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Application of a tetrazolium dye as an indicator of viability in anaerobic bacteria

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Abstract

The use of the redox dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) for evaluating the metabolic activity of aerobic bacteria has gained wide application in recent years. In this study, we examined the utility of CTC in capturing the metabolic activity of anaerobic bacteria. In addition, the factors contributing to abiotic reduction of CTC were also examined. CTC was used in conjunction with the fluorochrome 5-(4,6-dichlorotriazinyl) aminofluorescein (DTAF), that targets bacterial cell wall proteins, to quantitate the active fraction of total bacterial numbers. Facultative anaerobic bacteria, including *Escherichia coli* grown fermentatively, and *Pseudomonas chlorophis*, *P. fluorescens*, *P. stutzeri*, and *P. pseudoalcaligenes* subsp. *pseudoalcaligenes* grown under nitrate-reducing conditions, actively reduced CTC during all phases of growth. Greater than 95% of these cells accumulated intracellular CTC-formazan crystals during the exponential phase. Obligate anaerobic bacteria, including *Syntrophus aciditrophicus* grown fermentatively, *Geobacter sulfurreducens* grown with fumarate as the electron acceptor, *Desulfovibrio desulfuricans* subsp. *desulfuricans* and *D. halophilus* grown under sulfate-reducing conditions, *Methanobacterium formicicum* grown on formate, H₂ and CO₂, and *Methanobacterium thermoautotrophicum* grown autotrophically on H₂ and CO₂ all reduced CTC to intracellular CTC-formazan crystals. The optimal CTC concentration for all organisms examined was 5 mM. Anaerobic CTC incubations were not required for quantification of anaerobically grown cells. CTC-formazan production by all cultures examined was proportional to biomass production, and CTC reduction was observed even in the absence of added nutrients. CTC was reduced by culture fluids containing ferric citrate as electron acceptor following growth of either *G. metallireducens* or *G. sulfurreducens*. Abiotic reduction of CTC was observed in the presence of ascorbic acid, cysteine hydrochloride, dithiothreitol, NADH, NADPH, Fe(II)Cl₂, sodium thioglycolic acid and sodium sulfide. These results suggest that while CTC can be used to capture the metabolic activity of anaerobic bacteria, care must be taken to avoid abiotic reduction of CTC. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: 5-Cyano-2,3-ditolyl tetrazolium chloride (CTC); Anaerobic; Microbial activity

1. Introduction

The use of tetrazolium redox dyes for the direct

visual determination of bacterial metabolic activity has gained increased recognition in recent years (Zimmerman et al., 1978; Fukui and Takii, 1989; Rodriguez et al., 1992; Roslev and King, 1993; Thom et al., 1993; McFeters et al., 1995). Tetrazolium redox dyes scavenge electrons from microbial oxidation/reduction reactions and are intracellularly

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reduced to brightly colored formazan precipitates by the electron transport system (ETS) components or dehydrogenases in metabolically-active microorganisms (Kaprelyants and Kell, 1993a; Smith and McFeters, 1997). These colored precipitates can be accurately detected within individual cells by direct microscopy or quantified in cell extracts by spectrophotometry (Zimmerman et al., 1978; Fukui and Takii, 1989; Rodriguez et al., 1992; Roslev and King, 1993; Kaprelyants and Kell, 1993a; Smith and McFeters, 1997; Severin et al., 1985; Yu and McFeters, 1994; Walsh et al., 1995; Smith and McFeters, 1996).

5-Cyano-2,3-ditoyl tetrazolium chloride (CTC) is one of the recently introduced redox dyes that has gained wide application in ecological and environmental studies. CTC in its native form is colorless and is reduced by metabolically-active bacteria to an intracellular red/orange fluorescent formazan product. CTC has been used in conjunction with the fluorochrome stain 4',6-diamidino-2-phenylindole (DAPI) that targets cellular nucleic acids for quantitatively assessing total bacterial numbers and determining the viable or active fraction of those bacteria (Rodriguez et al., 1992; Bovill et al., 1994; Jørgensen et al., 1994; Smith et al., 1994; Huang et al., 1995; Pyle et al., 1995). Several procedures using CTC have been developed and used to detect active bacteria in aquatic environments, groundwater, soils, and other environmental samples (Rodriguez et al., 1992; Pyle et al., 1995; Schaule et al., 1993; Coallier et al., 1994; Winding et al., 1994; Gasol et al., 1995; Yu et al., 1995; Yamaguchi and Nasu, 1997; Bhupathiraju et al., 1999).

Although it is well established that CTC can be used to detect the metabolic activity of aerobic bacteria, it was unclear whether CTC could be used to detect the metabolic activity of anaerobic bacteria. Because of the potential for the reduction of tetrazolium redox dyes by strong chemical reductants, their utility in assessing anaerobic microbial activity or microbial activity in low redox potential environments was thought to be doubtful (Rodriguez et al., 1992; Schaule et al., 1993; Karner and Fuhrman, 1997). However, few studies have actually examined the use of tetrazolium redox dyes to assess metabolic activity of anaerobic bacteria. Fukui and Takii (1989) reported that *D. desulfuricans* and *Desul-*

fotomaculum orientis grown under sulfate-reducing conditions are capable of reducing the tetrazolium redox dyes triphenyl tetrazolium chloride (TTC) and 2(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT). Walsh et al. (1995) reported that INT and CTC could be used to detect the metabolic activity of a thermophilic sulfate-reducing consortium. More recently, Smith and McFeters (1997) demonstrated that cultures of *E. coli* grown fermentatively or with fumarate or nitrate as the electron acceptors are able to reduce CTC. Results from these studies suggest that tetrazolium salts could potentially be used to detect the metabolic activity of bacteria under some anaerobic conditions.

In this study, diverse groups of facultative and obligate anaerobic bacteria were examined for their ability to reduce CTC, in order to determine if CTC can be used to capture the metabolic activity of a wide range of anaerobic bacteria. Since anaerobic bacteria grow under reducing conditions we also examined the effects of exogenous reductants on CTC.

2. Materials and methods

2.1. Media and culture conditions

Cultures were grown in a basal medium that contained the following components (g l^{-1}): NaCl, 4.0; NH_4Cl , 1.0; KCl, 0.1; KH_2PO_4 , 0.1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.04; 50 mM Tris-HCl buffer (pH 7.4), 5 ml trace metal solution (Tanner, 1997) and 10 ml vitamin solution (Tanner, 1997). The growth medium for *D. halophilus* also contained 1.2 M NaCl. Substrates and electron acceptors used for growth of cultures are listed in Table 1. *Syntrophus aciditrophicus* was grown in the basal medium of McNerney et al. (1979). *Geobacter sulfurreducens* was grown in the basal medium of Caccavo et al. (1994). *Geobacter metallireducens* was grown in ferric citrate medium (Lovley et al., 1993). For growth of *D. desulfuricans* subsp. *desulfuricans*, *D. halophilus*, *M. thermoautotrophicum*, *M. formicum*, and *S. aciditrophicus* the basal medium was reduced using cysteine-HCl (3mM). Methods for the preparation and use of anaerobic media were those of Balch and Wolfe (1976). With

Table 1
CTC-formazan produced by aerobic and anaerobic cultures

Culture	Growth substrates (e ⁻ donor/e ⁻ acceptor)	CTC-formazan Produced ^a (nmoles/mg cells)
<i>E. coli</i> K-12 (Strain W3110)	Glucose (5.5 mM)/O ₂ ^b	270±70
	Glucose (5.5 mM)/NO ₃ (26 mM) ^{b,c}	1100±300
	Glucose (5.5 mM) ^{b-d}	1200±60
	Glucose (30 mM) ^{c-e}	50±5
<i>P. chlorophis</i> (ATCC 17810)	Glucose (5.5 mM)/O ₂ ^b	750±120
	Glucose (5.5 mM)/NO ₃ (26 mM) ^{b,c}	370±40
<i>P. fluorescens</i> (ATCC 33512)	Glucose (5.5 mM)/O ₂ ^b	470±60
	Glucose (5.5 mM)/NO ₃ (26 mM) ^{b,c}	260±60
<i>P. pseudoalcaligenes</i> subsp. <i>pseudoalcaligenes</i> (ATCC 17440)	Acetate (3 mM)/O ₂ ^b	10±1
	Acetate (3 mM)/NO ₃ (9 mM) ^{b,c}	70±10
	Acetate (30 mM)/O ₂ ^c	150±5
	Acetate (30 mM)/NO ₃ (30 mM) ^{c,e}	60±5
<i>P. stutzeri</i> (ATCC 11607)	Glucose (5.5 mM)/O ₂ ^b	1010±10
	Glucose (5.5 mM)/NO ₃ (26 mM) ^{b,c}	280±150
	Acetate (3 mM)/O ₂ ^b	40±1
	Acetate (3 mM)/NO ₃ (9 mM) ^{b,c}	70±5
<i>S. aciditrophicus</i> (ATCC 700169)	Crotonate (10 mM) ^{d-f}	60±5
<i>G. sulfurreducens</i> (ATCC 51573)	Acetate (10 mM)/Fumarate (30 mM) ^{e,f}	80±10
<i>D. desulfuricans</i> subsp. <i>desulfuricans</i> (ATCC 27774)	Lactate (40 mM)/SO ₄ (20 mM) ^{c,e}	20±1
<i>M. thermoautotrophicum</i> (ATCC 29096)	H ₂ (80%)/CO ₂ (20%) ^g	— ^g
<i>M. formicicum</i> (ATCC 33274)	Formate (10 mM), H ₂ (80%)/CO ₂ (20%) ^g	— ^g

^a Data are averages of triplicates±SD.

^b Cultures were grown in chemostat reactors.

^c Cultures were grown in basal medium with a head space of N₂ (100%).

^d Substrates were used fermentatively.

^e Cultures were grown in batch reactors.

^f Cultures were grown in basal medium with a head space of N₂ (80%) and CO₂ (20%).

^g —, not available. Although significant formazan was produced, it could not be extracted for quantification by the procedure used.

exception to *M. thermoautotrophicum* which was incubated at 62°C, all cultures were incubated at 37°C.

2.2. Assessment of metabolic activity

The metabolic activity of the cultures was determined using the tetrazolium redox dye CTC

(Polyscience, Inc., Warrington, PA) and the counter-stain DTAF (Molecular Probes, Inc., Eugene, OR). Cultures were grown either in a batch reactor or in a chemostat reactor. Samples from batch-grown cultures were routinely taken during mid-exponential phase of growth and/or during different stages of growth. Samples from chemostat-grown cultures were taken during steady-state conditions. Samples

from batch-grown cultures and/or chemostat-grown cultures were subjected to direct epifluorescent microscopy (DEM)-based CTC cell enumeration and spectrophotometric CTC reduction assays (see below). In order to evaluate the effect of CTC concentration on CTC-reduction by different cultures, batch-grown cultures were harvested during exponential growth and subjected to CTC cell enumeration assays (see below), using different concentrations of CTC. Cell growth was quantified spectrophotometrically by measuring the increase in absorbance at 600 nm according to accepted methods (McInerney et al., 1979). Unless otherwise noted all experiments were performed in triplicate. Cell free assays and assays with autoclaved (121°C, 15 min) and/or formaldehyde-treated (3.7% final concentration, treated for 30 min) cultures were used as controls.

2.3. DEM-CTC cell enumeration

CTC cell enumerations were performed using a procedure modified from Rodriguez et al. (1992). The enumerations were performed in phosphate-buffered-saline-I ([PBS I] 10 mM Na-phosphate buffer [pH 7.4]; 138 mM NaCl; 2.7 mM KCl) containing 5 mM CTC, in a total volume of 0.5 ml. Unless otherwise mentioned the enumerations were performed under aerobic conditions and without the addition of exogenous nutrients. The enumerations were initiated by the addition of cell culture (1 to 10 μ l) to the CTC reaction mixture, and samples were incubated at 37°C for 4 h under agitation in the dark. After incubation, samples were counter-stained with 1 ml of the protein stain DTAF ([1 mg ml⁻¹] dissolved in phosphate-buffered-saline-II [PBS II; 50 mM Na₂HPO₄, 145 mM NaCl; pH 9.0]) (Sherr et al., 1987; Bloem et al., 1995) and incubated at room temperature for 30 min under agitation in the dark. Stained cells were filtered onto black polycarbonate membrane filters (Poretics Co., Livermore, CA; 0.22 μ m-pore-diameter; 25 mm filter diameter) and washed twice with 50 ml of PBS II buffer. After the second wash, the filters were rinsed with distilled water and dried under vacuum. Following the addition of several drops of mounting solution (1 M TRIS [pH 8.6]-buffered glycerol (1:1 (v/v))) containing 2% 1,4 diazobicyclo [2,2,2] octane as a

quench retardant) the filters were then transferred to clean slides and examined immediately by DEM using an Olympus BH2-RFCA microscope equipped with epifluorescence illumination (11-W mercury burner). Cells that retained the green fluorescence (DTAF) and that contained the bright intracellular orange formazan (CTC-formazan) deposits were considered active, while those that retained only the green fluorescence were considered inactive. A minimum of ten randomly chosen fields were examined under a total magnification of $\times 1250$, and cell numbers were estimated as described previously (Bhupathiraju et al., 1999).

2.4. Spectrophotometric CTC-reduction assays

In order to measure the specific mass of CTC-formazan produced by cultures, cell suspensions of either chemostat-grown cultures or batch-grown cultures were used. Cell suspensions were prepared by harvesting cultures by centrifugation (1200 \times g; 20 min), washing the cell pellet twice with PBS I buffer, and resuspending washed cells in PBS I buffer. For anaerobic cultures, cell preparations were done using strict anaerobic techniques, and the washed cells were suspended in anoxic PBS I buffer. Different aliquots of the cell suspension were then used in the spectrophotometric CTC reduction assays which were performed essentially as described above except in a total volume of 2.5 ml. All experiments were performed in triplicate. CTC-formazan extraction's were performed using a procedure modified from Rodriguez et al. (1992). After incubation, cells were collected on white polycarbonate membrane filters (Poretics Co., Livermore, CA; 0.22 μ m-pore-diameter; 25 mm filter diameter). The filters were then transferred to vials containing 2.5 ml of 95% ethanol. The vials were sealed and the CTC-formazan was extracted into ethanol, by agitating the vials for 12 h at room temperature. The contents of the vials were then filtered through a 0.22 μ m-pore-diameter filter (Poretics Co., Livermore, CA), and the amount of CTC-formazan in the filtrate was determined by measuring the absorbance spectrophotometrically at 450 nm and using a molar extinction coefficient of 1.625×10^4 M⁻¹ cm⁻¹ (Smith and McFeters, 1997). The amount of CTC-formazan in the filtrate was normalized to cell dry weight.

Spectrophotometric measurements were made using a Coleman 55 spectrophotometer (Perkin Elmer Corporation, Maywood, IL). Dry weights of cell suspensions were determined using standard methods (Koch, 1995).

2.5. Effect of chemical reductants on CTC

In order to evaluate the effect of reductants on CTC, anoxic solutions of ascorbic acid, cysteine hydrochloride, dithiothreitol, Fe(II)Cl₂, NADH, NADPH, sodium sulfide or sodium thioglycolic acid were added to CTC solutions (5 mM) prepared in anoxic PBS I buffer. All manipulations were done in an anaerobic chamber (Coy Laboratories Product, Ann Arbor, MI). After incubation at room temperature for 1 h, the samples were filtered through a 0.22 μm-pore-diameter filter and the CTC-formazan was extracted into 95% ethanol. The amount of CTC-formazan in the ethanolic extracts was determined spectrophotometrically as described above.

3. Results

3.1. CTC reduction by facultative and obligate anaerobic bacteria

Cultures of *P. pseudoalcaligenes* subsp. *pseudoalcaligenes* reduced CTC when grown with acetate and either oxygen or nitrate as the terminal electron acceptors (Fig. 1 A,B). Cultures reduced CTC during all phases of growth, with greater than 98% of the cells accumulating CTC-formazan crystals intracellularly during the exponential phase of growth. Cultures of *E. coli* also reduced CTC when grown on glucose with oxygen or nitrate as electron acceptors (Table 1), or when grown fermentatively on glucose, with 95% of the cells accumulating CTC-formazan crystals intracellularly during the exponential phase of growth (Fig. 1C). No CTC-formazan crystals were detected in cell-free or killed controls. The mass of CTC-formazan produced by cultures of *P. pseudoalcaligenes* subsp. *pseudoalcaligenes* and *E. coli* was in linear proportion to live cell mass (Fig. 1). The addition of exogenous carbon source did not significantly increase the mass of CTC-formazan produced by cultures of *P. pseudoalcaligenes* subsp.

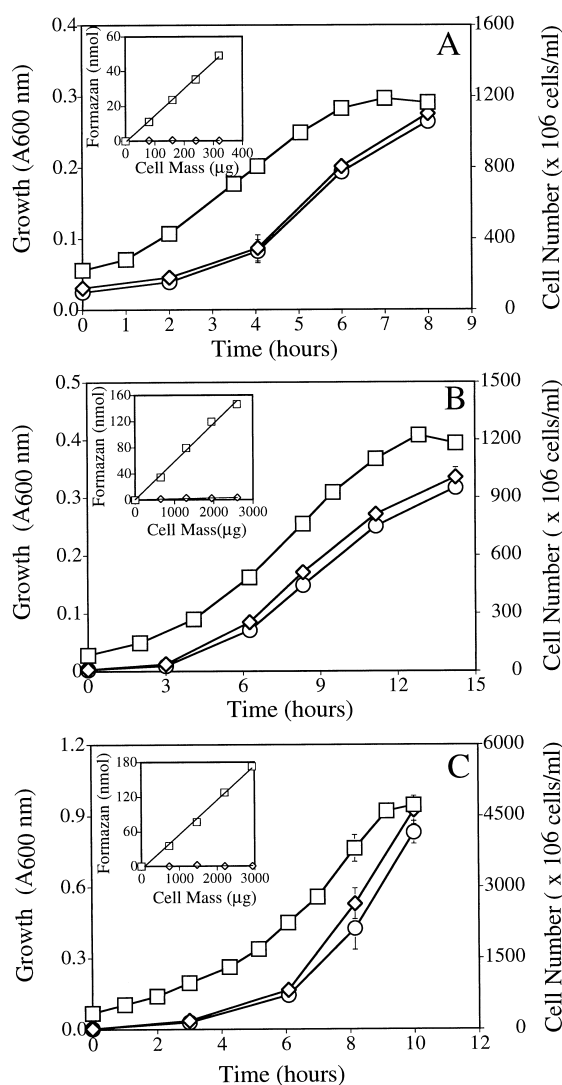


Fig. 1. Growth curves of facultative anaerobic bacteria following absorbance and metabolic activity using CTC. (A) *P. pseudoalcaligenes* subsp. *pseudoalcaligenes* grown with acetate as the carbon source and oxygen as the electron acceptor; (B) *P. pseudoalcaligenes* subsp. *pseudoalcaligenes* grown with acetate as the carbon source and nitrate as the electron acceptor; (C) *E. coli* grown fermentatively on glucose. Symbols: □, absorbance; ◇, total cell number; ○, active cell number. Insets: CTC-formazan formation as a function of cell concentration. Symbols: □, live cells; ◇, killed cells. Data are averages of triplicates ± SD. Some error bars are present within the symbols.

pseudoalcaligenes and *E. coli* (data not shown). Cultures of *P. fluorescens* and *P. chlorophis* also reduced CTC in a similar manner when grown with

glucose as the carbon source and either oxygen or nitrate as the electron acceptors (Table 1). Cultures of *P. stutzeri* grown aerobically or under nitrate-reducing conditions with either glucose or acetate as the carbon source also actively reduced CTC (Table 1).

No consistent pattern of formazan production with respect to growth conditions was observed with the facultative bacteria examined (Table 1). With chemostat-grown *P. chlorophis*, *P. fluorescens*, and *P. stutzeri*, the mass of CTC-formazan produced per unit biomass was substantially higher in cultures grown on glucose aerobically compared to cultures grown anaerobically with nitrate as the electron acceptor. Similarly, batch-grown *P. pseudoaerogenes* subsp. *pseudoaerogenes* produced more formazan per unit biomass when grown on acetate aerobically than when grown reducing nitrate. In contrast, chemostat-grown *P. pseudoaerogenes* subsp. *pseudoaerogenes* and *P. stutzeri* produced more formazan per unit biomass when grown on acetate with nitrate as the electron acceptor. Similarly, chemostat-grown *E. coli* also produced more formazan per unit biomass when grown reducing nitrate.

Cultures of *S. aciditrophicus* reduced CTC when grown fermentatively on crotonic acid, *G. sulfurreducens* reduced CTC when grown on acetate with fumarate as electron acceptor, and *D. desulfuricans* subsp. *desulfuricans* reduced CTC when grown on lactate with sulfate as electron acceptor (Table 1). Each of these cultures accumulated intracellular CTC-formazan crystals during all phases of growth (Fig. 2). In addition, cultures of *D. halophilus*, a halophilic sulfate-reducing bacterium, also reduced CTC to intracellular CTC-formazan (data not shown). CTC-formazan crystals that appeared attached to the cell exterior were occasionally observed with cultures of *S. aciditrophicus*. The mass of CTC-formazan produced by cultures of *S. aciditrophicus*, *G. sulfurreducens* and *D. desulfuricans* subsp. *desulfuricans* was linear with cell mass (Fig. 2). The addition of exogenous carbon source did not significantly increase the amount of CTC-formazan produced by any of these cultures (data not shown). No CTC-formazan was detected in cell free or killed controls.

M. formicicum grown on formate, H_2 and CO_2 ,

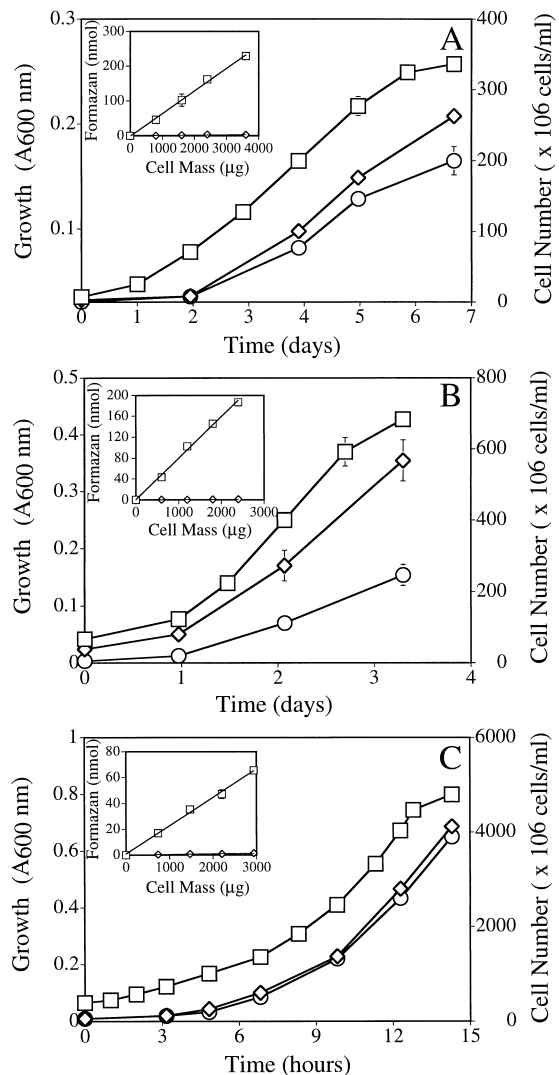


Fig. 2. Growth curves of obligate anaerobic bacteria following absorbance and metabolic activity using CTC. (A) *S. aciditrophicus* grown fermentatively on crotonic acid; (B) *G. sulfurreducens* grown with acetate as the electron donor and fumarate as the electron acceptor; (C) *D. desulfuricans* subsp. *desulfuricans* grown with lactate as the electron donor and sulfate as the electron acceptor. Symbols: \square , absorbance; \diamond , total cell number; \circ , active cell number. Insets: CTC-formazan formation as a function of cell concentration. Symbols: \square , live cells; \diamond , killed cells. Data are averages of triplicates \pm SD. Some error bars are present within the symbols.

and *M. thermoautotrophicum* grown autotrophically on H_2 and CO_2 also reduced CTC (Table 2). Typically two or more intracellular CTC-formazan

Table 2
CTC reduction by methanogenic cultures^a

Culture	Cells reducing CTC to CTC-formazan ($\times 10^5$ cells/ml)	Total number of cell ($\times 10^5$ cells/ml)	CTC-formazan forming cells (%)
<i>M. thermoautotrophicum</i> (ATCC 29096)	760 \pm 110	1720 \pm 190	44
<i>M. formicicum</i> (ATCC 33274)	400 \pm 3	710 \pm 23	56

^a CTC cell enumeration assays were done after cultures were incubated for 72 h. Data are averages of triplicates \pm SD.

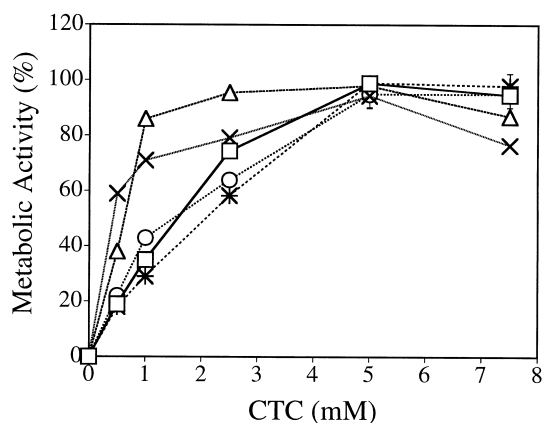


Fig. 3. CTC reduction as a function of CTC concentration. Symbols: \square , *P. pseudoalcaligenes* subsp. *pseudoalcaligenes*; Δ , *E. coli*; \times , *S. aciditrophicus*; \circ , *G. sulfurreducens*; $*$, *D. desulfuricans*. Data are averages of triplicates \pm SD. Some error bars are present within the symbols.

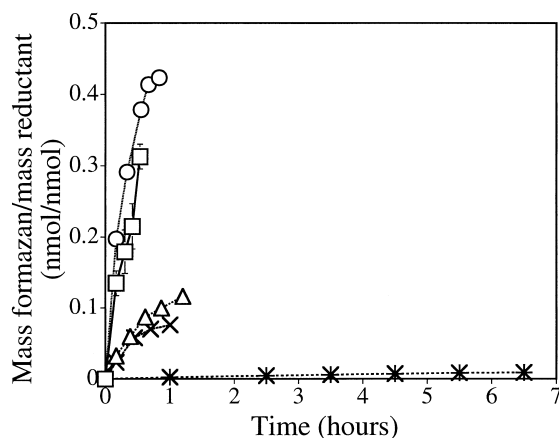


Fig. 4. Abiotic reduction of CTC by chemical reductants. Symbols: \circ , Ascorbic acid; \square , Sodium sulfide; Δ , NADH; \times , NADPH; $*$, Cysteine hydrochloride. Data are averages of triplicates \pm SD. Some error bars are present within the symbols.

crystals were observed per each cell. While no CTC reduction was observed in cell-free assays and assays using formaldehyde-killed cultures of *M. formicicum* and *M. thermoautotrophicum*, intracellular CTC-formazan crystals were occasionally observed in assays using autoclaved cultures of *M. thermoautotrophicum*.

CTC reduction as evidenced by intracellular CTC-formazan formation was observed over a wide range of CTC concentrations in the absence of exogenous nutrient additions and with aerobic CTC incubations (Fig. 3). With exception to *E. coli* and *S. aciditrophicus*, CTC reduction by all cultures was low ($< 50\%$) at CTC concentrations below 2.5 mM. At 5 mM CTC concentration, greater than 95% of cells from all the cultures examined accumulated intracellular CTC formazan crystals without added nutrients, and maintenance of anaerobic conditions during CTC incubations did not significantly increase the number of CTC-reducing cells. At CTC concentrations above 7.5 mM, the intracellular CTC-formazan accumulation caused swelling of cells for some of the cultures examined. The percentage of CTC-formazan accumulating cells decreased drastically at CTC concentrations above 10 mM (data not shown).

3.2. Extracellular and abiotic reduction of CTC

Extracellular reduction of CTC was observed with some obligate anaerobic bacteria under specific growth conditions. Extracellular reduction of CTC was observed when cysteine-sulfide was used as a reducing agent in the growth medium for *S. aciditrophicus* and *D. desulfuricans* subsp. *desulfuricans*, whereas little or none was observed when cysteine alone was used as the reducing agent in the growth medium. Sulfide alone rapidly reduced CTC (Fig. 4),

suggesting that the observed extracellular CTC reduction was mainly due to sulfide. Similarly, although no extracellular CTC reduction was observed with *G. sulfurreducens* grown on acetate with fumarate as the electron acceptor, significant extracellular CTC reduction was observed when ferric citrate was used as the electron acceptor. Addition of cells in culture medium to the CTC reaction mixture almost immediately resulted in the formation of red/orange CTC-formazan precipitates in solution and prevented the enumeration of metabolically-active cells. A similar result was observed with cultures of *G. metallireducens* grown in ferric citrate medium. Formation of CTC-formazan precipitates also occurred in cell-free culture liquid (prepared by filtering culture liquid through a sterile 0.22 μm -pore-diameter filter) from ferric citrate grown cultures of *G. sulfurreducens* and *G. metallireducens*. Formation of CTC-formazan precipitates decreased substantially with heat-treated (boiled for 5 min) cell-free culture liquid. No CTC-formazan precipitates were detected when uninoculated ferric citrate medium was used, but CTC was abiotically reduced by Fe(II)Cl_2 (Table 3), suggesting that the ferrous iron produced from the use of ferric citrate as electron acceptor may be responsible for the CTC

reduction. Extracellular reduction of CTC was also detected with cultures of *M. formicicum* and *M. thermoautotrophicum* grown on formate, H_2 and CO_2 , but was not further investigated. Significant extracellular reduction of CTC was also consistently observed in CTC cell enumeration assays containing CTC in excess of 7.5 mM.

Abiotic reduction of CTC was observed with a variety of chemical reductants (Table 3). Abiotic CTC reduction was dependent on pH, incubation time, and reductant concentration. The CTC reduced after a 1 h incubation with ascorbic acid, Fe(II)Cl_2 , NADH, NADPH, and sodium sulfide was substantially higher than that observed with other reductants (Table 3). Abiotic reduction of CTC was also observed with dithiothreitol and sodium thioglycolic acid (Table 3). The reduction of CTC was most rapid with ascorbic acid and sodium sulfide (Fig. 4), with formazan formation clearly apparent within minutes after addition of these chemicals to the CTC solution. Reduction of CTC with cysteine-HCl at neutral pH was slow, and the amount of CTC-formazan produced was low under the conditions examined (Table 3, Fig. 4). The amount of CTC-formazan produced with both cysteine-HCl and dithiothreitol increased substantially after 24 h incubations (data

Table 3
Abiotic reduction of CTC^a

Reductant (mM)		CTC-formazan produced (nmoles)	Mass formazan produced/mass reductant used (nmoles/nmoles)
Ascorbic acid	(2.5)	2340 \pm 10	0.370
	(5)	2890 \pm 30	0.230
Cysteine-hydrochloride	(5)	30 \pm 10	0.003
	(10)	45 \pm 10	0.002
Dithiothreitol	(5)	50 \pm 10	0.004
	(10)	75 \pm 1	0.003
Fe(II)Cl_2	(0.1)	80 \pm 3	0.320
	(1)	760 \pm 60	0.300
NADH	(1.4)	490 \pm 4	0.140
	(2.8)	510 \pm 50	0.073
NADPH	(0.67)	120 \pm 2	0.072
Sodium sulfide	(5)	1550 \pm 680	0.125
	(10)	3390 \pm 1350	0.136
Sodium thioglycolic acid	(5)	15 \pm 2 ^b	0.001
	(10)	25 \pm 3 ^b	0.001

^a CTC reduction assays were performed as described in Materials and methods. Except otherwise noted CTC-formazan was measured after 1 h. Data are averages of triplicates \pm SD.

^b CTC-formazan was measured after 24 h.

not shown). Little or no CTC reduction was observed with cysteine-HCl at a pH below 4.0 even after 24 h of incubation.

4. Discussion

Data from this study suggest that a variety of anaerobic bacteria are capable of reducing CTC to CTC-formazan. CTC reduction was observed during all phases of growth, the amount of CTC-formazan produced by all of the organisms examined was proportional to produced cell mass, and no CTC reduction was observed in killed controls. These results suggest that CTC can be used to quantitate the metabolic activity of a variety of anaerobic bacteria. Anaerobically grown cultures of *P. pseudoalcaligenes* subsp. *pseudoalcaligenes*, *P. chlorophis*, *P. fluorescens*, and *P. stutzeri* effectively reduced CTC without any evidence of extracellular CTC reduction. Cultures of *E. coli* reduced CTC when grown either fermentatively or under nitrate-reducing conditions in accordance with the observations of Smith and McFeters (1997). While all of these cultures reduced CTC when grown under either aerobic and anaerobic conditions, it is unclear whether aerobic or anaerobic growth enhances CTC reduction since no consistent pattern of formazan production with respect to growth conditions was observed with these facultative bacteria.

Although this study demonstrated CTC reduction by a dissimilatory metal-reducing bacterium (*G. sulfurreducens*) grown with fumarate as an electron acceptor, it was not possible to use CTC to enumerate Fe(III)-grown metal-reducers due to interference from extracellular CTC reduction. The formation of CTC-formazan precipitates observed in cell-free culture fluids of *G. sulfurreducens* and *G. metallireducens* grown with ferric citrate suggests that CTC may be reduced by ferrous iron, a product of microbial Fe(III) reduction. The observed abiotic reduction of CTC by Fe(II)Cl₂ in this study further supports this hypothesis. The fact that CTC reduction decreased substantially in heat-treated cell-free culture fluids would then be explained by the oxidation of ferrous to ferric iron during the aerobic heat treatment. These findings indicate that CTC may not

be suitable for evaluating the metabolic activity of bacteria under iron-reducing conditions.

S. aciditrophicus, an obligately anaerobic syntrophic bacterium effectively reduced CTC. It has been previously proposed that *b*-type and *c*-type cytochromes are involved in the electron-transport system (ETS) of crotonate-grown cultures of *S. buswellii* (Auburger and Winter, 1996), a syntrophic bacterium very closely related phylogenetically to *S. aciditrophicus* (Bhupathiraju, 1996). Cellular dehydrogenases present in *S. aciditrophicus* (Bhupathiraju, 1996) or components of the ETS may to be involved in CTC reduction, although direct evidence for ETS in this organism has not been demonstrated. The extracellular deposition (CTC-formazan crystals attached to cell exterior) of CTC-formazan crystals which was occasionally observed with cultures of *S. aciditrophicus* is puzzling. One possible explanation for this phenomenon could be the dissolution and the diffusion of the CTC-formazan through the cell membrane and the subsequent re-precipitation on the cell exterior, as has previously been proposed for formazans produced from tetrazolium salts methylthiazolyldiphenyl tetrazolium (MTT) and INT (Thom et al., 1993), however, it is not clear why this phenomenon would occur with *S. aciditrophicus* and not with the other cultures tested. Alternatively, the external CTC-formazan crystals could be the result of CTC reduction by cell-surface associated redox enzymes (Severin et al., 1985; Stellmach, 1984), or components of the ETS that may be oriented to the cell exterior.

The sulfate-reducing bacteria (SRB) *D. desulfuricans* subsp. *desulfuricans* and *D. halophilus* effectively reduced CTC in this study. The reduction of the tetrazolium salts TTC and INT by SRB has been previously demonstrated (Fukui and Takii, 1989), as has the reduction of CTC by a sulfate-reducing consortium (Walsh et al., 1995). The presence of electron transfer proteins in SRB is well established (Odom and Peck, 1984). Fukui and Takii (1989) speculated that INT reduction in SRB may be supported physiologically by the presence of cytochromes in the ETS. Although INT and CTC do not have identical reduction potentials, the same components involved in INT reduction may also facilitate CTC reduction in SRB. Alternatively, respiratory chain dehydrogenases or other enzymes present in

these SRB may be involved in CTC reduction. While CTC can be used to evaluate the metabolic activity of SRB, the sulfide produced from sulfate reduction could potentially cause interference with CTC cell enumeration assays since sulfide can abiotically reduce CTC.

CTC reduction was observed with both the mesophilic methanogenic bacterium *M. formicicum* and the thermophilic methanogenic bacterium *M. thermoautotrophicum*. Energy conservation in methanogenic bacteria involves a type of ETS (Müller et al., 1993). Since cytochromes are not present in H₂-grown methanogens (Kühn et al., 1983; Jussofie and Gottschalk, 1986) other components such as dehydrogenases must be involved in CTC reduction by H₂-grown *M. formicicum* and *M. thermoautotrophicum*. The observation of CTC-formazan crystals in autoclaved cultures of *M. thermoautotrophicum*, suggests that some heat-stable components in *M. thermoautotrophicum* are also able to reduce CTC. Heat-stable metalloenzymes such as corrinoids, which are found in high levels in methanogens, have been implicated in reductive dechlorination of chlorinated aliphatic compounds (Krone et al., 1989; Gantzer and Wackett, 1991). It is possible that these metalloenzymes may reduce CTC even when the methanogenic host cell is non-viable.

CTC reduction by all cultures examined in this study was sensitive to CTC concentration in the CTC cell enumeration assays. The highest numbers of CTC-formazan accumulating cells were obtained when using 5 mM CTC, within the same concentration range reported in previous studies for aerobic bacteria (Rodriguez et al., 1992; Bovill et al., 1994; Kaprelyants and Kell, 1993b; Cappelier et al., 1997; del Giorgio et al., 1997). The percentage of CTC-reducing cells decreased substantially at CTC concentrations above 5 mM with most of the cultures examined. This may be due to the toxicity of CTC itself (Ullrich et al., 1996) or the impurities associated with commercially available CTC, as has been suggested in other studies (Rodriguez et al., 1992; Coallier et al., 1994). The increased levels of extracellular CTC-formazan deposits observed at CTC concentrations exceeding 7.5 mM may be due to leakage of intracellular CTC-formazan deposits. Indeed, swelling and rupturing of cells due to

excessive deposition of intracellular CTC-formazan crystals were observed with some cultures at the higher CTC concentrations used. Thus, concentrations above 5 mM should be avoided when enumerating metabolically-active bacteria using CTC.

Previous studies have reported increased numbers of CTC-reducing cells and increased CTC-formazan deposition within cells with nutrient additions (Rodriguez et al., 1992; Smith and McFeters, 1996; Bovill et al., 1994; Smith et al., 1994). At 5 mM CTC, 95–99% of the anaerobically grown cells of *P. pseudoalcaligenes*, subsp. *pseudoalcaligenes*, *E. coli*, *S. aciditrophicus*, *G. sulfurreducens* and *D. desulfuricans* subsp. *desulfuricans* accumulated intracellular CTC formazan crystals without the addition of exogenous nutrients and the amount of CTC-formazan produced did not increase significantly with the addition of exogenous nutrients. This suggests that the addition of exogenous nutrients may not be necessary in CTC reduction assays of laboratory-grown cultures when optimum concentrations (~5 mM) of CTC are used. However, it is unclear whether nutrient addition would affect CTC reduction in samples that may be nutrient deficient. Interestingly, the anaerobically grown cultures tested in this study were capable of reducing CTC and accumulating intracellular CTC-formazan as effectively when cell enumeration assays were conducted with short aerobic incubations (following anaerobic cell preparation) as when strict anaerobic conditions were maintained throughout. This suggests that the component(s) involved in CTC reduction in these organisms may not be particularly oxygen sensitive over the short term. This is not highly unusual since some dehydrogenases do not appear to be oxygen sensitive, even in strict anaerobes (Wofford et al., 1986).

Abiotic reduction of CTC was observed with a variety of reductants including ascorbic acid, cysteine hydrochloride, dithiothreitol, Fe(II)Cl₂, NADH, NADPH, sodium sulfide, and sodium thioglycolic acid. While the abiotic reduction of CTC by ascorbic acid, dithionite, thiosulfate and some undefined media components has been previously reported (Rodriguez et al., 1992; Smith and McFeters, 1997; Smith and McFeters, 1996; Bovill et al., 1994; Boucher et al., 1994), abiotic reduction of CTC by

cysteine hydrochloride, dithiothreitol, Fe(II)Cl₂, NADH, NADPH, and sodium sulfide has not been previously demonstrated. Although the direct transfer of electrons from reduced coenzymes to tetrazolium dyes was generally thought to be unlikely (Stellmach, 1984; Kugler, 1982; Van Noorden and Tas, 1982), the reduction of CTC by NADH and NADPH observed in this study suggests that direct transfer of electrons from reduced coenzymes to CTC is possible. Enhancement in microbial CTC reduction by exogenously supplied NADH and NADPH has been previously reported (Kaprelyants and Kell, 1993b; Lopez-Amoros et al., 1995), and may have been partly due to abiotic reduction by these coenzymes. The abiotic reduction of CTC in this study was dependent on reductant concentration, pH and incubation time. These results suggest that exogenous and endogenous reductants are able to abiotically reduce CTC, which could interfere with and complicate results from CTC reduction assays.

The use of cysteine-sulfide (Balch and Wolfe, 1976) as a reducing agent in the growth medium used for some obligate anaerobic bacteria interfered with CTC reduction assays by causing extracellular reduction of CTC. While both cysteine and sulfide abiotically reduced CTC to CTC-formazan, the use of cysteine alone as the reducing agent in the growth medium did not interfere with the enumeration of CTC-reducing bacteria. This may be due to the moderate reduction potential of cysteine relative to sulfide under the experimental conditions examined. This is supported by the observation that the abiotic reduction of CTC by cysteine was slow and the amount of CTC-formazan produced was low when compared to that observed with sulfide alone. Little or no CTC reduction was observed with cysteine under highly acidic conditions. This suggests that the protonated form of cysteine exhibits lower reactivity toward CTC. These results suggest that cysteine could potentially be used as a reducing agent without causing interference in CTC cell enumeration assays provided the pH of the culture medium is maintained below neutrality and the incubation times are short.

In summary, the use of CTC for evaluating metabolic activity of anaerobic bacteria is promising, but care should be taken when interpreting data obtained from the CTC reduction assays since several chemical agents and endogenous reductants can

also reduce CTC. Direct microscopy of cell preparations as well as cell-free media preparations aid in determining CTC-applicability to experimental design. If the use of high levels of reducing agents in anaerobic culture medium is unavoidable, the use of cysteine-HCl as the reductant is recommended, as the abiotic reduction of CTC with cysteine-HCl appears to be slow. Also, we have observed significant variations from lot to lot in the texture and quality of commercially available CTC, which could potentially affect results from CTC reduction assays. If comparative studies are to be performed using CTC reduction assays, it is recommended that measurements be taken using CTC from the same lot. High concentrations of CTC are to be avoided in CTC reduction assays as CTC appears to be toxic to prokaryotes. A CTC concentration of 5 mM appears to be the optimal for enumerating the metabolic activity of bacteria.

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References

- Auburger, G., Winter, J., 1996. Activation and degradation of benzoate, 3-phenylpropionate and crotonate by *Syntrophus buswellii* strain GA. Evidence for electron-transport phosphorylation during crotonate respiration. Appl. Microbiol. Biotechnol. 44, 807–815.
- Balch, W.E., Wolfe, R.S., 1976. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. Appl. Environ. Microbiol. 32, 781–791.
- Bhupathiraju, V.K., Hernandez, M., Krauter, P., Alvarez-Cohen, L.,

1999. A new direct microscopy based method for evaluating in-situ bioremediation. *J. Haz. Materials* (in press).
- Bhupathiraju, V.K., 1996. Physiology and taxonomy of fermentative halophilic anaerobic bacteria and a novel anaerobic syntrophic bacterium. Ph.D. thesis. University of Oklahoma, Norman.
- Bloem, J., Veninga, M., Shepherd, J., 1995. Fully automated determination of soil bacterium numbers, cell volumes, and frequencies of dividing cells by confocal laser scanning microscopy and image analysis. *Appl. Environ. Microbiol.* 61, 926–936.
- Boucher, S.N., Slater, E.R., Chamberlain, A.H.L., Adams, M.R., 1994. Production and viability of coccoid forms of *Campylobacter jejuni*. *J. Appl. Bacteriol.* 77, 303–307.
- Bovill, R.A., Shallcross, J.A., Mackey, B.M., 1994. Comparison of the fluorescent redox dye 5-cyano-2,3-ditolyl tetrazolium chloride with *p*-iodonitrotetrazolium violet to detect metabolic activity in heat-stressed *Listeria monocytogenes* cells. *J. Appl. Bacteriol.* 77, 352–358.
- Caccavo, Jr. F., Lonergan, D.J., Lovley, D.R., Davis, M., Stolz, J.F., McInerney, M.J., 1994. *Geobacter sulfurreducens* sp. nov., a hydrogen- and acetate-oxidizing dissimilatory metal-reducing microorganism. *Appl. Environ. Microbiol.* 60, 3752–3759.
- Cappelier, J.M., Lazaro, B., Rossero, A., Fernandez-Astorga, A., Federighi, M., 1997. Double staining (CTC–DAPI) for detection and enumeration of viable but non-culturable *Campylobacter jejune* cells. *Veterinary Research (Paris)* 28, 547–555.
- Coallier, J., Prevost, M., Rompre, A., 1994. The optimization and application of two direct viable count methods for bacteria in distributed drinking water. *Can. J. Microbiol.* 40, 830–836.
- del Giorgio, P.A., Prairie, Y.T., Bird, D.F., 1997. Coupling between rates of bacterial production and the abundance of metabolically active bacteria in lakes, enumerated using CTC reduction and flow cytometry. *Microbiol. Ecol.* 34, 144–154.
- Fukui, M., Takii, S., 1989. Reduction of tetrazolium salts by sulfate-reducing bacteria. *FEMS Microbiol. Ecology* 62, 13–20.
- Gantzer, C.J., Wackett, L.P., 1991. Reductive dechlorination catalyzed by bacterial transition-metal coenzymes. *Environ. Sci. Technol.* 25, 715–722.
- Gasol, J.M., del Giorgio, P.A., Massana, R., Duarte, C.M., 1995. Active versus inactive bacteria: size-dependence in a coastal marine plankton community. *Mar. Ecol. Prog. Ser.* 128, 91–97.
- Huang, C.-T., Yu, F.P., McFeters, G.A., Stewart, P.S., 1995. Nonuniform spatial patterns of respiratory activity within biofilms during disinfection. *Appl. Environ. Microbiol.* 61, 2252–2256.
- Jørgensen, F., Nybroe, O., Knøchel, S., 1994. Effect of starvation and osmotic stress on viability and heat resistance of *Pseudomonas fluorescens* AH9. *J. Appl. Bacteriol.* 77, 340–347.
- Jussolie, A., Gottschalk, G., 1986. Further studies on the distribution of cytochromes in methanogenic bacteria. *FEMS Microbiol. Lett.* 37, 15–18.
- Kaprelyants, A.S., Kell, D.B., 1993a. Dormancy in stationary-phase cultures of *Micrococcus luteus*: flow cytometric analysis of starvation and resuscitation. *Appl. Environ. Microbiol.* 59, 3187–3196.
- Kaprelyants, A.S., Kell, D.B., 1993b. The use of 5-cyano-2,3-ditolyl tetrazolium chloride and flow cytometry for visualisation of respiratory activity in individual cells of *Micrococcus luteus*. *J. Microbiol. Methods* 17, 115–122.
- Karner, M., Fuhrman, J.A., 1997. Determination of active marine bacterioplankton: a comparison of universal 16S rRNA probes, autoradiography, and nucleotide staining. *Appl. Environ. Microbiol.* 63, 1208–1213.
- Koch, A.L., 1995. Growth measurements. In: *Methods for General and Molecular Bacteriology*, American Society for Microbiology, Washington, DC, pp. 248–277.
- Krone, U.E., Laufer, K., Thauer, R.K., Hogenkamp, H.P.C., 1989. Coenzyme F₄₃₀ as possible catalyst for reductive dehalogenation of chlorinated C₁-hydrocarbons in methanogenic bacteria. *Biochemistry* 28, 10061–10065.
- Kugler, P., 1982. Quantitative dehydrogenase histochemistry with exogenous electron carriers (PMS, MPMS, MB). *Histochemistry* 75, 99–112.
- Kühn, W., Fiebig, K., Hippe, H., Mah, R.A., Huser, B.A., Gottschalk, G., 1983. Distribution of cytochromes in methanogenic bacteria. *FEMS Microbiol. Lett.* 20, 407–410.
- Lopez-Amoros, R., Mason, D.J., Lloyd, D., 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.
- Lovley, D.R., Giovannoni, S.J., White, D.C., Champine, J.E., Phillips, E.J.P., Gorby, Y.A., Goodwin, S., 1993. *Geobacter metallireducens* gen. nov. sp. nov., a microorganism capable of coupling the complete oxidation of organic compounds to the reduction of iron and other metals. *Arch. Microbiol.* 159, 336–344.
- McFeters, G.A., Yu, F.P., Pyle, B.H., Stewart, P.S., 1995. Physiological assessment of bacteria using fluorochromes. *J. Microbiol. Methods* 21, 1–13.
- McInerney, M.J., Bryant, M.P., Pfennig, N., 1979. Anaerobic bacterium that degrades fatty acids in syntrophic association with methanogens. *Arch. Microbiol.* 122, 129–135.
- Müller, V., Blaut, M., Gottschalk, G., 1993. Bioenergetics of methanogenesis. In: Ferry, J.G. (Ed.), *Methanogenesis: Ecology, Physiology, Biochemistry and Genetics*, Chapman and Hall, New York, pp. 360–406.
- Odom, J.M., Peck, Jr. H.D., 1984. Hydrogenase, electron transfer proteins and energy coupling in the sulfate reducing *Desulfovibrio*. *Annu. Rev. Microbiol.* 38, 551–592.
- Pyle, B.H., Broadaway, S.C., McFeters, G.A., 1995. Factors affecting the determination of respiratory activity on the basis of cyanoditolyl tetrazolium chloride reduction with membrane filtration. *Appl. Environ. Microbiol.* 61, 4304–4309.
- Rodriguez, G.G., Phipps, D., Ishiguro, K., Ridgway, H.F., 1992. Use of a fluorescent redox probe for direct visualization of actively respiring bacteria. *Appl. Environ. Microbiol.* 58, 1801–1808.
- Roslev, P., King, G.M., 1993. Application of a tetrazolium salt with a water-soluble formazan as an indicator of viability in respiring bacteria. *Appl. Environ. Microbiol.* 59, 2891–2896.
- Schaule, G., Flemming, H.-C., Ridgway, H.F., 1993. Use of 5-cyano-2,3-ditolyl tetrazolium chloride for quantifying plank-

- tonic and sessile respiring bacteria in drinking water. *Appl. Environ. Microbiol.* 59, 3850–3857.
- Severin, E., Stellmach, J., Nachtigal, H.M., 1985. Fluorimetric assay of redox activity in cells. *Anal. Chim. Acta* 170, 341–346.
- Sherr, B.F., Sherr, E.B., Fallon, R.D., 1987. Use of monodispersed, fluorescently labeled bacteria to estimate in situ protozoan bacterivory. *Appl. Environ. Microbiol.* 53, 958–965.
- Smith, J.J., McFeters, G.A., 1997. Mechanisms of INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride), and CTC (5-cyano-2,3-ditolyl tetrazolium chloride) reduction in *Escherichia coli* K-12. *J. Microbiol. Methods* 29, 161–175.
- Smith, J.J., McFeters, G.A., 1996. Effects of substrates and phosphate on INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride) and CTC (5-cyano-2,3-ditolyl tetrazolium chloride) reduction in *Escherichia coli*. *J. Appl. Bacteriol.* 80, 209–215.
- Smith, J.J., Howington, J.P., McFeters, G.A., 1994. Survival, physiological response, and recovery of enteric bacteria exposed to a polar marine environment. *Appl. Environ. Microbiol.* 60, 2977–2984.
- Stellmach, J., 1984. Fluorescent redox dyes: 1. Production of fluorescent formazan by unstimulated and phorbol ester- or digitonin-stimulated Ehrlich ascites tumor cells. *Histochemistry* 80, 137–143.
- Tanner, R.S., 1997. Cultivation of bacteria and fungi. In: *Manual of Environmental Microbiology*, American Society for Microbiology, Washington, DC, pp. 52–60.
- Thom, S.M., Horobin, R.W., Seidler, E., Barer, M.R., 1993. Factors affecting the selection and use of tetrazolium salts as cytochemical indicators of microbial viability and activity. *J. Appl. Bacteriol.* 74, 433–443.
- Ullrich, S., Karrasch, B., Hoppe, H.-G., Jeskulke, K., Mehrens, M., 1996. Toxic effects on bacterial metabolism of the redox dye 5-cyano-2,3-ditolyl tetrazolium chloride. *Appl. Environ. Microbiol.* 62, 4587–4593.
- Van Noorden, C.J.F., Tas, J., 1982. The role of exogenous electron carriers in NAD(P)-dependent dehydrogenase cytochemistry studied in vitro and with a model system of polyacrylamide films. *J. Histochem. Cytochem.* 30, 12–20.
- Walsh, S., Lappin-Scott, H.M., Stockdale, H., Herbert, B.N., 1995. An assessment of the metabolic activity of starved and vegetative bacteria using two redox dyes. *J. Microbiol. Methods* 24, 1–9.
- Winding, A., Binnerup, S.J., Sorensen, J., 1994. Viability of indigenous soil bacteria assayed by respiratory activity and growth. *Appl. Environ. Microbiol.* 60, 2869–2875.
- Wofford, N.Q., Beaty, P.S., McInerney, M.J., 1986. Preparation of cell-free extracts and the enzymes involved in fatty acid metabolism in *Syntrophomonas wolfei*. *J. Bacteriol.* 167, 179–185.
- Yamaguchi, N., Nasu, M., 1997. Flow cytometric analysis of bacterial respiratory and enzymatic activity in the natural aquatic environment. *J. Appl. Microbiol.* 83, 43–52.
- Yu, F.P., McFeters, G.A., 1994. Rapid in situ assessment of physiological activities in bacterial biofilms using fluorescent probes. *J. Microbiol. Methods* 20, 1–10.
- Yu, W., Dodds, W.K., Banks, M.K., Skalsky, J., Strauss, E.A., 1995. Optimal staining and sample storage time for direct microscopic enumeration of total and active bacteria in soil with two fluorescent dyes. *Appl. Environ. Microbiol.* 61, 3367–3372.
- Zimmerman, R., Iturriaga, R., Becker-Birck, J., 1978. Simultaneous determination of the total number of aquatic bacteria and the number thereof involved in respiration. *Appl. Environ. Microbiol.* 36, 926–935.