. The viabilities of *Pseudomonas fluorescens* (19) and coccoid *Campylobacter jejuni* (5) have likewise been determined by this technique. Flow cytometry has been used to enumerate *Micrococcus luteus* following CTC incubation (21). The CTC reduction technique has been applied to the examination of bacterial biofilms in situ on solid surfaces (49, 50). CTC is becoming more widely used in microbial ecology and physiology investigations, and we are learning more about its characteristics as an indicator of bacterial respiratory activity (6, 38, 40, 44).

The procedure that we have described incorporates the advantages of membrane filtration for sample concentration, incubation of the membrane on an absorbent pad saturated with CTC medium for assessment of respiratory activity, and reaction with an FA directly on the membrane for detection of a specific organism, which was, in this case, *E. coli* O157:H7. A similar method, using an indirect FA reaction, has been applied successfully to cultures of *Salmonella typhimurium* and *Klebsiella pneumoniae*. The time taken from sampling to completion of epifluorescence microscopy ranges from 3 to 4 h, which means that our procedure is significantly more rapid than many other methods. Discrimination of the specific respiring cells is relatively straightforward when the FA is conjugated with a fluorochrome, such as fluorescein isothiocyanate, which gives contrasting fluorescence with the appropriate epifluorescence microscopy filters. Results obtained by the CTC-FA technique compared favorably with plate counts with nonselective R2A medium.

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