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Factors Affecting the Determination of Respiratory Activity on the Basis of Cyanoditolyl Tetrazolium Chloride Reduction with Membrane Filtration

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Deficiencies in traditional bacterial enumeration techniques which rely on colony formation have led to the use of total direct counting methods, such as the acridine orange direct count technique for the enumeration of planktonic bacteria. As total direct counts provide no information on the viability or activity of the organisms, demonstration of respiratory activity with the fluorochrome cyanoditolyl tetrazolium chloride (CTC) has been employed. We have modified this technique by performing filtration prior to CTC incubation. Cells captured on a polycarbonate membrane were incubated on absorbent pads saturated with medium containing CTC. Following counterstaining with DAPI (4′,6-diamidino-2-phenylindole) total and respiring cells were enumerated by epifluorescence microscopy. Factors affecting CTC reduction by *Klebsiella pneumoniae*, *Salmonella typhimurium*, and *Escherichia coli* K-12 were investigated. With *K. pneumoniae*, nutrient additions to the CTC medium did not increase the number of respiring cells detected. CTC reduction by all three organisms decreased in response to an increase of the pH of the CTC medium above pH 6.5. Increasing phosphate concentrations contributed to this inhibitory effect. CTC-membrane filter counts of *K. pneumoniae*, *S. typhimurium*, and *E. coli* K-12 and of bacteria in well water corresponded closely with plate counts (r = 0.987). The results show that careful attention should be given to the composition of CTC-containing media which are used to enumerate respiring bacteria. With an appropriate medium, reliable enumeration of respiring bacteria can be achieved within a few hours.

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Traditional plate counting techniques may considerably underestimate the total number of viable or active bacteria in environmental samples (17) because of cellular injury (15) and the viable but nonculturable physiological state (6, 10, 26). Viable but nonculturable bacteria give positive direct viable count results when the method developed by Kogure et al. (13) is employed but fail to form colonies on solid media. Other possible causes of underestimation by plate counts include cell clumping and attachment to particulate material (8).

In response to the deficiencies inherent in plate counting methods, bacteria have been counted by direct microscopic techniques (3, 4, 7, 18). While these methods permit total cell enumeration, they do not give reliable estimates of viable or metabolically active cell numbers (1, 16, 19, 32). Incubation with 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT) and other tetrazolium salts has been used to detect actively respiring bacteria in aquatic environments, groundwater, and sediments from the subsurface (23, 30, 31, 33, 36).

Rodriguez et al. (25) introduced a novel method for direct visualization of actively respiring bacteria. Suspensions of groundwater, seawater, and wastewater bacteria were incubated with 5-cyano-2,3-di-4-tolyl-tetrazolium chloride (CTC), captured on black polycarbonate membrane filters, counterstained with 4′,6-diamidino-2-phenylindole (DAPI), and enumerated by epifluorescence microscopy. In the present study, we modified the CTC procedure (25) by incubating with CTC following capture of the bacteria by membrane filtration (MF) rather than performing CTC incubation in broth medium and then filtering. This MF-CTC approach is similar to that of Desmonts et al. (5), who adapted the direct viable count procedure by performing the incubation directly on polycarbonate membranes to detect viable *Salmonella* spp. in seawater.

Initially, we observed that the use of certain media and conditions for the CTC incubation decreased the number of cells reducing CTC. Subsequently, factors affecting the detection of respiring bacteria by this alternative MF-CTC method have been examined, including the effects of incubation medium, time, and temperature and the responses to various pH and phosphate concentrations with cultures of *Klebsiella pneumoniae*, *Salmonella typhimurium*, and *Escherichia coli* K-12. These cultures, and natural bacteria in domestic well water, were enumerated by the modified MF-CTC technique. Counts of these bacteria obtained by the MF-CTC method have been compared with plate counts. With appropriate incubation conditions, MF-CTC results correlated well with plate counts.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *K. pneumoniae* Kp1, isolated from a drinking water distribution system, was provided by D. Smith, South Central Connecticut Regional Water Authority, New Haven. A culture of *S. typhimurium* SL3201 (FIRN biotype) was obtained from B. A. D. Stocker at Stanford University. *E. coli* K-12 was supplied by N. D. Reed at Montana State University.

Cultures were streaked on MacConkey agar and tryptone-lactose-yeast extract agar to determine purity and harvested from tryptone-lactose-yeast extract in 20% glycerol–2% peptone as frozen (−70°C) stocks. 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. Identity of bacteria was confirmed by API 20E test strips (bioMérieux).

Frozen stock cultures were inoculated into 100-ml volumes of medium containing 0.3% Casamino Acids and 0.03% yeast extract (CA-YE) (28) and incubated at room temperature with shaking at 100 rpm for 18 to 24 h to obtain cells in mid- to late logarithmic phase.

Cell suspension preparation. All solutions used for MF procedures were prefiltered (0.22-μm pore-size filter, type GS; Millipore) and autoclaved prior to use, taking care to rinse dispensers and containers to remove microscopic particles. Phosphate-buffered saline (PBS; 10.03 mM PO_4, 0.85% NaCl, pH 7.5) was prepared from a sterile 100× stock solution. An aliquot of culture was diluted 10^{-1} or 10^{-2} in PBS and agitated on a Vortex mixer at full speed for 1 min

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(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. With *E. coli* K-12, the effects of cell suspension diluents on subsequent CTC reduction were examined by diluting the culture in sterile water, phosphate-buffered water without magnesium sulfate (21), physiological saline (PS; 0.85% NaCl), or PBS. These suspensions were prepared and held at room temperature for up to 1 h before filtration or plating.

Well water samples. Samples (ca. 3 liters) were collected in sterile polyethylene containers from a domestic faucet with an untreated well water supply after running ca. 20 liters of water to waste. The samples were transported to the laboratory at air temperature within 1 h, refrigerated on arrival for up to 3 h, and blended for 5 min on ice at half-speed (ca. 12,000 rpm; Tekmar Ultra-Turrax) immediately before examination. Volumes of 20 to 40 ml were filtered for direct counts, and dilutions were prepared in sterile water for plate counting.

Membrane filter CTC reduction assay. A 5- or 10-ml sample of the final cell suspension was filtered through a black polycarbonate 0.2-μm-pore-size membrane filter (25 mm in diameter; Nuclepore). The membrane filter was transferred to a 25-mm-diameter absorbent pad (Millipore) which had been saturated with 0.6 ml of medium containing CTC (Polysciences) at the desired concentration and incubated at room temperature protected from light for 0.5 to 4.0 h.

Fixation and DAPI counterstaining. After incubation, the membrane filter was removed from the pad, 0.1 ml of 37% formaldehyde (Baker) was dispensed onto the pad, and the membrane filter was replaced and fixed for 5 min. The membrane filter was then returned to the filter funnel apparatus, covered with 0.5 ml of either 1.0 or 10 μg of DAPI (Sigma) solution per ml, and incubated for 5 min at room temperature. The DAPI solution was removed by applying vacuum to the filter funnel apparatus; then the filter was removed and placed on a 47-mm-diameter absorbent pad (Millipore) in a petri dish to dry. Dried filters were refrigerated for up to 14 days, and no significant change in either DAPI- or CTC-positive cell numbers was observed over this time.

CTC assay media. Various media were used because it was found that CTC reduction may be affected by using different media. CA-YE medium was prepared as described above. R2A broth (R2A) containing phosphate was prepared as described elsewhere (24) without agar. R2A broth without phosphate, half-strength R2A broth, and half-strength R2A broth without phosphate were prepared according to the same formulation. Trypticase soy broth (Difco) was prepared according to the manufacturer's directions.

The pH of 20 mM phosphate buffer was adjusted by dissolving different proportions of Na_2HPO_4 and KH_2PO_4 in PS. To determine the effect of varying the phosphate concentration in PS at a constant pH, 100 mM solutions of Na_2HPO_4 and KH_2PO_4 in PS were mixed in a 7:3 ratio and adjusted to pH 6.5 with Na_2HPO_4 or KH_2PO_4. The 100 mM PBS solution was diluted with PS to obtain phosphate concentrations from 10 to 100 mM. The pH of 3-[*N*-morpholino]propanesulfonic acid (MOPS) buffer was adjusted by adding KOH in various amounts. PS and sterile reagent-grade water (Milli-Q UV Plus) were also used for CTC incubations. CTC was dissolved directly in these media at a concentration of 2.0 or 3.5 mM or at 1.28 mM when deionized distilled water was used. The 2 mM concentration is in the lower end of the range suggested by Rodriguez et al. (25), and 1.28 mM is equivalent to the concentration of 4 mg/ml, which is a commonly used concentration for INT. For the experiments with different buffers, a 20 mM stock solution of CTC was prepared and diluted in the medium to obtain the required final concentration. All CTC solutions were prepared on the day of use.

Epifluorescence microscopy enumeration. Immediately prior to microscopic examination, each dried filter was mounted on a microscope slide in low-fluorescence immersion oil (Cargille type A). A coverslip was placed on top and pressed down to remove excess oil or air and flatten the polycarbonate membrane. The target number of cells observed in each microscope field ocular grid on a membrane filter was 10 to 100, and at least 10 fields were counted to obtain a total of at least 200 cells.

Bacteria on the filters were examined by epifluorescence microscopy (Leitz Ortholux II), using light filters for DAPI (Leitz filter block B2; excitation, 350 to 410 nm; dichroic mirror, 455 nm; suppression, 470 nm) 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). DAPI-stained cells were counted to determine the total number, and CTC-positive cells were counted to determine the number of actively respiring cells. The number of cells per milliliter was calculated from the average number of cells per ocular grid, the filter area, and the grid area plus the dilution and sample volume filtered. The proportion of CTC-positive (active) cells was calculated as a percentage of the DAPI-stained (total) cells.

Plate counts. A modified drop plate count procedure was used (22), with tryptone-lactose-yeast extract agar incubated for 24 h at 35°C used for enumerating enterobacteria cultures and R2A agar incubated for 5 to 7 days at room temperature used for detecting bacteria in well water.

RESULTS

The overall aim of this study was to determine the optimal conditions for enumerating respiring cells, using a CTC reduction assay on membrane-filtered cell suspensions and environmental water samples. In preliminary experiments, about 60% of actively growing *K. pneumoniae* cells collected on filters and incubated on pads saturated with either R2A or half-strength R2A medium without phosphate rapidly reduced CTC, with detectable CTC-formazan formation, within 15 min of the start of incubation. A DAPI concentration of 10 μg/ml was used because it gave more consistent results than a 1-μg/ml solution. Staining with 10 μg of DAPI per ml for 5 min, the procedure used in this study, is consistent with the procedure used in many other studies (12). The resulting DAPI stain appeared luminescent blue-green with the B2 filters and green with the H filters and was not visible with the N2.1 filters. CTC-formazan appeared dark red with the N2.1 filters, pale red with the H filters, and pale red-orange with the B2 filters. As there were sometimes two or more CTC-formazan crystals in some cells, and the crystal(s) in some CTC-positive cells could be observed only with the N2.1 filters, it was necessary to switch from one filter combination to another to accurately count the CTC-reducing cells.

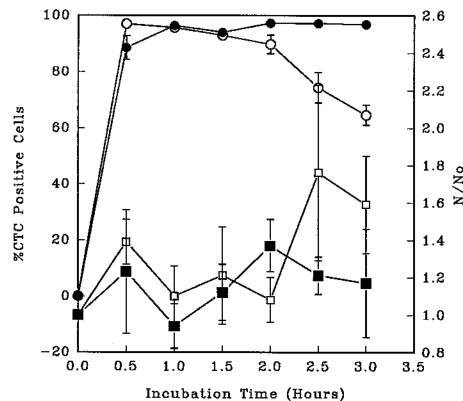

FIG. 1. Effects of incubation temperature on CTC reduction by *K. pneumoniae* Kp1 with CA-YE medium containing 2 mM CTC. Percent CTC-positive cells following incubation at room temperature (●) or 35°C (○); N/N_0 following incubation at room temperature (■) or 35°C (□). Bars indicate standard errors (n = 3).

Effects of incubation time and temperature. In an effort to optimize the numbers of CTC-reducing cells detected, incubation with CTC dissolved in nutrient media without added phosphate was investigated at room temperature and at 35°C. Incubation of *K. pneumoniae* with 2 mM CTC in CA-YE at room temperature or 35°C for up to 3 h showed that, with incubation at 35°C, the proportion of respiring cells decreased, while the total cell number increased (Fig. 1). Similar results were observed when half-strength R2A without PO_4 was used as the incubation medium: 98.1% ± 0.4% of cells reduced CTC at room temperature compared with 75.8% ± 13.8% at 35°C. More than 95% of *K. pneumoniae* cells incubated at room temperature on CA-YE for 1 to 3 h (Fig. 1) reduced CTC, with an increase in cell numbers of less than 1.2-fold. When no microcolonies were formed, the observed increase in total cell numbers during incubation may be attributed to enhanced DAPI staining, because the cells appeared brighter than those stained before the CTC incubation regardless of the CTC medium. This may have been due to changes in cell permeability or affinity of nucleic acids for DAPI.

Room temperature incubation for 3 h on either half-strength R2A medium without phosphate or CA-YE medium resulted in a higher proportion of cells reducing CTC. With half-strength R2A medium without phosphate and room tempera-

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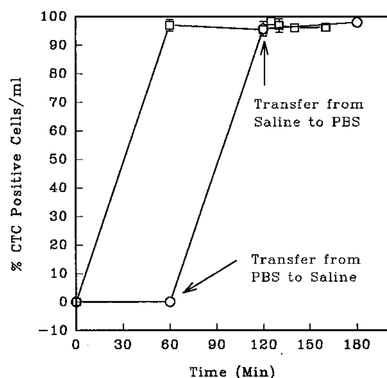

FIG. 2. Inhibition of CTC reduction in *K. pneumoniae* Kp1 by incubation with PBS (pH 7.5). Cells captured on membrane filters were transferred from PBS to PS at 60 min (○) or from saline to PBS at 120 min (□). Both media contained 2 mM CTC. Error bars indicate standard deviation (n = 3).

ture incubation, cell numbers increased 1.5-fold. With the 35°C incubations it was observed that microcolonies began to form within 2.0 to 3.0 h. It was also found that many of the cells in microcolonies did not contain CTC-formazan crystals when examined microscopically. Some individual cells, particularly those that became enlarged, did not contain fluorescent CTC-formazan deposits. The apparent failure of some recently reproduced cells in microcolonies to reduce CTC at 35°C resulted in the decrease observed in the proportion of cells that were CTC positive after 2 h of incubation. Winding et al. (33) also noted that some cells in microcolonies of soil bacteria did not contain CTC-formazan crystals.

Effects of phosphate and pH. When *K. pneumoniae* cells which had been grown in CA-YE and diluted in PBS were collected by MF and incubated initially on PS containing 2 mM CTC, ca. 95% reduced CTC within 60 min (Fig. 2). After 120 min, these filters were transferred to PBS containing CTC (PBS-CTC) and incubated for a further 60 min with no change in the proportion of cells reducing CTC. In contrast, identically prepared cells incubated initially on PBS-CTC failed to reduce CTC within the first 60 min. The filters were then transferred to PS containing CTC (PS-CTC). After an additional 30 min (total incubation time, 90 min), the proportion of cells which reduced CTC had reached >95%, which was the same as for the filters which were initially incubated on PS-CTC. The proportion of cells reducing CTC on the filters exposed to the PBS-CTC for 60 min which were then switched to PS-CTC for 120 min remained at >95% after a total of 180 min. It was apparent from these experiments that the phosphate in PBS inhibited CTC-formazan formation. It also appeared that phosphate in the medium did not cause quenching or the loss of CTC crystals from the cells.

The inhibitory effect of PBS was initially attributed solely to the phosphate component, because a similar phenomenon resulting in the inhibition of CTC reduction had been observed with the R2A media which contained phosphate. Experiments in which the phosphate concentration in the CTC reaction medium was varied from 0 through ca. 32 mM, using three organisms (Fig. 3), were performed. CTC reduction by each of the three bacteria examined was increasingly inhibited with higher phosphate concentrations. The inhibition was observed to be greatest for *K. pneumoniae* and *S. typhimurium*, occurring to a lesser extent with *E. coli* K-12. Subsequent experiments revealed that this effect was related to the pH of the medium containing CTC, as well as to the phosphate concentration. This effect was also most pronounced with the *Klebsiella* and *Salmonella* spp. used in this study.

Experiments in which *K. pneumoniae* suspensions were filtered and incubated on PBS (containing 2 mM CTC) adjusted to pH 6.5, 7.0, 7.5, and 8.0, followed by staining directly or rinsing with PBS (pH 6.5) and then staining, were performed to elucidate the effects of pH differences on CTC reduction. Initial incubation at pH values of >6.5 inhibited CTC reduction, and this inhibition was not reversed by rinsing with pH 6.5 PBS. This again confirmed that the apparent decrease in CTC-formazan-containing cells was not due to either quenching or solubilization by phosphate in the incubation medium or rinse buffer.

The pH values of the media used to obtain the results presented in Fig. 3 indicate that CTC reduction was significantly inhibited for all three species at pH values above 6.5. For *E. coli* K-12, there also appeared to be a relationship between inhibition of CTC reduction and phosphate concentration. The gradual decline in the percentage of *S. typhimurium* cells reducing CTC at phosphate concentrations of >15.79 mM, when the pH was constant at 7.8, suggested that both more than 16 mM phosphate and pH values of >7.0 may be inhibitory to CTC-formazan formation.

Further experiments were performed to determine if the observed inhibition of CTC reduction was due primarily to pH or to the concentration or form of phosphate in the CTC reaction medium. The pH of PBS-CTC containing 20 mM PO_4 was adjusted by varying the proportions of Na_2HPO_4 and KH_2PO_4 in PS while maintaining a phosphate concentration of 20 mM. CTC reduction by *K. pneumoniae* and *S. typhimurium* was inhibited at pH values of >7.0 (Fig. 4a). A less pronounced effect was observed with *E. coli* K-12. Similar results were obtained by using 40 mM MOPS buffer in which the pH was varied by adding KOH (Fig. 4b), although inhibition of CTC reduction by *K. pneumoniae* occurred between pH 6.5 and 7.0. The effect on *E. coli* K-12 of increasing the pH of MOPS medium (Fig. 4b), which contained no phosphate, was much greater than that found with PBS (Fig. 4a).

To quantify the effect of phosphate on CTC reduction, increasing phosphate concentrations from 0 to 100 mM were prepared at pH 6.5 in saline. The proportion of *K. pneumoniae* Kp1 cells which reduced CTC diminished at phosphate concentrations above 50 mM (Fig. 5). CTC reduction by *E. coli* K-12 and *S. typhimurium* decreased only slightly with increasing phosphate concentration. The results shown in Fig. 4 and 5

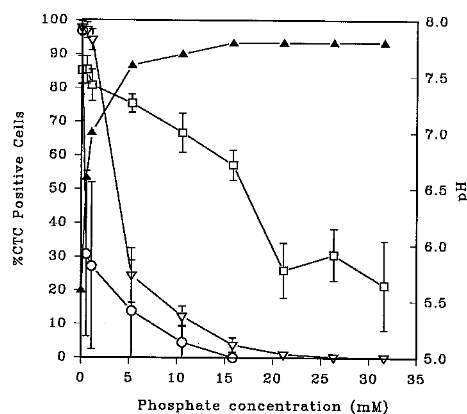

FIG. 3. Effect of varying the phosphate concentration in PS, and resultant pH, on CTC reduction with 2 mM CTC. ○, *K. pneumoniae* Kp1; □, *E. coli* K-12; ▽, *S. typhimurium* SL3201; ▲, pH.

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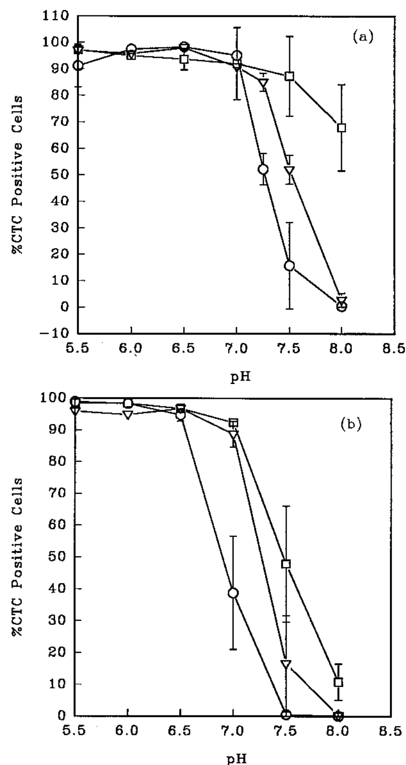

FIG. 4. Effect of varying the pH on CTC reduction with 2 mM CTC: PBS containing 20 mM PO_4 (a) or 40 mM MOPS buffer (b). ○, *K. pneumoniae* Kp1; □, *E. coli* K-12; ▽, *S. typhimurium* SL3201.

indicate that both medium pH and phosphate concentration may affect CTC-formazan formation, depending on the bacterial species or strain under consideration. We found that the mean pH of PS containing CTC (2 mM) was 5.9 with a standard deviation of 0.3 (n = 17; range, 5.2 to 6.3). It may be concluded that the CTC caused a decrease in pH in an unbuffered medium such as PS, while buffering by either phosphate or other medium components caused the pH to remain at higher values, resulting in less CTC reduction.

Effects of diluents. It was also found that CTC reduction by *E. coli* K-12 was affected by the diluent in which cells were

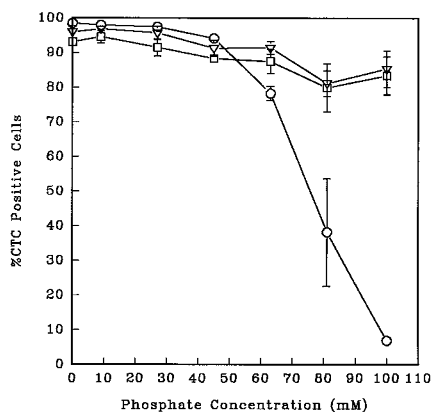

FIG. 5. Effect of varying the phosphate concentration at pH 6.5 on CTC reduction with 2 mM CTC in PS. Symbols are the same as given in the legend to Fig. 4.

TABLE 1. Regression of CTC counts against plate counts for samples from cultures of *K. pneumoniae*, *S. typhimurium*, and *E. coli* K-12 and domestic well water

Sample	No. of samples	Intercept	Slope	r
K. pneumoniae	17	0.67	0.87	0.77
S. typhimurium	13	−0.27	1.06	0.95
E. coli K-12	13	1.38	0.73	0.64
Well water	7	1.84	0.43	0.51
Overall	50	0.23	0.96	0.99

suspended prior to filtration and incubation. When the suspension was prepared with phosphate-buffered water (0.3 mM phosphate) or sterile water, 98.4% ± 0.9% (n = 3) and 97.4% ± 0.6% of the cells, respectively, reduced CTC. Suspension in PS or PBS (10 mM phosphate) resulted in decreases to 76.7% ± 5.2% and 71.9% ± 6.6% CTC-positive cells, respectively, and these results were much more variable than those with phosphate-buffered or sterile water. This suggests that, at least for this strain of *E. coli*, the cell diluent used prior to filtration may significantly affect subsequent CTC reduction. All subsequent experiments with this organism were performed following dilution in water.

Comparison of MF-CTC data with plate counts. CTC reduction by the three enterobacterial cultures was subsequently determined by incubating cells collected by MF on absorbent pads saturated with PS containing 2 mM CTC, and half-strength R2A medium without phosphate containing 3.5 mM CTC was used to enumerate respiring bacteria in well water samples. Well water bacteria were incubated for 3 h at room temperature with 3.5 mM CTC in various media, using half-strength R2A without phosphate as the control. Compared with 100% for half-strength R2A without phosphate, MF-CTC counts (n = 6) were 66.2% ± 9.8% with half-strength R2A containing phosphate, 59.8% ± 14.1% with PS, and 72.4% ± 13.8% when reagent-grade water was used as the CTC medium. For this reason, half-strength R2A without phosphate was chosen as the medium for CTC incubation of well water samples.

The resultant counts of respiring, and therefore presumably active or viable, cells were compared with plate count data (Table 1) by calculating the linear regressions. The regressions obtained for *K. pneumoniae* and *S. typhimurium* indicated good agreement between the numbers of actively growing cells which reduced CTC and those able to form colonies on agar. The regression coefficients and slopes for *E. coli* K-12 and well water bacteria were somewhat lower. This may have been related to the relatively narrow range of cell counts obtained for each sample type, which were within 1 log, i.e., ca. 10^5 CFU/ml for the diluted cultures and ca. 10^3 CFU/ml for the well water samples (Fig. 6). The single "outlier" well water sample significantly affected the statistical analysis for the seven data points (Table 1) but did not affect the analysis when the well water results were combined with the results for *K. pneumoniae* cultures and well water samples were analyzed together to extend the range over several logs. The overall regression for the *K. pneumoniae* and well water data indicated good correspondence between plate counts and CTC reduction results (Fig. 6). With the phosphate-free medium and the well water samples, total DAPI-stained cell numbers increased only slightly on average (mean N/N_0 = 1.18) during the 3-h room temperature incubation with 3.5 mM CTC. This result,

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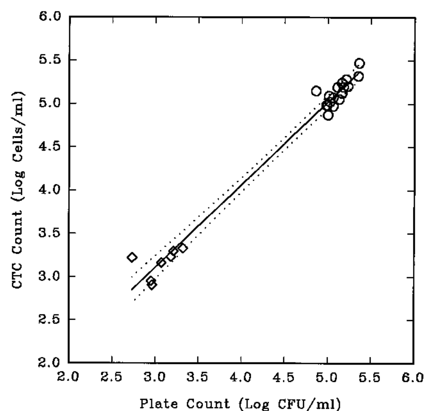

FIG. 6. Comparison of number of cells reducing CTC and those forming colonies on agar media. ○, *K. pneumoniae* Kp1 diluted in PBS before CTC incubation and plating on tryptone-lactose-yeast extract agar; ◇, well water samples filtered directly for CTC incubation and diluted in sterile water for plating on R2A agar. The solid line represents the regression (intercept = 0.24; slope = 0.96; r = 0.92), and dotted lines indicate the 99% confidence interval.

and the observation of no microcolonies, shows that few of the cells divided during the incubation.

DISCUSSION

The procedure described here facilitates incubation of bacterial cells on membrane filters with media containing CTC to determine the number of cells involved in respiration. With laboratory cultures, we found that media containing low nutrient concentrations, and even some lacking added nutrients, were more effective than enriched media for the detection of CTC reduction. These results conflict with those of Rodriguez et al. (25) and Smith et al. (29), who found that nutrient-containing media were most effective. These discrepancies could have occurred because laboratory-cultivated bacteria were used primarily in this study, whereas bacteria in groundwater, temperate seawater, and wastewater (25) or a polar marine environment (29) were used in the other studies. However, Rodriguez et al. (25) used 50% R2A medium containing CTC for the environmental samples and incubated for 4 h at 28°C on a shaker, so it is possible that the increased enumeration of "respiring plus substrate" cells compared with respiring cells in unamended samples could have partially resulted from cell multiplication during incubation. It is also possible that the bacterial strains and species present in those environmental samples (25) responded differently than the species used in this study. In addition, incubation on a medium containing CTC following filtration could have performance characteristics different from those of CTC incubation followed by filtration, as has been done in other investigations (25, 29).

In future applications of this method, certain factors which affect the performance characteristics of this assay need to be optimized. The diluent used prior to filtration, if this step is necessary, could be chosen to eliminate possible effects such as those observed with *E. coli* K-12. A medium with a final pH of around 6.0 to 6.5 may improve CTC reduction results. It is important to determine the pH after dissolving the CTC because the latter tends to lower the pH of weakly buffered media. When nutrient amendment is considered necessary, phosphate-containing components should be minimized and other medium constituents should be checked to ensure that they do not adversely affect CTC-formazan formation. It is desirable that the CTC incubation time and temperature be selected to maximize CTC reduction while total cell numbers are maintained close to those observed immediately after filtration. Similarly, it is preferable to avoid microcolony formation as cells in microcolonies or enlarged, possibly dividing cells may fail to exhibit characteristic CTC reduction. For counterstaining after CTC incubation, the concentration of DAPI may need to be varied to optimize total cell counts. When the CTC methods for any previously untested sample type are evaluated, it is advisable to perform initial total cell counts for comparison with counts after incubation so that significant cell multiplication during incubation can be detected. The data in this report suggest that some increases in cell number may be the result of enhanced DAPI staining rather than cell growth. However, microcolony formation during incubation may indicate that multiplication has occurred.

In the experiments described and discussed here, we have used low concentrations of CTC (≤2 to 3.5 mM) to minimize the cost of each enumeration. Rodriguez et al. (25) suggested that concentrations of 2 to 5 mM may yield the best results and recommended 5 mM for environmental samples. Other concentrations in this range could therefore be evaluated for the particular samples being examined to determine the lowest effective concentration. A major advantage of the MF-CTC technique is that the amount of CTC used for each test is the same because the sample is filtered and the filter is incubated on a pad saturated with the CTC medium. With environmental samples containing few bacteria, it may be necessary to examine volumes greater than 25 ml, and this would significantly increase the amount of CTC used per test if the CTC was added to the sample for incubation prior to filtration. Filtering before CTC incubation also allows the observation of microcolonies which may form during incubation, particularly with enriched media or with incubation at higher temperatures. When microcolonies which were not present before incubation are observed, steps can be taken to modify the incubation conditions to prevent this.

The CTC method is becoming increasingly popular as a more rapid and reliable alternative to plate counting. For example, the technique has been used to quantify planktonic and sessile respiring bacteria in drinking water (27), soil (33), and enteric bacteria in a polar marine environment (29). The procedure has also been used to study the viability of *Pseudomonas fluorescens* (9) and coccoid *Campylobacter jejuni* (2) and in flow cytometry studies of *Micrococcus luteus* (11). The technique has recently been adapted for the in situ examination of bacterial biofilms on solid surfaces (34, 35). Responses of *E. coli* to starvation in seawater have been monitored by using CTC with flow cytometry (14).

When appropriate combinations of diluent, medium, CTC concentration, incubation time and temperature, DAPI concentration, and epifluorescence microscopy filters are used, reliable results from the CTC reduction method for detecting total and viable bacteria in water and other liquid samples can be obtained. The alternative CTC reduction method proposed here produced results which corresponded closely with enumerations by plate counts for the three enteric bacterial species and the well water samples included in the study.

In conclusion, an alternative method for enumerating respiring bacteria in liquid samples has been developed, and some factors affecting its performance have been investigated. A sample was filtered through a 0.2-μm-pore-size black polycarbonate membrane, and the filter membrane was placed on an absorbent pad saturated with PS containing 2 mM CTC. Following incubation, cells collected on the membrane filter were counterstained with DAPI, mounted, and examined by epifluorescence microscopy, using appropriate filters to visualize

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the total number of cells stained with DAPI and the proportion containing fluorescent CTC-formazan crystals. The MF-CTC method described here has been combined successfully with a fluorescent-antibody procedure (20). This permits the rapid detection and enumeration of specific respiring bacteria.

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REFERENCES

1. **Back, J. P., and R. G. Kroll.** 1991. The differential fluorescence of bacteria stained with acridine orange and the effects of heat. J. Appl. Bacteriol. **71:**51–58.
2. **Boucher, S. N., E. R. Slater, A. H. L. Chamberlain, and M. R. Adams.** 1994. Production and viability of coccoid forms of *Campylobacter jejuni*. J. Appl. Bacteriol. **77:**303–307.
3. **Daley, R. J.** 1979. Direct epifluorescence enumeration of native aquatic bacteria: uses, limitations, and comparative accuracy, p. 29–45. *In* J. W. Costerton and R. R. Colwell (ed.), Native aquatic bacteria: enumeration, activity, and ecology. ASTM STP 605. American Society for Testing and Materials, Philadelphia.
4. **Daley, R. J., and J. E. Hobbie.** 1975. Direct counts of aquatic bacteria by a modified epifluorescent technique. Limnol. Oceanogr. **20:**875–882.
5. **Desmonts, C., J. Minet, R. Colwell, and M. Cormier.** 1992. An improved filter method for direct viable count of *Salmonella* in seawater. J. Microbiol. Methods **16:**195–201.
6. **Elliot, E. L., and R. R. Colwell.** 1985. Indicator organisms for estuarine and marine waters. FEMS Microbiol. Rev. **32:**61–79.
7. **Francisco, D. E., R. A. Mah, and A. C. Rabin.** 1973. Acridine orange epifluorescent technique for counting bacteria in natural waters. Trans. Am. Microsc. Soc. **92:**416–421.
8. **Herbert, R. A.** 1990. Methods for enumerating microorganisms and determining biomass in natural environments. Methods Microbiol. **22:**1–39.
9. **Jørgensen, F., O. Nybroe, and S. Knøchel.** 1994. Effects of starvation and osmotic stress on viability and heat resistance of *Pseudomonas fluorescens* AH9. J. Appl. Bacteriol. **77:**340–347.
10. **Kaprelyants, A. S., J. C. Gottschal, and D. B. Kell.** 1993. Dormancy in non-sporulating bacteria. FEMS Microbiol. Rev. **104:**271–286.
11. **Kaprelyants, A. S., and D. B. Kell.** 1993. Dormancy in stationary-phase cultures of *Micrococcus luteus*: flow cytometric analysis of starvation and resuscitation. Appl. Environ. Microbiol. **59:**3187–3196.
12. **Kepner, R. L., Jr., and J. R. Pratt.** 1994. Use of fluorochromes for direct enumeration of total bacteria in environmental samples: past and present. Microbiol. Rev. **58:**603–615.
13. **Kogure, K., U. Simidu, and N. Taga.** 1979. A tentative direct microscopic method for counting living marine bacteria. Can. J. Microbiol. **25:**415–420.
14. **Lopez-Amorós, R., D. J. Mason, and D. Lloyd.** 1995. Use of two oxonols and a fluorescent tetrazolium dye to monitor starvation of *Escherichia coli* in seawater by flow cytometry. J. Microbiol. Methods **22:**165–176.
15. **McFeters, G. A.** 1990. Enumeration, occurrence, and significance of injured indicator bacteria in drinking water, p. 478–492. *In* G. A. McFeters (ed.), Drinking water microbiology: progress and recent developments. Springer-Verlag New York Inc., New York.
16. **McFeters, G. A., A. Singh, S. Byun, P. R. Callis, and S. Williams.** 1991. Acridine orange staining reaction as an index of physiological activity in *Escherichia coli*. J. Microbiol. Methods **13:**87–97.
17. **Peele, E. R., and R. R. Colwell.** 1981. Applications of a direct microscopic method for enumeration of substrate-responsive marine bacteria. Can. J. Microbiol. **27:**1071–1075.
18. **Pettipher, G. L.** 1986. Review: the direct epifluorescent filter technique. J. Food Technol. **21:**535–546.
19. **Pugsley, A. P., and L. M. Evison.** 1974. A membrane filtration staining technique for detection of viable bacteria in water. Water Treat. Exam. **23:**205–214.
20. **Pyle, B. H., S. C. Broadaway, and G. A. McFeters.** 1995. A rapid, direct method for enumerating respiring enterohemorrhagic *Escherichia coli* O157:H7 in water. Appl. Environ. Microbiol. **61:**2614–2619.
21. **Pyle, B. H., and G. A. McFeters.** 1989. Iodine sensitivity of bacteria isolated from water systems treated with iodine. Can. J. Microbiol. **35:**520–523.
22. **Pyle, B. H., S. K. Watters, and G. A. McFeters.** 1994. Physiological aspects of disinfection resistance in *Pseudomonas cepacia*. J. Appl. Bacteriol. **76:**142–148.
23. **Quinn, J. P.** 1984. The modification and evaluation of some cytochemical techniques for the enumeration of metabolically active heterotrophic bacteria in the aquatic environment. J. Appl. Bacteriol. **57:**51–57.
24. **Reasoner, D., and E. E. Geldreich.** 1985. A new medium for the enumeration and subculture of bacteria from potable water. Appl. Environ. Microbiol. **49:**1–7.
25. **Rodriguez, G. G., D. Phipps, K. Ishiguro, and H. F. Ridgway.** 1992. Use of a fluorescent redox probe for direct visualization of actively respiring bacteria. Appl. Environ. Microbiol. **58:**1801–1808.
26. **Roszak, D. B., and R. R. Colwell.** 1987. Survival strategies of bacteria in the natural environment. Microbiol. Rev. **51:**356–379.
27. **Schaule, G., H.-C. Flemming, and H. F. Ridgway.** 1993. Use of 5-cyano-2,3-ditolyl tetrazolium chloride for quantifying planktonic and sessile respiring bacteria in drinking water. Appl. Environ. Microbiol. **59:**3850–3857.
28. **Singh, A., B. H. Pyle, and G. A. McFeters.** 1989. Rapid enumeration of viable bacteria by image analysis. J. Microbiol. Methods **10:**91–101.
29. **Smith, J. J., J. P. Howington, and G. A. McFeters.** 1994. Survival, physiological response, and recovery of enteric bacteria exposed to a polar marine environment. Appl. Environ. Microbiol. **60:**2977–2984.
30. **Stubberfield, L. C. F., and P. J. A. Shaw.** 1990. A comparison of tetrazolium reduction and FDA hydrolysis with other measures of microbial activity. J. Microbiol. Methods **12:**151–162.
31. **Tabor, P. S., and R. A. Neihof.** 1982. Improved method for determination of respiring individual microorganisms in natural waters. Appl. Environ. Microbiol. **43:**1249–1255.
32. **Van Es, F. B., and L.-A. Meyer-Reil.** 1982. Biomass and metabolic activity of heterotrophic marine bacteria. Adv. Microb. Ecol. **6:**111–170.
33. **Winding, A., S. J. Binnerup, and J. Sørensen.** 1994. Viability of indigenous soil bacteria assayed by respiratory activity and growth. Appl. Environ. Microbiol. **60:**2869–2875.
34. **Yu, F. P., and G. A. McFeters.** 1994. Rapid in situ assessment of physiological activities in bacterial biofilms using fluorescent probes. J. Microbiol. Methods **20:**1–10.
35. **Yu, F. P., and G. A. McFeters.** 1994. Physiological responses of bacteria in biofilms to disinfection. Appl. Environ. Microbiol. **60:**2462–2466.
36. **Zimmerman, R., R. Iturriaga, and J. Becker-Birck.** 1978. Simultaneous determination of the total number of aquatic bacteria and the number thereof involved in respiration. Appl. Environ. Microbiol. **36:**926–935.

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