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Application of a Tetrazolium Salt with a Water-Soluble Formazan as an Indicator of Viability in Respiring Bacteria

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The tetrazolium salt sodium 3′-{1-[(phenylamino)-carbonyl]-3,4-tetrazolium}-bis (4-methoxy-6-nitro)benzenesulfonic acid hydrate (XTT) was examined for use as a colorimetric indicator of viability in respiring bacteria. XTT was reduced to an orange, water-soluble formazan product by *Methylosinus trichosporium* OB3b, *Pseudomonas putida*, *Escherichia coli*, and *Bacillus subtilis*. Formazan production was proportional to live cell biomass, and XTT was reduced by all cultures in the absence of added electron-coupling agents. XTT reduction by *M. trichosporium* OB3b was linear over several hours and was stimulated by the presence of an exogenous substrate (methanol). Addition of cyanide to cultures incubated under oxic conditions gave an initial 10-fold increase in XTT reduction. Viability of bacteria incubated in the absence of exogenous carbon substrates was measured as XTT reduction and compared with viability estimates from plate counts. Results obtained with the two methods were generally comparable, but the XTT assay was superior when cell recovery on plates was low. Incubation of *E. coli* for 7 days in the absence of exogenous carbon substrates decreased viability by 90%, whereas the corresponding decreases for cultures of *M. trichosporium* OB3b, *P. putida*, and *B. subtilis* were less than 40%.

Bacteria are often exposed to fluctuations in their local environment that can affect the survival of individual organisms and influence the dynamics of microbial communities. The effects of stress on bacterial viability have consequently attracted considerable interest and stimulated studies of strategies for survival of nutrient deprivation (e.g., 5, 9, 10, 12, 14). However, although of obvious importance, definitions and measurements of bacterial viability remain controversial (e.g., 19, 23). Estimates of viability based on the capacity of organisms to divide and form colonies have been questioned, and the application of techniques that focus on cell metabolic activity rather than cell growth has been recommended.

In some bacteria, respiration can provide a suitable measure of cell metabolic activity and give information about the effects of different conditions on cell viability. Respiration activity in microorganisms has previously been estimated by using direct, as well as indirect, methods, including assays of electron transport system (ETS) activity (17, 31). As an indicator of reducing systems like the ETS, tetrazolium salts have proven useful in a number of enzymatic assays (1). Some tetrazolium salts, predominantly 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) and recently the fluorescent compound 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), have also been used to enumerate respiring bacteria in cultures and environmental samples (20, 27, 31). Bacteria with an active ETS reduce these redox dyes from a water soluble tetrazolium salt to a colored, water-insoluble formazan product. The formazan accumulates inside the cells and is identified subsequently by microscopy. Attempts have also been made to establish colorimetric assays for the overall respiratory activity of microorganisms in environmental samples and cultures by dissolving and quantifying the amount of formazan produced (3, 6, 13, 20, 29). However, these assays are complicated by the insolubility of the formazan product in water, which necessitates addition of organic solvents to dissolve the product. In addition, accumulation during the assay of large intracellular formazan deposits could affect cell activity.

A new tetrazolium salt, sodium 3′-{1-[(phenylamino)-carbonyl]-3,4-tetrazolium}-bis (4-methoxy-6-nitro)benzenesulfonic acid hydrate (XTT), with a corresponding water-soluble formazan has been synthesized recently (18). This tetrazolium salt appeared promising as an indicator of viability in assays with eucaryotic cells, including in vitro screening of chemotherapeutic drugs, estimation of filarial viability, and susceptibility of yeasts to antifungal drugs (4, 24, 28). These assays have required addition of an electron-coupling agent, such as phenazine methosulfate or menadione, to give satisfactory color accumulation.

Here we show initial results from the application of XTT as a colorimetric indicator of bacterial ETS activity and viability. Formazan production was proportional to live bacterial biomass, and assays with XTT as the only artificial electron acceptor gave good production of water-soluble formazan by cultures of *Methylosinus trichosporium* OB3b, *Pseudomonas putida*, *Escherichia coli*, and *Bacillus subtilis*.

MATERIALS AND METHODS

Media and culture conditions. *M. trichosporium* OB3b was grown in nitrate minimal medium (NMM), pH 7.0, containing 10 mM KNO_3, 6.1 mM Na_2HPO_4, 3.9 mM KH_2PO_4, 0.8 mM Na_2SO_4, 0.2 mM $MgSO_4$, and 0.05 mM $CaCl_2$. Trace elements were added from a concentrated stock solution to give the following final concentrations in the medium: 2 μM $ZnCl_2$, 2 μM $CuCl_2$, 1 μM NaBr, 0.5 μM Na_2MoO_2, 2 μM $MnCl_2$, 1 μM KI, 2 μM H_3BO_3, 1 μM $CoCl_2$, and 1μM $NiCl_2$. Iron was added as filter-sterilized $FeSO_4$ in 1 M HCl to give a concentration of 50 μM in NMM. *M. trichosporium* OB3b was grown on methane in batch cultures with an initial atmosphere of 20% CH_4 and 80% air. *P. putida*, *E. coli*, and *B. subtilis* were grown in glucose-yeast extract medium

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(GYM) consisting of ammonium mineral medium (AMM) supplemented with 20 mM glucose and 0.5% yeast extract. AMM contained inorganic nitrogen as 10 mM NH_4Cl but was otherwise similar to the nitrate minimal medium described above. *M. trichosporium* OB3b cultures (0.8 liter) were grown in 4-liter Erlenmeyer flasks, and *P. putida*, *E. coli*, and *B. subtilis* cultures (0.2 liter) were grown in 1-liter Erlenmeyer flasks. All cultures were grown at 30°C on a shaker at 120 rpm. Cells were harvested in the exponential phase by centrifugation (8,000 × *g*, 10 min), washed twice, and resuspended in media as specified below.

XTT assay. The tetrazolium salt XTT (Polysciences, Warrington, Pa.) was dissolved in medium a few hours before use and heated to 50°C in a water bath to ensure solution. A 10 mM stock solution was prepared (using an effective molecular weight of 764.5 for the XTT hydrate) and stored at 40°C in the dark until use. The assays were started by mixing cell suspensions with the XTT solution at a 1:1 ratio giving, unless stated otherwise, a final XTT concentration of 5 mM and a cell biomass of approximately 300 μg (dry weight) ml^{-1}. Cell-free assays and cell suspensions containing 3% glutaraldehyde were used as controls. All XTT assays were carried out in 12-ml serum bottles sealed with green neoprene stoppers. Bottles were incubated horizontally at 35°C and 120 rpm on a gyratory shaker (New Brunswick Scientific, N.J.). At each time point, a 0.5-ml culture volume was removed from the serum bottles and mixed with 0.5 ml of autoclaved, deionized water. The mixture was vortexed and then centrifuged (8,000 × *g*, 8 min) to pellet the cells. The formazan product was then measured in the supernatant at 470 nm on a spectrophotometer (LKB Ultrospec 4050, Gaithersburg, Md.) with medium without XTT as a reference.

Iron added as $FeSO_4$ to the regular growth medium interfered with the XTT assay by chemically reducing the dye. Iron was added instead as a ferric citrate complex in XTT assays in which methanol was used as a carbon substrate and omitted in assays without added carbon substrates. Media used for cell growth and incubations prior to the XTT assay contained iron as $FeSO_4$.

Effect of XTT concentration on formazan production. Methane-grown *M. trichosporium* OB3b cells were switched to growth on 2 mM methanol, harvested 20 h later and resuspended in NMM. Cell suspensions were mixed with XTT in NMM (1 ml:1 ml) to give XTT concentrations between 0.5 and 7 mM. Methanol was used as a substrate and was added to all bottles at an initial concentration of 2 mM. Formazan was measured initially and after 12 h of incubation.

Effect of biomass on formazan production. Methane-grown *M. trichosporium* OB3b cells were harvested and resuspended in NMM at different cell densities. Cell suspensions were mixed with XTT in NMM (1 ml:1 ml) to give biomasses between 78 and 440 μg (dry weight) ml^{-1}. Cells were incubated with no added carbon substrate, and formazan was measured initially and after 8 h of incubation.

Stimulation and inhibition of formazan production. Methane-grown *M. trichosporium* OB3b cells were switched to growth on 2 mM methanol, harvested 20 h later, resuspended in NMM, and mixed with XTT in NMM (2 ml:2 ml). *M. trichosporium* OB3b cells were incubated with 3% glutaraldehyde, with medium only, with 200 μM KCN, or with methanol in the assay. Methanol was added at the beginning of the experiment and after 8 and 14 h to give a calculated concentration of 4 mM at each time point. Formazan was measured initially and after 4, 8, 12, and 24 h of incubation.

Viability determined by XTT reduction. Cultures of *M. trichosporium* OB3b, *P. putida*, *E. coli*, and *B. subtilis* were incubated for 7 days in the absence of exogenous carbon substrates to affect cell viability. Cells from cultures in the exponential growth phase were harvested, washed twice, and resuspended in inorganic mineral medium to densities of approximately 170 μg (dry weight) ml^{-1}. *M. trichosporium* OB3b was resuspended in NMM; the three other cultures were resuspended in AMM. These bacteria are subsequently referred to as carbon-starved cells. Triplicate samples of 30 ml from each culture were transferred to 160-ml serum bottles and incubated with an oxic headspace at 35°C on a gyratory shaker at 120 rpm. Autoclaved AMM without added cells was used as a control. Samples for measurement of XTT reduction were removed from the serum bottles after 0, 2, and 7 days. The subsamples (4 ml) were centrifuged, suspended in 1 ml of mineral medium, and mixed with 1 ml of XTT solution. Formazan production was measured over 8 h.

Viability determined by plate counts. Samples for spread plate counts were taken from the carbon-starved cultures after 0, 2, and 7 days of incubation. After serial dilutions, *P. putida*, *E. coli*, and *B. subtilis* cultures were plated onto GYM agar plates and incubated at 30°C. Colonies were counted after 2 and 4 days. *M. trichosporium* OB3b cultures were diluted and plated onto NMM agar plates and incubated at 30°C with an initial atmosphere of 30% methane in air. The methane atmosphere was changed weekly, and colonies were counted after 2 and 3 weeks of incubation.

RESULTS

XTT assay. The methanotroph *M. trichosporium* OB3b was used to evaluate the effects of different incubation conditions on XTT reduction. Initial experiments gave good formazan production with XTT as the only artificial electron acceptor present in the medium, and addition of electron-coupling agents was not required for XTT reduction. All of the subsequent results are from assays carried out at 35°C in the absence of electron couplers. Lower temperatures apparently decreased the solubility of the tetrazolium salt.

Absorption spectra of XTT formazan produced by *M. trichosporium* OB3b showed an absorption maximum between 460 and 480 nm, whereas the corresponding absorption by unreacted XTT was low (data not shown). A wavelength of 470 nm was used in the assays for measurements of XTT formazan.

Production of formazan was dependent on the initial concentration of XTT and showed a sigmoidal relationship (Fig. 1). A strong concentration effect was observed between 0.5 and 3 mM XTT, followed by a decreased dependency at higher concentrations. An XTT concentration of 5 mM was chosen for the assay to give approximately zero-order kinetics.

Effect of biomass on formazan production. XTT reduction was directly proportional to the live biomass of *M. trichosporium* OB3b (Fig. 2). The relationship was linear for cell densities of up to 440 μg (dry weight) ml^{-1} or approximately 5×10^8 cells ml^{-1}. Cells used in the assay were harvested in the mid-exponential phase and incubated with XTT in the absence of exogenous carbon substrates. The results suggest that under these conditions a linear relationship exists between formazan production and the numbers of respiring bacteria.

Stimulation and inhibition of formazan production. The effect of substrate and cyanide addition on XTT reduction

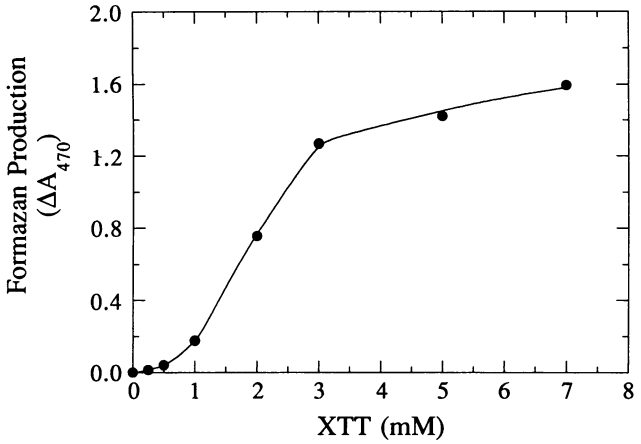

FIG. 1. Effect of XTT concentrations on production of formazan by *M. trichosporium* OB3b. Cells were incubated with 2 mM methanol and different initial concentrations of XTT. Formazan was measured after 12 h. Datum points are means of duplicate determinations.

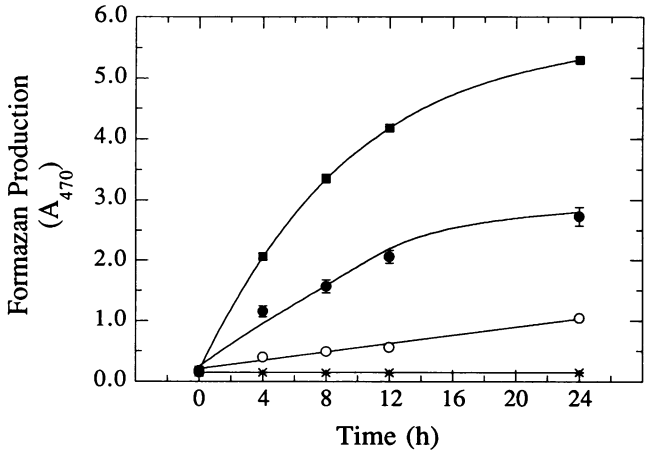

FIG. 3. Time courses of formazan production by *M. trichosporium* OB3b incubated with 5 mM XTT and 4% glutaraldehyde (×), with no added carbon substrate (○), with 4 mM methanol (●), and with 200 μM cyanide (■). Datum points are means of triplicate determinations (± standard errors). Some error bars are contained within the symbols.

was examined in a 24-h assay with *M. trichosporium* OB3b (Fig. 3). Linear formazan production was observed for cells incubated for 24 h in the absence of added carbon substrates. In our studies, XTT reduction by carbon-starved *M. trichosporium* OB3b was generally measured over 8 h but the results suggest that exceeding this limit may not alter the linearity of formazan production (Fig. 3). This could be advantageous in assays with low cell numbers or in situations with cells having low metabolic activity.

M. trichosporium OB3b showed a significant increase in XTT reduction when incubated in the presence of methanol, which was added initially and after 8 and 14 h of incubation (Fig. 3). However, the rate of formazan production changed over time, presumably because of exhaustion of methanol in the medium between 14 and 24 h. High initial concentrations of methanol were not used, to avoid potentially inhibitory levels.

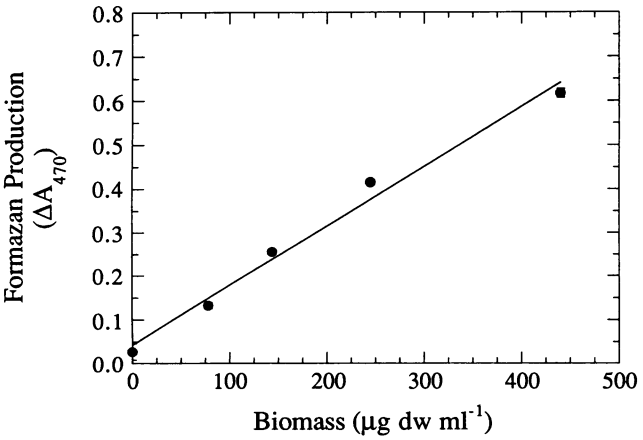

FIG. 2. Formazan production by *M. trichosporium* OB3b as a function of biomass. Cells were harvested during exponential growth, immediately resuspended at the indicated cell densities, and incubated with 5 mM XTT for 8 h. Datum points are means of triplicate determinations (± standard errors). Some error bars are contained within the symbols.

Addition of 200 μM KCN to cells incubated with no added carbon substrate gave a pronounced increase in XTT reduction, with 10-fold-higher formazan production after 8 h than cells incubated without cyanide (Fig. 3). Stimulation of formazan production by cyanide was curvilinear, and 78% of the final amount of formazan was produced during the first 12 h of incubation. *M. trichosporium* OB3b incubated with XTT in the presence of glutaraldehyde (3%) resulted in complete inhibition of formazan production, suggesting that nonenzymatic XTT reduction was low in the assay (Fig. 3).

Viability of carbon-starved bacteria. Viability of *M. trichosporium* OB3b, *P. putida*, *E. coli*, and *B. subtilis* cells during carbon starvation was examined by comparing formazan production and plate counts in the same cultures. A general correspondence between the two methods of estimating viability was observed (Table 1). Relative changes in formazan production for a given strain were generally comparable to relative changes in plate counts. However, at the onset of starvation, *B. subtilis* showed low recovery on plates and *M. trichosporium* OB3b displayed low and variable plate counts throughout this study. The observed variability for *M. trichosporium* OB3b was supported by comparing plate counts and direct microscopic counts for exponential-phase cultures, which occasionally gave much lower counts on plates than expected on the basis of microscopic findings (data not shown).

Different responses to carbon deprivation were observed among the cultures as expressed in changes in formazan production and plate counts. The viability of *E. coli* decreased rapidly, and 90% of the initial formazan production activity was lost after 7 days. In contrast, *P. putida* and *B. subtilis* showed formazan production and plate count increases after 2 days of carbon starvation which were then followed by a decrease in viability. After 7 days of carbon starvation, *M. trichosporium* OB3b and *P. putida* had lost less than 40% of their XTT reduction activity while *B. subtilis* showed higher activity than at the beginning of the experiment.

There was an initial fivefold difference in the rate of formazan production among the four cultures (despite the same biomass), with *E. coli* and *B. subtilis* having the highest

TABLE 1. Viability of carbon-starved bacteria determined by XTT reduction and plate counts

Culture	Starvation time (days)	Formazan production		Plate count	
		ΔA_{470}[a,b]	Relative[c]	10^8 CFU/ml[b]	Relative[d]
Cell-free control	0	0.028 ± 0.009		ND[e]	
	2	0.025 ± 0.015		ND	
	7	0.015 ± 0.019		ND	
M. trichosporium OB3b	0	0.315 ± 0.022	1	0.36 ± 0.19	1
	2	0.245 ± 0.008	0.78	0.22 ± 0.09	0.70
	7	0.237 ± 0.008	0.75	0.12 ± 0.03	0.33
P. putida	0	0.282 ± 0.053	1	3.13 ± 0.55	1
	2	0.360 ± 0.029	1.28	3.87 ± 0.29	1.24
	7	0.177 ± 0.018	0.63	1.75 ± 0.10	0.56
E. coli	0	0.975 ± 0.080	1	4.40 ± 1.59	1
	2	0.553 ± 0.087	0.57	2.33 ± 0.18	0.53
	7	0.101 ± 0.028	0.10	0.55 ± 0.10	0.13
B. subtilis	0	0.194 ± 0.007		ND	
	2	0.595 ± 0.012	1	1.00 ± 0.12	1
	7	0.348 ± 0.006	0.58	0.58 ± 0.07	0.58

[a] Formazan production was measured over 8 h as described in Materials and Methods.
[b] Means for triplicate cultures ± standard errors are shown.
[c] Relative values of formazan production (FP) were calculated for each culture as $FP_{day\ t}/FP_{day\ 0}$; relative values for *B. subtilis* were calculated as $FP_{day\ t}/FP_{day\ 2}$.
[d] Relative values of plate counts (PC) were calculated for each culture as $PC_{day\ t}/PC_{day\ 0}$; relative values for *B. subtilis* were calculated as $PC_{day\ t}/PC_{day\ 2}$.
[e] ND, not detectable.

and lowest initial activities, respectively. After 7 days of carbon starvation, this relationship had reversed and *E. coli* showed the lowest and *B. subtilis* showed the highest viability based on formazan production.

DISCUSSION

In the present study, we examined the possibility of using the tetrazolium salt XTT as an indicator of bacterial viability. Biological reduction of tetrazolium salts has been correlated previously with ETS activity in respiring cells and thus used as an index of metabolic activity (7, 15, 31). Our results suggest that XTT can be applied as a simple colorimetric indicator of bacterial ETS activity and used to estimate the viability of carbon-starved bacteria. Unlike viability estimates obtained via spread plate counts or most-probable-number techniques, the current method provides direct information about metabolic activity during the incubation, while the former methods estimate the capacity for cell recovery after exposure of the organisms to different experimental conditions.

XTT has been used previously in a number of assays with eucaryotic cells (4, 21, 24, 28). In these experiments, addition of electron-coupling agents, such as phenazine methosulfate and menadione, was required to get satisfactory formazan production. This requirement may have been due to the limited membrane transport of XTT to the site of reduction in eucaryotic cells (e.g., mitochondria) (30). In a recent study of neutrophil bactericidal activity, it was concluded that electron-coupling agents were also required for bacterial XTT reduction (25). Unfortunately, the use of electron couplers can complicate interpretations of XTT reduction, as these compounds are known to increase background absorption and to reduce XTT in cell-free controls (data not shown; 21). In addition, the electron coupler phenazine methosulfate can also affect reproducibility and cause crystallization in assays with XTT (24).

In contrast to Stevens and Olsen (25), who concluded that XTT alone was not reduced by *E. coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Brucella abortus*, we found that *M. trichosporium* OB3b, *P. putida*, *E. coli*, and *B. subtilis* reduced XTT in assays without added electron couplers. A concentration of 5 mM XTT was found to be well suited for measurements of formazan production by these cultures. This is comparable to the optimal concentrations of the tetrazolium salt CTC, which has a water-insoluble formazan product (20).

In *M. trichosporium* OB3b, methanol oxidation proceeds via methanol, formaldehyde, and formate dehydrogenases, which donate electrons to the electron transport chain at various levels (2). The observed increase in XTT reduction in the presence of methanol (Fig. 3) could therefore be explained by a general increase in ETS activity. Addition of cyanide to cultures of *M. trichosporium* OB3b also increased XTT reduction. On the basis of the known effects of cyanide on the respiratory chain in *M. trichosporium* OB3b (2), it is likely that inhibition of the terminal oxidase resulted in increased electron transport to XTT, which then served as the dominant terminal electron acceptor. A similar stimulation has been reported for the tetrazolium salt INT, with which cyanide concentrations between 0.2 and 1 mM gave a 25 to 30% increase in formazan production by phytoplankton homogenates (8). Although cyanide additions may provide a means for increasing the sensitivity of the XTT assay, it is important to note that the resulting formazan production by *M. trichosporium* OB3b was nonlinear (Fig. 3). In the absence of cyanide, formazan production by carbon-starved *M. trichosporium* OB3b was linear over 24 h (Fig. 3). The constant rate of XTT reduction over time and the observed increase in formazan production with increasing XTT concentrations (Fig. 2) did not suggest any inhibitory effect of XTT or its formazan product on XTT reduction. Similarly, no apparent toxic effects of XTT were seen in assays with human tumor cells (24).

Estimates of viability for carbon-starved cells of *P. putida*, *E. coli*, and *B. subtilis* based on formazan production and plate counts were highly correlated ($r > 0.99$). Relative changes in plate counts were apparently paralleled by similar relative changes in formazan production. These data also suggest that XTT reduction for a given organism changed very little during carbon deprivation when calculated on a per-cell basis (formazan production divided by plate counts). Although different among the three cultures, this ratio was approximately constant for carbon-starved bacteria during the 7-day sampling period. How this relationship is affected by sporulation or whether it changes with extended carbon starvation is not known.

The low plate counts measured for *M. trichosporium* OB3b during this study and the resulting underestimation of the number of viable cells are consistent with known limitations of this method when used as an indicator of viability (23). In contrast, the viability of *M. trichosporium* OB3b cells estimated on the basis of formazan production corresponded well to viability measured as the capacity for methane oxidation after carbon starvation (22). These results indicated that estimates of viability based on plate counts were questionable for *M. trichosporium* OB3b; instead, the XTT assay appeared useful as an alternative. With formazan production as an indicator of viability, the initial response of *M. trichosporium* OB3b to carbon deprivation suggests that also methanotrophs may survive extended periods (weeks) in the absence of exogenous carbon substrates.

In summary, the present study shows that the XTT assay can be used as a simple colorimetric indicator of metabolic activity and as a tool for estimating viability in cultures with respiring bacteria. Careful comparison of XTT reduction with viability estimates obtained with other methods (e.g., plate counts) can also be potentially helpful in investigations of viable (i.e., metabolically active) but nonculturable bacteria (e.g., 11, 16, 23, 26). In environmental studies, XTT can potentially substitute for some tetrazolium salts used previously to quantify overall ETS activity (e.g., 3, 6, 13, 29). Further applications of the XTT assay in environmental and culture studies can thus alleviate some problems associated with the use of electron-coupling agents and tetrazolium salts with water-insoluble formazan products.

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