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Use of 5-Cyano-2,3-Ditolyl Tetrazolium Chloride for Quantifying Planktonic and Sessile Respiring Bacteria in Drinking Water

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Direct microscopic quantification of respiring (i.e., viable) bacteria was performed for drinking water samples and biofilms grown on different opaque substrata. Water samples or biofilms developed in flowing drinking water were incubated with the vital redox dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and R2A medium. One hour of incubation with 0.5 mM CTC was sufficient to obtain intracellular reduction of CTC to the insoluble fluorescent formazan (CTF) product, which was indicative of cellular respiratory (i.e., electron transport) activity. This result was obtained with both planktonic and biofilm-associated cells. Planktonic bacteria were captured on 0.2-μm-pore-size polycarbonate membrane filters and examined by epifluorescence microscopy. Respiring cells containing CTF deposits were readily detected and quantified as red-fluorescing objects on a dark background. The number of CTC-reducing bacteria was consistently greater than the number of aerobic CFU determined on R2A medium. Approximately 1 to 10% of the total planktonic population (determined by counterstaining with 4,6-diamidino-2-phenylindole) were respirometrically active. The proportion of respiring bacteria in biofilms composed of drinking water microflora was greater, ranging from about 5 to 35%, depending on the substratum. Respiring cells were distributed more or less evenly in biofilms, as demonstrated by counterstaining with 4,6-diamidino-2-phenylindote. The amount of CTF deposited in single cells of *Pseudomonas putida* that formed monospecies biofilms was quantified by digital image analysis and used to indicate cumulative respiratory activity. These data indicated significant cell-to-cell variation in respiratory activity and reduced electron transport following a brief period of nutrient starvation. The results of this study demonstrate that CTC reduction is a rapid and sensitive method for quantification and localization of viable bacteria in drinking water and other environmental samples. The method is particularly well suited for exploration of cellular activity in surface biofilms.

The microbiological quality of drinking water is an issue of global concern. Among the possible sources of microbial contamination are surface-associated biofilms, which are common in drinking water systems (4, 8, 12, 13, 24, 32). The methods required for evaluation of the bacteriological quality of potable water are often based on cultivation of planktonic bacteria in a sample (e.g., heterotrophic plate counts [HPCs]; 4). However, subculture techniques often require lengthy incubation times of several days or more; thus, there is a need for more rapid and convenient monitoring methods for quantitative assessment of the viability of microorganisms. As virtually all aerobic heterotrophic bacteria possess electron transport systems, artificial electron acceptors, such as the tetrazolium salts (1, 2, 7), have been used to detect electron transport (i.e., respiratory) activity as an indicator of cellular viability. Triphenyltetrazolium chloride (27) and 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (7, 10, 20) have been used for this purpose. The intracellular occurrence of colored crystals of the water-insoluble formazan product provides an indication of in vivo respiratory activity. However, such crystals are often difficult to detect by bright-field microscopy and thus have limited the general use and acceptance of this method.

Fluorescent dyes are much more readily detected than nonfluorescent dyes. Snyder and Greenberg (30) developed a system to detect viable microorganisms based on the enzymatic hydrolysis of diacetylfluorescein to the fluorescent product. A serious disadvantage of this method, however, is that hydrolysis of the compound is not directly related to carbon processing or cellular energy metabolism (e.g., electron transport or oxidative phosphorylation).

Rodriguez et al. (26) recently reported use of the redox-sensitive dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) for microscopic detection of respiring bacteria in aquatic environments. An aqueous solution of CTC is nearly colorless and nonfluorescent, while the corresponding formazan product (reduced CTC [CTF]) fluoresces in the red range at approximately 620 nm when excited at 420 nm. Soluble CTC is readily reduced to the water-insoluble fluorescent CTF product via the microbial electron transport system and indicates mainly respiratory activity. CTF is deposited intracellularly like other formazans in a time-dependent manner and provides an indication of cumulative respiratory activity. Rodriguez et al. have demonstrated the suitability of CTC reduction for direct microscopic detection of actively respiring planktonic and biofilm bacteria in axenic laboratory cultures and native environmental samples containing mixed populations.

Data presented in this report corroborate and extend the work of Rodriguez et al. (26) and demonstrate the usefulness of CTC for rapid detection and quantification of actively respiring (i.e., viable) bacteria in domestic drinking water samples containing mixed populations. New findings are

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presented on the optimization of the CTC staining procedure and incubation times. Comparisons of the CTC procedure with the standard HPC method are explored. Additional data are provided concerning the detection of respiring bacteria in attached monospecies biofilms and the influence of nutrient starvation on CTC reduction.

MATERIALS AND METHODS

Chemicals. The redox dye CTC was purchased from Polysciences, Inc. (Eppelheim, Germany), and the DNA-binding fluorochrome 4,6-diamino-2-phenylindole (DAPI) was from Sigma Chemical Co. (St. Louis, Mo.). The fluorescent dye acriflavine (AF), a mixture of 3,6-diamino-10-methylacridinium chloride and 3,6-diaminoacridine, was obtained from Aldrich-Chemie (Steinheim, Germany).

Drinking water system and flow cell for adhesion experiments. A 100-liter stainless steel (SS) tank was installed on a tap of the Stuttgart, Germany, municipal drinking water distribution system. The purpose of the tank was to break line pressure and eliminate fluctuations in flow normally associated with this city water distribution system. The tank effluent fed a polymethylene methacrylate (PMMA) flow cell (217 by 100 by 65 mm) designed to permit study of the adhesion and biofouling properties of aerobic, heterotrophic microbial populations native to the drinking water system. The flow rate to the cell was approximately 2.0 liters/h. Thirty biofilm coupons were placed in the flow cell, and after each experiment the cell was mechanically cleaned with a brush and thoroughly rinsed to remove any residual biofilm. The chemical and microbiological characteristics of the Stuttgart drinking water were as follows: temperature (in the flow cell), approximately 12°C; free chlorine, undetectable; pH, 7.9 ± 0.05; dissolved organic carbon, 1.05 ± 0.05 mg/liter; total hardness, 91.8 ± 2.4 mg of CaO per liter; total bacterial cell number (TCN) determined by epifluorescence microscopy, 10^5 to 10^6/ml; CFU on standard 1 medium (4) after 48 h at 20°C, 0 to 10 CFU/ml. The above analyses were performed in accordance with standard procedures set forth by the German government for analysis of drinking water (4).

Water samples. Water samples were collected in sterile glass bottles from the flow cell and immediately analyzed chemically and microbiologically.

Test surfaces (coupons). Test coupons measured 2.0 cm long by 1.0 cm wide. All coupons were new and thoroughly cleaned before exposure to drinking water in the flow cell. The coupons consisted of the following materials: SS (69% Fe, 18% Cr, 11% Mo, ≤0.07% C), copper pipe (Cu; 2.54 cm in diameter), high-density polyethylene pipe (DIN 8075; approximately 10 cm in diameter, 9-mm wall thickness), and PMMA (3 mm thick). SS, Cu, high-density polyethylene, and PMMA materials are permitted for use in drinking water systems in Germany.

TCNs. TCNs in water samples were determined by using the autofluorescent dye AF modified as described by Bergström et al. (5). Water samples were filtered through a Nucleopore black polycarbonate membrane (pore diameter, 0.2 μm). Coupons with biofilm were covered directly with a freshly filtered (pore diameter, 0.2 μm) 1.0 mM AF solution. After incubation for 10 min, the AF solution was vacuum filtered through the Nucleopore filter. Coupons were rinsed lightly with filtered water and air dried. Counts were determined with a Zeiss Universal microscope fitted with an HBO 50 W mercury short-arc lamp. For AF-stained bacteria, the following Zeiss filter set was employed: a BP450-490 exciter, an FT510 dichroic beam splitter, and an LP520 barrier filter. An Epiplan 100×/1.25 NA oil immersion objective lens was used. Bacteria in 20 visual fields (each 100 by 100 μm) were enumerated, and then the mean and standard deviation (SD) were calculated. Low SD values were associated with more homogeneous cell distributions, whereas higher SD values indicated microcolonies or otherwise patchy distribution.

HPCs. Aerobic HPCs were obtained by spread plating appropriate dilutions of water samples on R2A medium (23), which was also used by Rodriguez et al. (26). For comparison, standard 1 broth (Merck) was used in accordance with the German government drinking water regulation (4). Plates were incubated at 20°C for 48 h before enumeration of colonies.

Preparation of biofilm-coated slides. Biofilms of *Pseudomonas putida* 54g were grown on plastic (polyester) microscope slides partially submerged in a stirred-batch reactor (SBR). *P. putida* 54g was isolated from groundwater extracted from a gasoline-contaminated aquifer in Seal Beach, Calif. (25). The SBR consisted of a 1-liter glass beaker in which the microscope slides were vertically suspended around the periphery of the inside wall. The upper, dry end of each slide was fastened to a metal clip connected to a circular stainless steel plate, which served as the cover for the SBR. Approximately 500 ml of growth medium (10% R2A broth) was placed in the beaker such that the lower three-fourths of each slide was submerged. The entire reactor assembly and contents, except the slides, were sterilized by autoclaving prior to inoculation. Slides were sterilized separately by UV irradiation at 258 nm. Following installation of the sterile slides and inoculation of the growth medium with strain 54g, the SBR contents were continuously stirred (200 rpm) with a magnetic bar. After 96 h at approximately 23°C, the biofilm-coated slides were removed from the SBR. Nonadherent or loosely attached bacteria were removed by three sequential washes with NPM buffer (10.0 mM $NaPO_4$, 1.0 mM $MgCl_2$, pH 7.0). Each washing step consisted of 5 min of submersion in 500 ml of continuously stirred (200 rpm) NPM buffer in a 1-liter beaker. The biofilm samples were subjected to digital image analysis as described below.

Staining of water samples with CTC. Water samples were amended with CTC and nutrient and then stained with DAPI in accordance with a modification of the procedure described by Rodriguez et al. (26). The water sample (5.0 ml) was added to R2A broth (4.0 ml) and 1 ml of CTC stock solution (5.0 mM). A final CTC concentration of 0.5 mM was sufficient to allow detection of respiring bacteria in all of the water samples tested and reduced the overall cost of the assay. The mixture was continuously stirred for 1 h in the dark at room temperature (about 23°C). After the sample was filtered through a Nuclepore black polycarbonate membrane (pore diameter, 0.2 μm) and air dried, respirometrically active cells were enumerated microscopically.

Water samples with high cell densities were diluted before R2A medium and CTC solution were added. For water samples with low cell densities (≤10^5 cells per ml) or those with low numbers of respiring bacteria, it was necessary to concentrate the cells as follows. A suitable volume of water was filtered through a Nuclepore black polycarbonate membrane, which was then transferred to a mixture of R2A medium (diluted 1:2) and CTC. After incubation, the filter was rinsed with particle-free water and returned to the filter device and the combined washings and sample were filtered through the membrane. An alternative procedure, developed by Pyle (22), is to incubate the filter with nutrient after the

accumulation step. This is performed in a sterile petri dish, and the filter paper is saturated with R2A medium and CTC. The filtration method is also suitable for water samples containing soluble reducing substances that must be removed to prevent abiotic CTC reduction.

The CTC-reducing bacteria in 20 visual fields were counted in a microscope. The following optical filter combination was used for CTF detection and enumeration: a Zeiss BP450-490 exciter and FT510 and LP590 barrier filters. For AF, the LP590 barrier filter was replaced with an LP520 filter.

Staining of biofilms on coupons with CTC and DAPI. Coupons with attached biofilms were incubated in a mixture of R2A medium (diluted 1:2 with ultrapure water) and CTC (final concentration, 5.0 mM) for 1 h, unless otherwise indicated. The coupons were then rinsed and air dried. To compare the positions of CTF-containing cells in biofilms, counterstaining with DAPI (2.0 μg/ml) for 20 min at room temperature was performed. The dye solution was freshly prepared weekly from a stock solution of DAPI (1.0 mg/ml) and stored in the dark at 4°C.

To estimate the biofilm TCN, one part of the coupon was stained with AF. Scotch tape was used to prevent diffusion of AF to other parts of the slide. Comparative investigations with DAPI and AF indicated higher total cell numbers with AF. Cells stained with AF tended to be brighter with higher contrast, depending on the nature of the substratum. Thus, to obtain optimal results each coupon was divided into two parts with Scotch tape, one for CTC-DAPI costaining and the other to estimate the TCN with AF. The Zeiss optical filter set used to view DAPI-stained cells consisted of a BP365 exciter, an FT395 dichroic beam splitter, and an LP420 barrier filter.

Staining of biofilms on slides with CTC and DAPI. Unstarved control biofilms were removed from the SBR and, after washing in NPM buffer as described above, transferred to 4.0 mM CTC prepared in HCMM2 mineral salts medium (25), incubated for 1 h, and counterstained with 1.0 μg of DAPI per ml for 20 min at 23°C. Slides were subsequently rinsed once with NPM buffer and air dried. Starved biofilms were prepared by incubation in HCMM2 medium lacking organic nutrients for 1 h. They were then transferred to fresh mineral salts solution containing CTC and incubated for an additional 1 h before counterstaining with DAPI.

Microscopic examination of biofilm slides. An Olympus Vanox AHBT3 microscope equipped with a 200 W mercury burner was used with a 100× oil immersion fluorescence objective. The optical filter combination for optimal viewing of CTC-treated preparations consisted of a blue 400 to 480-nm excitation filter in combination with a 590-nm barrier filter. The CTC- and DAPI-stained bacteria in the same preparation could be viewed simultaneously with a 365-nm excitation filter, a Y455 emission filter, and a 400-nm barrier filter. However, when cells were viewed under these conditions CTF fluorescence was less apparent.

Digital image analysis of *P. putida* biofilms on slides. The amount of CTF fluorescence observed in each cell indicated cumulative respiratory activity during the 1-h incubation period. The CTF fluorescence intensity of individual cells in starved and unstarved (control) biofilm preparations was determined by digital image analysis with the Olympus CUE-2 photometry program. A microscope-mounted, silicon-intensified series 5000 target television camera (COHU, Inc., San Diego, Calif.) was used to detect fluorescent bacteria. Television images were digitized by the CUE-2 system and stored in a computer memory. The fluorescence

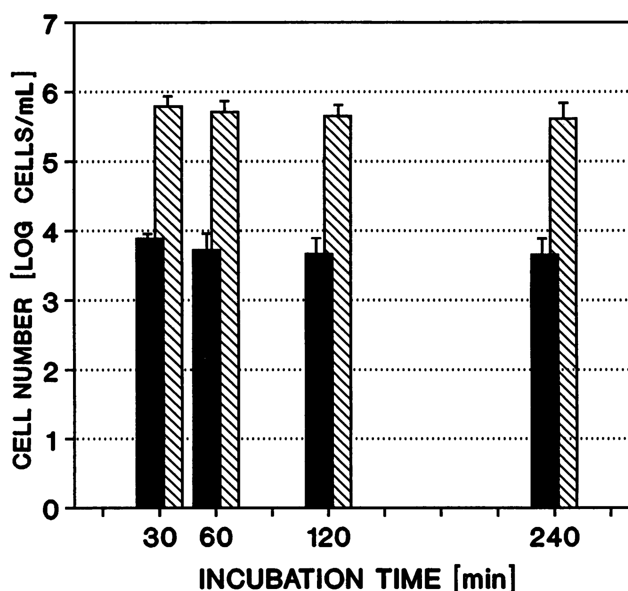

FIG. 1. Influence of incubation time on the number of bacteria in municipal drinking water that reduce CTC to CTF. Dark bars, numbers of CTC-reducing bacteria; light bars, TCNs; $n = 5$. Error bars denote ± 1 SD.

intensity of 100 randomly viewed cells in each preparation was quantified relative to that of an arbitrary (plastic) fluorescence standard. Fluorescence data for the starved and unstarved biofilm samples were divided into 10 equal fluorescence emission ranges based on the highest and lowest fluorescence values observed.

RESULTS

Influence of incubation time on CTC reduction by planktonic and sessile bacteria. The CTC assay was performed with planktonic and sessile bacteria with incubation times ranging from 0.5 to 4 h. For planktonic cells, 200 ml of drinking water was added to CTC and R2A medium and magnetically stirred. Duplicate 20-ml aliquots were collected and analyzed after each relevant incubation time. As shown in Fig. 1, there was no significant difference between any of the incubation times (0.5 to 4 h) with respect to either the TCN as determined by DAPI staining or the number of respiring (i.e., CTC-reducing) bacteria. Approximately 1.0% of the planktonic bacteria in this water sample reduced CTC and were respirometrically active. To ensure that the diffusion and uptake of CTC were not time limited, a 1-h incubation time was chosen for subsequent analyses. Similar results were obtained with different pure strains (15, 25a).

Further investigations were performed with 5-day-old biofilms developed on PMMA (data not shown) and SS coupons in the flowthrough chamber with drinking water. Five coupons were removed for each CTC assay and treated for 1 or 4 h with a mixture of CTC and one-half-strength R2A medium. In each assay, the TCN and the number of CTF-containing cells were determined microscopically. The mean TCN for the SS coupons after 1 h of incubation was $(9.3 \pm 0.4) \times 10^5$ cells per cm^2. After 4 h, the mean TCN was $(9.65 \pm 0.35) \times 10^5$ cells per cm^2. The numbers of CTC-reducing cells were $(2.34 \pm 0.8) \times 10^5$/cm^2, i.e., 25% of the TCN, after

1 h of incubation and $(2.54 \pm 0.7) \times 10^5/cm^2$, i.e., 26% of the TCN, after 4 h of incubation.

Enumeration of actively respiring planktonic bacteria in drinking water. The HPC method is the conventional approach for quantification of viable heterotrophic bacteria in drinking water and aquatic environmental samples. A disadvantage of the HPC method is, however, the long incubation time required for colony growth. Results are usually not obtained in less than 48 h, and often more than a week is needed before all colonies appear. The CTC method, as described in this and a previous report (26), may provide a much more sensitive and rapid approach for enumerating cell viability in drinking water. Thus, it was of interest to compare the CTC procedure with the HPC method.

Weekly cell enumeration data obtained in drinking water experiments carried out during a 6-month period (December 1991 to May 1992; $n = 30$) varied within about 1 order of magnitude. The TCNs for drinking water samples obtained from the flowthrough cell ranged from about 10^5 to 10^6/ml. About 10^4 to 10^5 of these cells were identified as respirometrically active (containing CTF deposits) after nutrient addition (R2A medium diluted 1:2). The corresponding HPCs (2-day incubation) ranged from about 10 to 10^3 CFU/ml. Thus, the CTC method yielded significantly greater estimates of cell viability than did standard R2A plate counts.

Biofilms on different materials. Bacterial growth in the suspended phase of drinking water distribution systems is thought to be negligible (8, 31). Most growth presumably occurs in surface biofilms, which represent a persistent contamination source for the water system. Thus, a comprehensive monitoring program for evaluation of drinking water quality should include sampling of surfaces (e.g., removable coupons) for detection and quantification of biofilm growth. A primary objective of this study was to develop a method for direct, rapid visualization and quantification of attached viable cells with CTC. Different materials were exposed to drinking water for several days and stained with CTC (see Materials and Methods). CTC-reducing bacteria could be readily detected and counted on smooth, low-fluorescence materials, such as polyethylene, SS, Cu, PMMA, and polycarbonate. However, the percentage of actively respiring cells in drinking water biofilms varied with the support material. The highest numbers were found on Cu (35% of the TCN were CTC reducing), PMMA (33%), and SS (19%), while on polyethylene only about 5% of the TCN were respiring. In the water phase, approximately 3% of all planktonic bacteria were CTC reducing. Quantification of cells containing CTF deposits on rough material (e.g., cement) or autofluorescent material (e.g., reverse osmosis membranes) was more difficult. Figure 2 compares CTC and TCN data for 7-day biofilms formed on different materials.

The colonization pattern on the various surfaces was heterogeneous (patchy) because of development of microcolonies. Red-fluorescing (CTF-positive) bacteria within one microcolony grown for 5 days on PMMA exposed to drinking water are depicted in Fig. 3a. The same microscopic field of view photographed with the DAPI optical filter combination is depicted in Fig. 3b.

Ratio of TCN to respiring cells in a developing biofilm. As demonstrated above, a greater proportion of biofilm bacteria than planktonic bacteria were actively respiring, regardless of the nature of the support material. To determine whether this trend was a function of biofilm age, coupons (SS and PMMA) were exposed to drinking water and removed after 4, 8, 16, 24, 48, 120, 144, and 168 h. The TCN and the number of CTC-reducing cells were determined at each time.

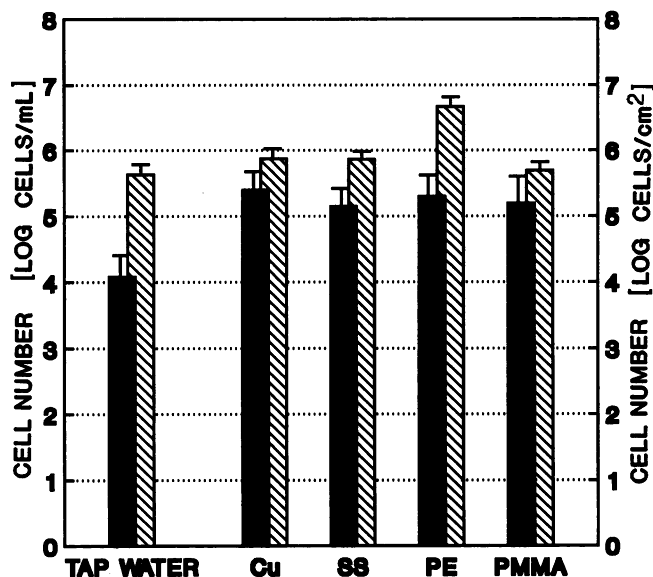

FIG. 2. Enumeration of bacteria (TCN and CTC reducing) in tap water and biofilms aged 1 week on Cu, SS, polyethylene, and PMMA. Dark bars, numbers of CTC-reducing bacteria; light bars, TCNs; $n = 5$. Error bars denote ± 1 SD.

The results for a biofilm on SS are shown in Fig. 4. At each sampling time, the number of CTC-reducing bacteria was about 10-fold less than the TCN. The number of active cells in the corresponding planktonic population was about 15-fold less than the TCN (Fig. 4).

Influence of nutrient starvation on the electron transport activity of *P. putida* in biofilms. Because CTC competes indirectly with molecular oxygen for reduction by the electron transport system, the amount of CTF deposited within a cell provides an indication of the cumulative respiratory (electron transport) history of that cell. Computer-based electronic (digital) imaging techniques were employed to quantify the amounts of CTF deposited in single cells of *P. putida* 54g in biofilms (see Materials and Methods). The relative amounts of CTF accumulated in unstarved (control) and starved, biofilm-associated cells of *P. putida* were compared (see Materials and Methods). The biofilms were starved for either 5 or 60 min prior to addition of CTC. In each preparation, 100 individual cells were analyzed digitally for fluorescence emmission relative to an arbitrary (plastic) fluorescence standard. As shown in Fig. 5, there was a dramatic decrease in CTC reduction in response to even a brief (5-min) period of nutrient starvation. The observed cellular activities in the unstarved control preparation fit an approximately normal distribution, indicating that individual bacteria that made up the biofilm respired at significantly different rates. In addition to a decline in the amount of CTF accumulated per cell, nutrient starvation also resulted in a very noticeable reduction in the proportion of respiring cells that could be visually detected by CTF fluorescence (Fig. 6).

DISCUSSION

The results of this study show that CTC is readily reduced by planktonic and sessile environmental microbial populations, such as those associated with municipal drinking water supplies. On the basis of comparisons presented in this report between the CTC method and the standard HPC

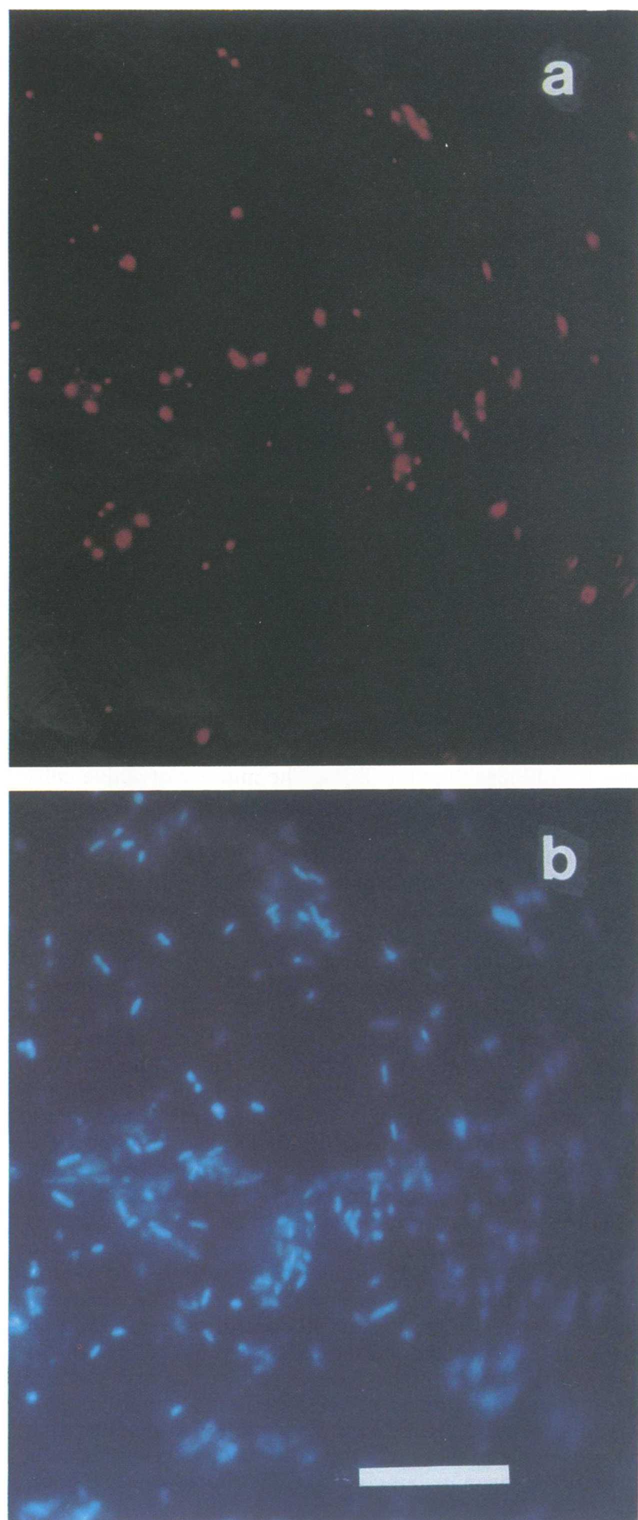

FIG. 3. (a) Epifluorescence photomicrograph of CTC-reducing (respiring) bacteria in a biofilm on a PMMA surface. The PMMA surface was exposed to flowing drinking water for 7 days. DAPI-counterstained cells in the same area are shown in panel b. Bar, 10 μm.

procedure, it appears that the former method could be used for rapid detection and enumeration of viable heterotrophic bacteria in drinking water supplies. If nutrients (e.g., R2A) are added with CTC, respirometrically active cells which readily utilize the nutrients are preferentially detected. Experiments were performed to determine whether the incubation time could be reduced from 4 h, as originally proposed by Rodriguez et al. (26). Reduction of the incubation time was thought to be prudent to reduce the likelihood of cell division and to decrease the overall time of the assay. Conceivably, dividing cells could significantly increase the number of respirometrically active bacteria observed, especially during lengthy incubation periods. An incubation time of 1 h with 0.5 mM CTC appears to be sufficient for quantification of active cells, whereas longer incubation times (4 h) do not yield significantly higher numbers. To prevent abiotic reduction of the CTC by dissolved reducing substances, cells can be washed prior to incubation with CTC and nutrients.

The numbers of CTC-reducing bacteria in drinking water samples was typically higher than HPCs. This discrepancy may reflect differences in the biological functions measured by the methods. In the HPC method, cell viability is defined as the ability of a bacterium to grow and form a visible colony. On the other hand, CTC reduction reflects the presence of a functional electron transport (i.e., respiratory) system or certain active dehydrogenases. Evidently, many respiring bacteria detected by the CTC reduction technique fail to produce visible colonies on agar media. The reason for this is unclear, but it may be that many respiring bacteria grow too slowly on R2A medium to form a colony in the incubation time allotted (48 h). Alternatively, it is possible that the native water samples contained essential micronutrients (absent in R2A plates) required for sustained bacterial growth and reproduction. Another possibility is that R2A medium contains inhibitory or stress-inducing substances that interfere with cell division processes but not respiration.

Presumably, dead bacteria and those with low levels of enzymatic activity (e.g., dormant cells or spores) are not detected by the CTC reduction method. However, it is not clear whether viable bacteria that are physiologically stressed (i.e., injured or viable but nonculturable) continue to reduce CTC. Thus, dead and dormant bacteria contribute to the difference between the number of CTC-reducing cells observed and the TCN as determined by DAPI (or AF) staining. If the density of active bacteria in a water sample is very low, enrichment by filtration can be applied, although high turbidities may preclude passage of large volumes of water through a 0.2-μm-pore-size filter.

It was shown that CTF accumulates in biofilm cells and renders them easily detectable in situ. In contrast to the nonfluorescent reduced form of triphenyltetrazolium chloride or 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride, red-fluorescent CTF deposits are readily perceived against the dark background of most surfaces. With computerized image analysis, it is possible to scan a colonized surface and rapidly quantify the respiratory activity of CTF-stained cells. As reported here, it is possible to focus on a single cell and quantify the amount of CTF per cell. This capability permits quantitative assessment of the cumulative respiratory history of an individual bacterium. Thus, CTC offers a new tool for nondestructive analysis of the architecture and distribution of physiological activity within a biofilm. Together with other modern methods for exploring cellular function, such as reporter gene analysis (3, 21), immunolabeling (33), and indoleacetic acid production

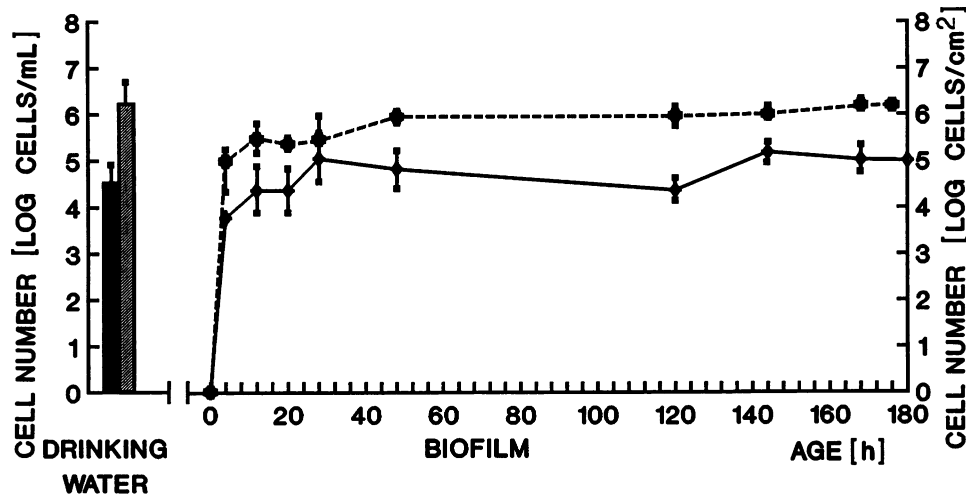

FIG. 4. Development of a biofilm on SS during 7 days of exposure to drinking water (■ and ◆, CTC-reducing cells; ▨ and ●, TCN). Error bars denote ± 1 SD.

(9), the CTC method could provide new information about how actively metabolizing bacteria behave and interact in biofilms. These cellular labeling methods offer particular advantages for the detection and differentiation of microorganisms with a scanning laser confocal microscope (17).

The electron transport activity of *P. putida* 54g bacteria that make up surface biofilms was significantly reduced in response to brief nutrient starvation. Interestingly, the data revealed considerable variation in the electron transport activities of individual bacteria that made up both the starved and unstarved biofilms. The reasons for such metabolic heterogeneity among individual bacteria are uncertain but might reflect nutrient competition or other local microscale effects, cell cycle differences, the degree of physiological stress, or cellular senescence. The fact that such differences in individual cellular respiration kinetics can be detected and measured suggests that similar differences in other metabolic functions (e.g., protein synthesis or growth rates) can also be expected on an individual-cell basis. These, in turn, might be correlated with CTC-reducing activity. Additional experiments are needed to resolve this issue.

It may not be feasible to employ CTC to explore microbial activity in very thick biofilms, in which low redox potentials could prevail, since CTC might undergo chemical (i.e., abiotic) reduction under such conditions. However, CTC has not been used to examine such biofilms; consequently, the extent or severity of this potential problem is not known. One possible strategy with thick biofilms might be to physically disperse the biofilm prior to CTC staining. This same approach is used in the HPC method, which also cannot be employed with intact biofilms.

Another method to determine physiologically active cells involves use of the DNA gyrase inhibitor nalidixic acid (16, 29). This antibiotic agent prevents cell division but not carbon processing and growth. Thus, only metabolically active cells elongate in the presence of nalidixic acid. The elongated bacteria can then be enumerated microscopically to estimate the number of active cells. In environmental samples, however, cells of many different lengths are frequently present from the outset. Thus, it is often difficult to determine which cells have undergone elongation in re-

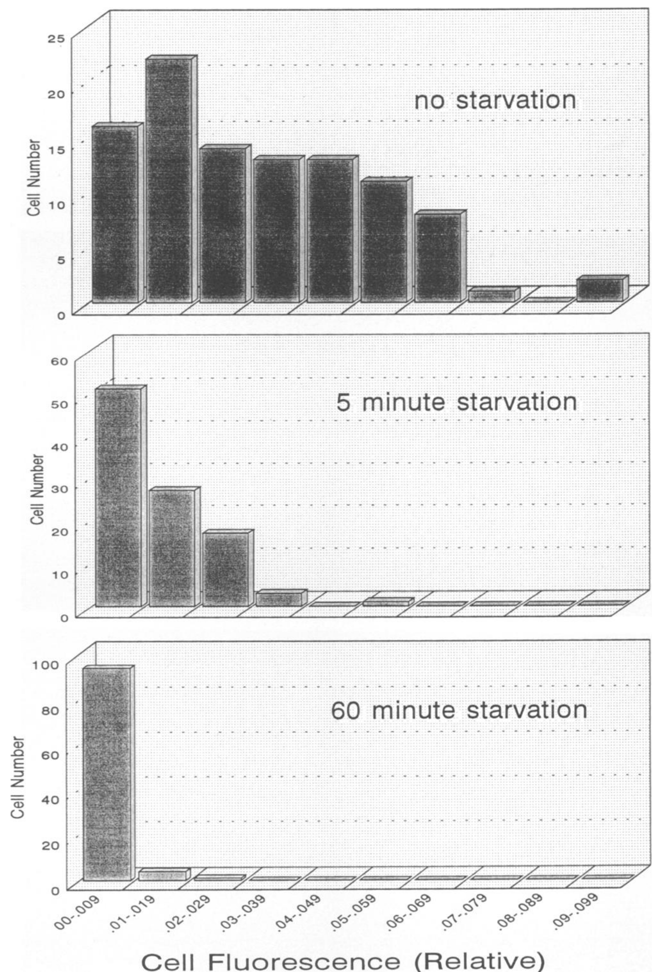

FIG. 5. Relative electron transport activities of individual cells of *P. putida* 54g in biofilms before and after nutrient starvation (see Materials and Methods for details). The relative fluorescence emissions of 100 random bacteria were quantified in each of the three biofilms by digital image analysis. The duration of starvation (5 or 60 min) refers to the time without nutrients prior to CTC addition.

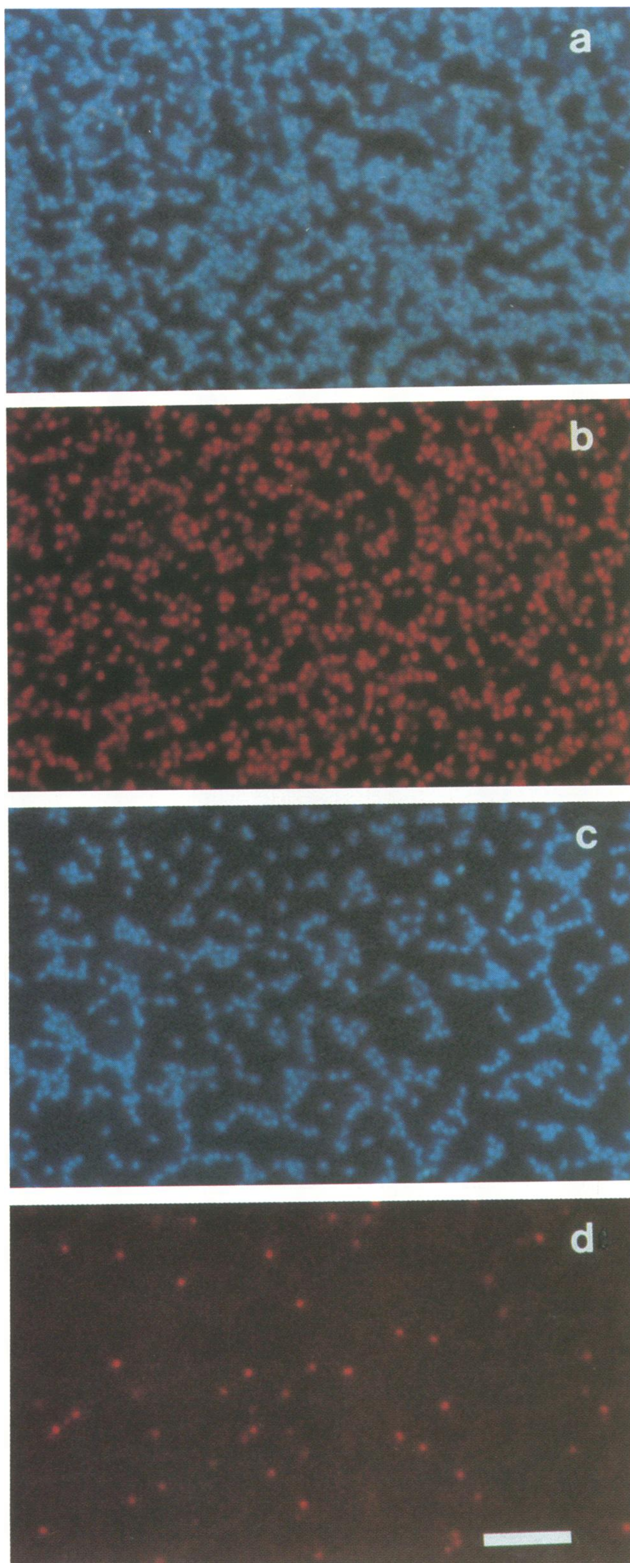

FIG. 6. Epifluorescence photomicrographs of *P. putida* biofilm before and after nutrient starvation. Panels: a, DAPI-stained cells in an unstarved control biofilm; b, CTC-stained, unstarved biofilm; c, DAPI-stained cells in a biofilm starved for 1 h; d, CTC-stained, starved biofilm. Bar, 10 μm.

sponse to the antibiotic. In addition, overlapping of elongated cells and aggregation may further complicate quantification by the nalidixic acid method. Compared with the nalidixic acid method, the CTC method described herein offers definite advantages in terms of ease of execution and interpretation of results.

Biofilm bacteria are generally more resistant to chemical biocides than are planktonic cells (11, 18, 19). Therefore, evaluation of biocide activity and other biofouling countermeasures should be performed with biofilm samples (e.g., coupon studies). The CTC methods described here and those of Rodriguez et al. (26) provide convenient and rapid approaches for quantification of the effect of a biocide in a given system. The CTC method might also be applicable for monitoring of inhibitory effects in bioreactors and activated-sludge treatment processes, analogous to earlier work with triphenyltetrazolium chloride (27) or triphenyltetrazolium chloride-malachite green (6).

The results of this study indicate the suitability of CTC for quantification of respirometrically active bacteria in both water samples and biofilms. Because of its simplicity, the method could be used as a quality control measure in drinking water microbiology for routine screening and monitoring of viable bacteria, yielding results within 1 to 2 h instead of several days. Further questions must be resolved concerning the sensitivity of CTC and related tetrazolium salts to oxygen competition (2, 28), system pH, redox potential (14), temperature, and other environmental factors.

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