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Improved Membrane Filtration Method Incorporating Catalase and Sodium Pyruvate for Detection of Chlorine-Stressed Coliform Bacteria†

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In vitro pure culture studies were conducted on three different strains of *Escherichia coli* (K-12, EPA 00244, and SWEI) to determine the effect of chlorination on catalase activity. In each case, stationary-phase cells exhibited significant ($P < 0.001$) reductions in enzyme activity following exposure to chlorine. Mean differences in activity between control and chlorine-stressed cells ranged from 8.8 to 20.3 U/mg of protein for *E. coli* SWEI and EPA 00244, respectively. Following initial enzyme studies, resuscitation experiments utilizing the membrane filtration technique were conducted on chlorinated sewage effluent. Five different amendments, including catalase (1,000 U per plate), heat-inactivated catalase (1,000 U per plate), sodium pyruvate (0.05%), a catalase-sodium pyruvate combination (1,500 U/0.01%), and acetic acid (0.05%), were tested for the ability to enhance detection of chlorine-stressed cells on M-fecal coliform (M-FC), mT7, M-Endo, and tryptone-glucose-yeast extract (TGY) media. Significant ($P < 0.001$) increases in recovery of fecal coliforms on M-FC, total coliforms on mT7 and M-Endo, and total heterotrophs on TGY were obtained on plates containing catalase, pyruvate, or the combination of these compounds. Supplementation with heat-inactivated catalase and acetic acid did not improve recovery of chlorine-stressed cells compared with recovery on nonamended media. Subsequent analysis of colonies from plates containing compounds which enhanced recovery indicated coliform verification percentages of >80% on M-FC, >90% on mT7, and >94% on M-Endo media. These data suggest that the addition of peroxide-degrading compounds to various standard recovery media may improve detection of both coliform and heterotrophic bacteria in chlorinated waters.

Chlorination is the most widely used method in the United States for disinfection of potable waters and municipal sewage discharges. Coliform bacteria are employed as indicator organisms to monitor chlorinated waters, since their detection may signify the presence of pathogens. It is well recognized that coliforms subjected to chlorine may become stressed, resulting in a reduced ability of these injured bacteria to proliferate on selective media employed for their detection (7, 10, 24, 33). Failure to detect coliforms can lead to an overly optimistic estimate of the safety of a water when, in essence, pathogens may still exist.

The inability to recover chlorine-injured coliforms on recommended M-fecal coliform (M-FC) and M-Endo media has been attributed, in part, to various surface-active ingredients in each medium (6, 13). Normally, indicator organisms are resistant to surfactants; however, damage to the cell membrane following chlorine exposure may result in an increased sensitivity to these compounds. Moreover, once membrane systems are damaged, chlorine may attack other components of the cell, including several enzymes and nucleic acids (20, 21). Reductions in oxygen uptake and respiration, cellular ATP concentration, and oxidative phosphorylation have also been shown to occur in *Escherichia coli* as the result of chlorine-damaged membranes (5, 10, 20).

Because of the location of certain enzymes involved in aerobic respiration, the possibility exists that these specialized proteins will be targets of chlorine attack. Several stress conditions reduce catalase activity in *E. coli* (8a, 9, 29, 30). Although numerous methods to detect chlorine-stressed coliforms from water have been developed, few of these modified techniques were designed with respect to chlorine damage of the catalase enzyme. A reduction in catalase activity may afford a secondary stress to bacteria, especially if the cells are forced to grow under aerobic incubation conditions. One consequence of reduced catalase activity may be the accumulation of toxic hydrogen peroxide (H_2O_2), to which injured bacteria apparently become increasingly sensitive (30, 35). The exogenous addition of catalase or nonenzyme peroxide-degrading compounds, such as sodium pyruvate, to various selective media has been reported to increase detection of stressed cells of *E. coli*, *Salmonella* spp., and coliform bacteria (8a, 25, 32). In these studies, the main function of pyruvate was to degrade H_2O_2, as opposed to functioning as an additional carbon source for the cells. Accordingly, the objectives of this study were to (i) investigate the effect of chlorine on catalase activity of *E. coli* in pure culture, (ii) assess the effectiveness of catalase and sodium pyruvate as amendments to standard recovery media for the detection of coliform and heterotrophic bacteria from chlorinated sewage effluents, and (iii) determine whether these amendments promote the growth of noncoliforms on selective media.

MATERIALS AND METHODS

Test organisms and cell preparation. *E. coli* EPA 00244 and K-12 (ATCC 10798) from departmental stock cultures and *E. coli* SWEI, an environmental isolate provided by S. M. Walsh (Westfield State College, Westfield, Mass.), were maintained at 4°C on nutrient agar (Difco Laboratories,

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Detroit, Mich.). Except where otherwise noted, all media were obtained from BBL Microbiology Systems, Cockeysville, Md.

Strains were transferred to 100 ml of Trypticase soy broth (TSB) and incubated on a rotary shaker (120 rpm) at 35°C for 18 to 24 h. One milliliter was serially diluted in 0.1% peptone (Difco) buffer, transferred to fresh TSB, and incubated for 18 h as described above. Following incubation, four 20-ml samples were harvested by centrifugation at 3,020 × *g* for 10 min and washed three times in 19 mM KH_2PO_4 buffer, pH 6.7, and the pellets were suspended in 10 ml of the same buffer. Two pellets were combined to yield separate 20-ml cell suspensions for subsequent use as control and chlorine-stressed cultures. All KH_2PO_4 buffer was rendered a chlorine demand-free buffer (CDFB) according to procedures outlined elsewhere (1).

Chlorine injury. Twenty milliliters of washed 18-h cultures was added to 480 ml of sterile CDFB and mixed for 1 to 2 min. A chlorine stock solution prepared fresh daily from dilution of commercially purchased Clorox (5.25% sodium hypochlorite) was volumeterically added to the buffer-cell mixture at desired concentrations (0.75 to 1.89 mg of chlorine as sodium hypochlorite per liter), and cells were stressed for 7.5 or 8.0 min with constant stirring. The reaction was terminated by the addition of 10 ml of 20% $Na_2S_2O_3$, with subsequent agitation for 10 min. A 250-ml sample was removed from the flasks, and cells were recovered by centrifugation at 3,020 × *g* for 15 min. Pellets were suspended in 25 ml of 0.05 M potassium phosphate buffer (9:1, K_2HPO_4-KH_2PO_4) containing 0.1 mM EDTA, pH 7.8. Control cells were exposed to CDFB under identical conditions and were harvested similarly. The chlorine exposure protocol provided consistently >90% injury and an average 3- to 4-log reduction in cell viability. Exposure concentrations of chlorine were calculated on the basis of a 5.25% solution of sodium hypochlorite. The assumption was made, on the basis of the studies of Fair et al. (18), that at least 85% of the free chlorine in CDFB was in the hypochlorous form at pH 7.0 Since the intention of this study was not to investigate the species of chlorine responsible for injury but rather to attain a cell injury level of at least 90%, no attempts were made to determine total or free chlorine levels at any time during exposure of cells.

Catalase activity measurement. Cells were disrupted by sonication utilizing a microtip-equipped Branson sonifier (model S110; Branson Instruments Inc., Stamford, Conn.) with intermittent application (30-s pulse, 30-s cooling cycle) at a power output of 7 A for 4 to 6 min in an ice-salt bath. Cell debris was removed by centrifugation at 30,000 × *g* for 1 h at 4°C. Extracts were dialyzed against 0.05 M potassium phosphate buffer (3:2, K_2HPO_4-KH_2PO_4), pH 7.0, for 18 to 24 h. One buffer change was made in each flask during the dialysis period. Dialyzed cell extracts were held at 4°C prior to catalase and protein analyses.

Cell extracts were assayed for total protein content according to the procedure of Lowry et al. (28) utilizing a commercially purchased diagnostic kit (Sigma Chemical Co., St. Louis, Mo.). Colorimetric measurements were made at 750 nm. Bovine serum albumin (Sigma) was employed as the standard. Catalase activity was determined according to the procedure described by Beers and Sizer (4). The decomposition of an ~30 mM solution of reagent-grade 30% H_2O_2 was followed directly by the decrease in A_{240}. Catalase activities of nonstressed (CDFB control) versus chlorine-stressed cultures were statistically compared by a one-tailed Student *t* test (14).

Medium amendment analyses. Initial experiments were conducted to determine the effect of addition of various concentrations of catalase and sodium pyruvate to selective and nonselective media on the recovery of coliform and heterotrophic bacteria from chlorinated sewage effluent. Tryptone-glucose-yeast extract (TGY) agar was used as the nonselective medium for the recovery of heterotrophic plate count (HPC) bacteria. Selective media for total and fecal coliforms included Standard Methods (1) M-Endo and M-FC broth, supplemented with 1.5% agar, and mT7 agar (Difco) (22). Some media were amended with catalase, sodium pyruvate, or both. Crystallized bovine liver catalase (Sigma) was dissolved in 0.05 M potassium phosphate buffer (3:2, K_2HPO_4-KH_2PO_4), pH 7.0, to desired concentrations. Enzyme solutions were filter sterilized with a 0.22-μm-pore-size membrane filter (GA-8; Gelman Sciences, Ann Arbor, Mich.), and 0.1-ml samples were aseptically transferred to the surfaces of the media to provide 500, 1,000, 2,000, and 4,000 U per plate. Sodium pyruvate (Eastman Kodak Co., Rochester, N.Y.) was added at desired concentrations (0.01, 0.05, 0.25, and 1.0%) directly to TGY and mT7 media prior to autoclaving and to M-Endo and M-FC media prior to boiling. The pHs of the sterile media were adjusted according to the specifications of the manufacturer. Media lacking supplements were designated nonamended controls.

Chlorinated sewage samples were collected from the effluent of the Morgantown Sewage Treatment Plant in sterile 2-liter Nalgene bottles containing 1 ml of 20% $Na_2S_2O_3$. Serial dilutions were prepared in 0.1% peptone buffer. Levels of coliform and HPC bacteria were determined by the standard membrane filtration technique (1) with type HA 0.45-μm-pore-size membrane filters (Millipore Corp., Bedford, Mass.). Filters were transferred to the surfaces of various media, and plates were incubated at 35°C (TGY, mT7, and M-Endo) or 44.5°C (M-FC) for 24 h prior to colony enumeration.

Data from initial experiments are presented as fold-increase values calculated from the following equation: fold increase = (mean CFU on amended media)/[mean CFU on nonamended (control) media]. Fold-increase values were utilized to evaluate the efficacy of various concentrations of amendments to recover target organisms on respective media. For each medium, comparisons were made between plate counts from nonamended (control) media versus amended (catalase and pyruvate) media by a one-tailed Student *t* test (14).

Enhanced-recovery studies. Data on compounds and concentrations which provided optimal recovery of chlorine-stressed bacteria were employed in subsequent studies for additional analysis of chlorinated sewage effluents. Media (nonamended and amended) for recovery of coliforms and HPC bacteria were as described above. In addition to these supplements, heat-denatured catalase (60°C for 15 min) and acetic acid (Fisher Scientific Co., Pittsburgh, Pa.) were utilized and served as controls for the active enzyme and sodium pyruvate, respectively. The degradation of H_2O_2 through oxidative decarboxylation of an α-keto acid such as pyruvic acid results in the formation of acetic acid and CO_2 as end products (16, 17). Addition of acetic acid to media and subsequent pH adjustment were as described above for sodium pyruvate. Chlorinated sewage samples were processed by the membrane filtration technique, and colonies were enumerated after a 24-h incubation at appropriate temperatures. Plate count data were analyzed by a one-way analysis of variance (14). Differences between cell recovery

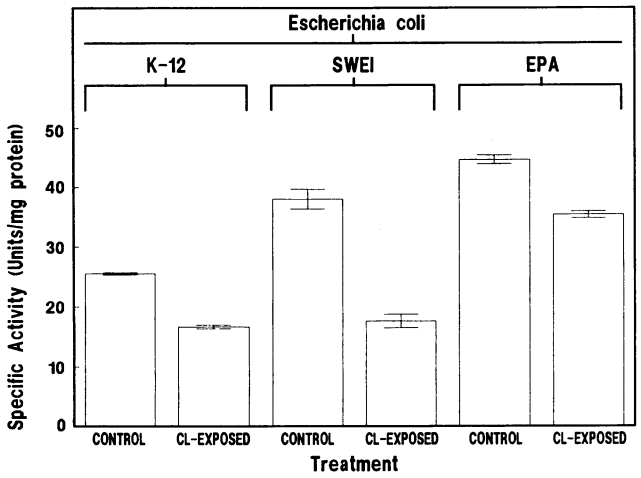

FIG. 1. Effect of chlorination on catalase activity of 18-h cultures of *E. coli* K-12, SWEI, and EPA 00244. Treatment: CONTROL, cells exposed to sterile CDFB, pH 6.7; CL-EXPOSED, cells exposed to 0.75 to 1.89 mg of chlorine as sodium hypochlorite (5.25%) per liter of CDFB, pH 6.7. Histograms represent the means of six determinations; bars represent 1 standard error of the mean.

on plates within each medium were determined by Duncan's new multiple range test (14).

All blue colonies on M-FC medium, representing presumptive fecal coliforms, were picked from filters of at least one replicate plate and inoculated simultaneously into tubes of brilliant green lactose bile broth and EC broth for verification. Similarly, all colonies producing a yellow color on mT7 and a metallic green sheen on M-Endo media were chosen from at least one replicate plate for total coliform verification in lauryl sulfate broth and brilliant green lactose bile broth. Tubes were incubated for 24 to 48 h under appropriate temperatures and scored positive when growth and gas formation were observed (1). Tubes exhibiting growth but no gas formation were scored negative for coliforms, and bacteria were identified by the API 20E system (Analytab Products, Plainview, N.Y.).

RESULTS

Catalase activity. Without exception, all *E. coli* strains exposed to chlorine exhibited significant ($P < 0.001$) reductions in the specific activity of catalase compared with activity in their respective controls (Fig. 1). *E. coli* SWEI was the most sensitive to chlorine, as indicated by a difference in the specific activities of control and chlorine-exposed cells of 20.3 U/mg of protein. In contrast, the calculated difference between activities of control and stressed cells of *E. coli* EPA 00244 and K-12 were 8.8 and 9.3 U/mg of protein, respectively.

Optimal recovery with catalase and pyruvate supplements. On all media, at least one concentration of catalase provided statistically better ($P < 0.001$) recovery of target organisms from chlorinated sewage effluent than the controls did (Table 1). Increased recovery of HPC bacteria on TGY was observed for all amended plates except those containing 4,000 U of catalase, with maximum detection on TGY containing 1,000 U of catalase. Similarly, fecal and total coliforms benefitted most by addition of 1,000 U of catalase to M-FC and M-Endo media, respectively. Amendments of 1,000 and 2,000 U of catalase provided optimal recovery of total coliforms on mT7 medium. The catalase concentration of 1,000 U per plate was used for all media in subsequent studies.

TABLE 1. Effect of various catalase concentrations in recovery media on detection of coliform and heterotrophic bacteria from chlorinated sewage effluent

Catalase (U/plate)	Fold increase (mean ± SEM)[a] in:			
	TGY	M-FC	mT7	M-Endo
500	$1.6 \pm 0.1^{b,c*}$	1.2 ± 0.2^{b}	2.2 ± 0.2^{b}	1.4 ± 0.1^{b}
1,000	$3.2 \pm 0.1^{c*}$	$2.0 \pm 0.1^{c*}$	$3.1 \pm 0.1^{c*}$	$1.8 \pm 0.1^{c*}$
2,000	$1.8 \pm 0.1^{c*}$	$1.6 \pm 0.1^{b,c*}$	$3.5 \pm 0.1^{c*}$	1.3 ± 0.1^{b}
4,000	1.2 ± 0.1^{b}	$1.6 \pm 0.1^{b,c*}$	$2.3 \pm 0.1^{b,c}$	1.4 ± 0.1^{b}

[a] Calculated by dividing each CFU/100 ml on the catalase-amended medium by the mean CFU/100 ml on the respective control medium. Values used in calculations were from at least two experiments with at least three replicate plates. A value of 1.0 indicates no difference in plate counts compared with those of the nonamended control. *, Values within each column are statistically different ($P < 0.001$) from those of the control medium.

[b,c] Values within the same column followed by the same superscript letter are not statistically different ($\alpha = 0.01$), as determined by Duncan's new multiple-range test.

One or more sodium pyruvate concentrations for all media permitted enhanced ($P < 0.001$) recovery of chlorine-stressed organisms compared with recovery from nonamended media (Table 2). In TGY medium, all pyruvate levels except 1.0% provided a 1.7-fold increase of HPC bacteria. The highest recovery of fecal and total coliform bacteria on M-FC and mT7 media was observed with plates containing 0.05% pyruvate, whereas 0.01 and 0.05% amendments provided optimal recovery on M-Endo medium. Conversely, supplementation of M-Endo with 1.0% pyruvate resulted in a decreased detection of total coliforms compared with the control. A concentration of 0.05% pyruvate was utilized for all media in subsequent experiments.

Nine separate combinations of various catalase-sodium pyruvate concentrations were tested in M-FC medium. With the exception of plates containing 500 U of catalase plus 0.05% pyruvate, all combinations provided statistically better ($P < 0.001$) recovery of chlorine-stressed fecal coliforms than the control medium did (Table 3). Maximal recovery was exhibited on plates containing 1,500 U of catalase plus 0.01% pyruvate, which was chosen as a supplement to all other media in subsequent experiments.

Comparative recovery studies with supplemented media. Those concentrations of catalase, sodium pyruvate, and a

TABLE 2. Effect of various sodium pyruvate concentrations in recovery media on detection of coliform and heterotrophic bacteria from chlorinated sewage effluent

Sodium pyruvate (%)	Fold increase (mean ± SEM)[a] in:			
	TGY	M-FC	mT7	M-Endo
0.01	$1.7 \pm 0.1^{b*}$	$2.1 \pm 0.1^{b*}$	2.0 ± 0.1^{c}	$1.7 \pm 0.1^{d*}$
0.05	$1.7 \pm 0.1^{b*}$	$2.7 \pm 0.1^{b*}$	$4.3 \pm 0.1^{b*}$	$1.6 \pm 0.1^{d*}$
0.25	$1.7 \pm 0.1^{b*}$	1.8 ± 0.1^{b}	2.3 ± 0.1^{c}	1.3 ± 0.1^{b}
1.0	1.1 ± 0.1^{c}	0.9 ± 0.1^{c}	$2.4 \pm 0.1^{c*}$	0.3 ± 0.1^{c}

[a] Calculated by dividing each CFU/100 ml on the pyruvate-amended medium by the mean CFU/100 ml on the respective control medium. Values used in calculations were from at least two experiments with at least three replicate plates. A value of 1.0 indicates no difference in plate counts compared with those of the nonamended control. *, Values are statistically different ($P < 0.001$) from those of the control medium.

[b,c,d] Values within the same column followed by the same superscript letter are not statistically different ($\alpha = 0.01$), as determined by Duncan's new multiple-range test.

TABLE 3. Effect of various concentrations of a catalase-sodium pyruvate combination in M-FC medium on the recovery of fecal coliforms from chlorinated sewage effluent

Catalase/pyruvate (U/%)	Fold increase (mean ± SEM)[a]
500/0.01	1.22 ± 0.04[b,c*]
500/0.05	1.07 ± 0.04[b,d]
500/0.1	1.29 ± 0.04[c,e*]
1,000/0.01	1.52 ± 0.04[f,g*]
1,000/0.05	1.24 ± 0.04[b,c,e*]
1,000/0.1	1.57 ± 0.04[f,g*]
1,500/0.01	1.66 ± 0.04[g*]
1,500/0.05	1.44 ± 0.08[e,f*]
1,500/0.1	1.56 ± 0.04[f,g*]

[a] Calculated by dividing each mean CFU/100 ml on the catalase-pyruvate-amended medium by the mean CFU/ml on the control medium. Values used in calculations were from six replicate plates. A value of 1.0 indicates no difference in plate counts compared with those of the nonamended control. *, Values are statistically different ($P < 0.001$) from those of the control medium. [b,c,d,e,f,g] Values followed by the same superscript letter are not statistically different ($\alpha = 0.01$), as determined by Duncan's new multiple-range test.

combination of both amendments which provided optimal recovery of bacteria were further evaluated with chlorinated sewage effluent. Recovery of HPC bacteria was statistically better ($P < 0.001$) on plates containing catalase, pyruvate, and the combination of both compounds (Fig. 2). No difference between the recovery on the control medium and that on medium amended with heated catalase or acetic acid was exhibited. Catalase addition provided optimal recovery of chlorine-stressed HPC bacteria (5.8×10^4 CFU/100 ml).

On M-FC media containing catalase, pyruvate, or both, recovery was statistically greater ($P < 0.001$) than that of other amendments tested and the control (Fig. 2). Plates amended with catalase provided the highest recovery of fecal coliforms (1.3×10^2 CFU/100 ml).

A significant increase ($P < 0.001$) in recovery of total coliforms on mT7 and M-Endo media was observed with plates amended with catalase, pyruvate, or both (Fig. 2). The addition of pyruvate to mT7 medium and catalase to M-Endo medium provided optimal recovery of chlorine-stressed cells, with mean plate counts of 6.6×10^3 and 1.2×10^4 CFU/100 ml, respectively. Overall, M-Endo medium proved superior to mT7 medium for the recovery of total coliform bacteria from chlorinated sewage.

Effect of supplements on coliform verification. On M-FC medium, verification percentages of blue colonies picked from plates containing supplements, with the exception of those containing acetic acid, were at least equal to those of the control (Table 4). A minimum of one colony from each plate which did not produce gas in EC broth was subjected to

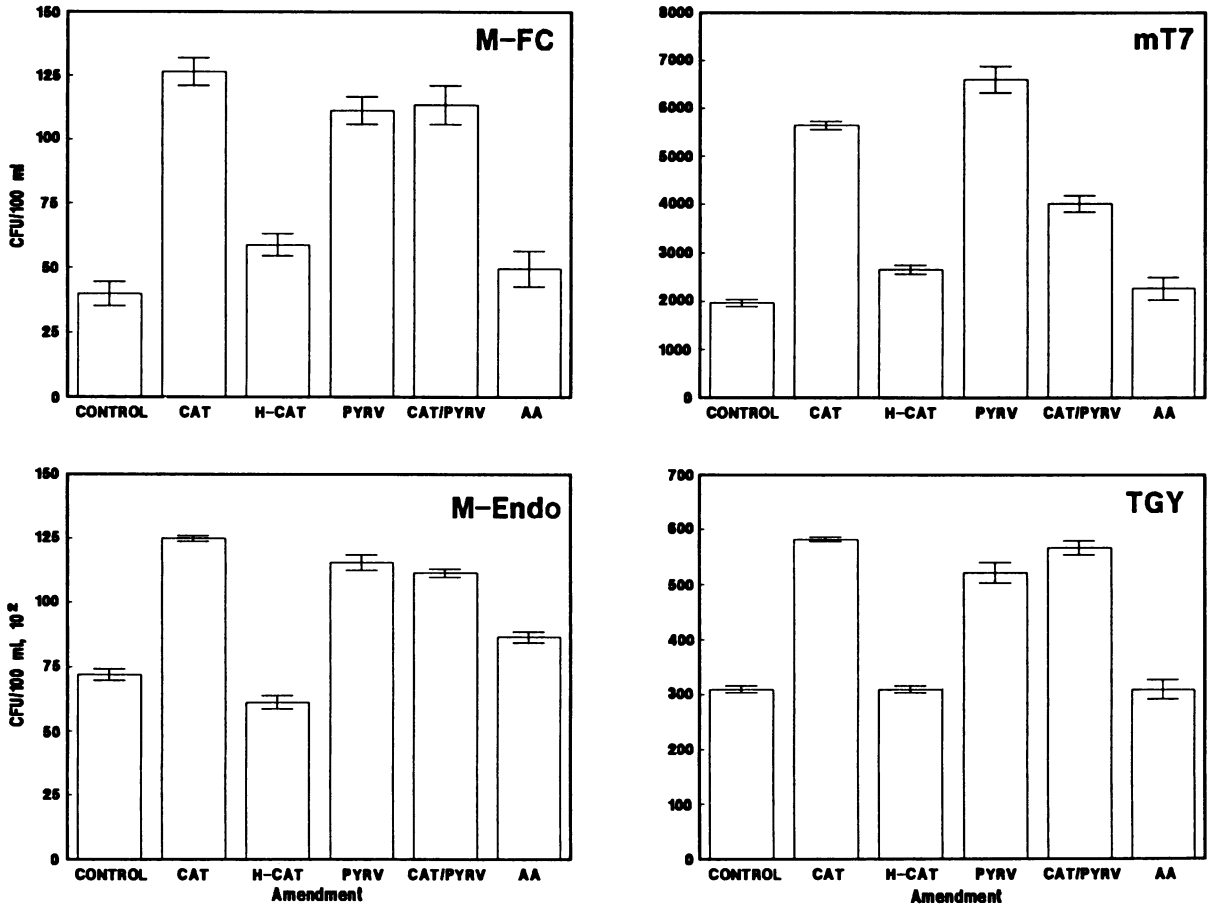

FIG. 2. Recovery of coliform and heterotrophic bacteria from chlorinated sewage effluent. Media: M-FC, fecal coliforms; mT7 and M-Endo, total coliforms; TGY, total heterotrophs. Amendments: CONTROL, nonamended; CAT, catalase (1,000 U); H-CAT, heated catalase (1,000 U); PYRV, sodium pyruvate (0.05%); CAT/PYRV, catalase (1,500 U) and sodium pyruvate (0.01%); AA, acetic acid (0.05%). Histograms represent the means of counts from four replicate plates; bars represent 1 standard error of the mean.

TABLE 4. Effect of addition of various compounds to M-FC, mT7, and M-Endo media on the verification percentage of coliform bacteria recovered from chlorinated sewage effluent

Amendment[a]	% Verification[b] in:		
	M-FC[c]	mT7[d]	M-Endo[d]
CONTROL	77	90	97
CAT	83	95	97
H-CAT	82	95	75
PYRV	84	90	97
CAT/PYRV	81	95	94
AA	62	85	94

[a] CONTROL, Nonamended; CAT, catalase (1,000 U); H-CAT, heated catalase (1,000 U); PYRV, sodium pyruvate (0.05%); CAT/PYRV, catalase (1,500 U) and sodium pyruvate (0.01%); AA, acetic acid (0.05%).

[b] Calculated by dividing the number of tubes scored positive in a verification medium (see Materials and Methods) by the total sample size and multiplying by 100.

[c] Medium for recovery of fecal coliform bacteria.

[d] Medium for recovery of total coliform bacteria.

identification with the API 20E system. All 11 randomly chosen colonies were identified as total coliform bacteria. *Enterobacter cloacae* was identified only once (from the control medium), whereas the remaining 10 colonies were *Citrobacter freundii*. The recovery of *C. freundii* was consistent on all M-FC plates and was not associated with any particular amendment.

Of the yellow colonies chosen from mT7 medium, 90% or more were verified as total coliforms, except on plates containing acetic acid, which had 85% verification (Table 4). On mT7 plates containing supplements which provided enhanced recovery of cells, the verification was not less than that calculated for the control medium. Identification of 11 colonies randomly picked from all plates which did not produce gas in brilliant green lactose bile broth was performed. Ten of the isolates were identified as *Klebsiella oxytoca*. The remaining isolate was identified as *Serratia oderifera* and was removed from mT7 plates containing pyruvate.

Verification of green-sheen colonies on all plates of M-Endo medium was 94% or better, except on plates containing heated catalase (Table 4). Eight colonies were chosen for identification because no gas production was observed in brilliant green lactose bile broth. Four isolates were identified as *K. oxytoca*, and three were identified as *C. freundii*, and there was a single isolate of an *Aeromonas* sp.

DISCUSSION

Coliform bacteria are primary indicator organisms employed for analysis of treated waters; therefore, it is essential that a method or medium provide optimal detection of both noninjured and chlorine-injured cells (34). Exposure to chlorine often renders a portion of the bacterial population injured, resulting in the inability of these indicator organisms to be recovered on recommended media (10). Several investigators have shown that supplementation of various selective media with catalase, pyruvate, or both can improve recovery of heat- and freeze-stressed *E. coli* (30, 32, 35), "*Salmonella senftenberg*" (37), *Staphylococcus aureus* (2, 8, 11, 31), and coliforms (25) which have presumably sustained damage to the catalase enzyme necessary for degrading H_2O_2 formed during growth. However, the main objectives of these studies were related to concerns associated with areas of food microbiology employing the standard pour- or surface-plating technique as the enumeration procedure. No information which addresses the effect of chlorine stress on catalase levels in coliform bacteria associated with treated waters exists. In this paper, we propose a method by which detection of chlorine-stressed coliform and HPC bacteria can be enhanced by incorporation of catalase, sodium pyruvate, or both into recovery media. This approach was based on the hypothesis that chlorine, which attacks the bacterial membrane, will likewise affect catalase enzyme activity in the cell. The result is a debilitated state which would restrict proliferation of cells on selective media while functioning under an aerobic metabolism.

Significant reductions in catalase activity were exhibited for three different strains of chlorine-stressed *E. coli* (Fig. 1). Catalase is a tetramer consisting of separate single polypeptide chains that associate with a prosthetic group (27, 43). Chlorine may oxidize either the protein portion of the enzyme molecule or the heme groups of the protoporphyrin IX prosthetic group. Oxidation of the protein structures can result in lower specific activity but not a complete loss of enzyme function. This condition may explain only reduced catalase activity in chlorine-stressed cells as the result of partial inactivation of the enzyme. Several investigators have suggested that the oxidation state of the heme prosthetic group plays a key role in catalase activity (3, 12, 22). If chlorine affected the oxidation state of the iron atom, then either temporary or permanent reduction in catalase activity would be expressed in stressed cells.

Reduced catalase activity in chlorine-stressed *E. coli* suggests that H_2O_2 toxicity may play a role in low recovery of indicator organisms from water. Addition of catalase or pyruvate significantly ($P < 0.001$) enhanced recovery of both coliform and heterotrophic bacteria from chlorinated sewage effluent when incorporated into M-FC, M-Endo, mT7, and TGY media. Similarly, supplementation of M-FC medium with a catalase-pyruvate combination significantly ($P < 0.001$) increased recovery of fecal coliforms.

Incorporation of catalase or pyruvate into standard media required minimal alterations of standard coliform detection procedures to enhance recovery of stressed cells. Other proposed methods to increase coliform recovery often involve extra procedural steps, including (i) preenrichment in nonselective broth or on minimal media with acclimation at reduced incubation temperatures (26, 41), (ii) a nonselective-selective medium overlay technique (39), (iii) preincubation on dilute selective media (19), and (iv) elimination of selective components from recovery media (36). It is difficult to accurately compare the present data with those of other workers who modified procedures to increase fecal-coliform recovery from chlorinated sewage, since all municipal waste effluents differ from one treatment plant to another. Although other methods have proven effective, the present method has the advantage of being simple, rapid, and accurate.

If enhanced recovery of stressed cells was not a function of H_2O_2 toxicity but rather was the result of increased substrate availability upon addition of catalase or pyruvate, then recovery on media containing compounds devoid of peroxide-degrading capability should produce similar results. However, data indicate that recovery on all media containing catalase, pyruvate, or both was significantly better than recovery on control (nonamended) media or on media supplemented with inactivated enzyme or acetic acid. Furthermore, if the added-substrate hypothesis were true, then equal recovery of cells would be most evident on nonselective TGY medium which can support growth of various heterotrophic bacteria. However, colony counts on

plates containing heated catalase or acetic acid were not different from those on control plates.

Supplementation of M-FC, mT7, and M-Endo media with catalase, sodium pyruvate, or both resulted in significantly better recovery of coliform bacteria from chlorinated sewage effluent. Subsequent verification of coliforms from these plates compared well with that from the control, suggesting that addition of these compounds did not promote the growth of noncoliform bacteria.

Our findings indicate that M-Endo medium provided better overall detection of total coliforms than mT7 medium did (Fig. 2). In contrast, McFeters et al. (34) and LeChevallier et al. (23) demonstrated that mT7 medium was superior to M-Endo medium for analysis of potable water containing primarily free chlorine as the active form of disinfectant. No attempts were made in our study to measure the species of chlorine present in the treated sewage, but the presence of organic matter in domestic sewage suggests that combined chlorine, such as chloramine, should compose a major portion of the chlorine species. Our data parallel those of Watters et al. (44) and Rice et al. (38), who observed better recovery with M-Endo-type media than with mT7 medium when enumerating chloramine-stressed coliforms. A second explanation of the better recovery on M-Endo medium may be related to the apparent ability of this medium to degrade H_2O_2 (J. P. Calabrese, Ph.D. dissertation, West Virginia University, Morgantown, 1988). The occurrence of H_2O_2 in surface waters is well documented (40, 42, 45). Several investigators have reported that high concentrations of H_2O_2 are characteristic of waters containing appreciable levels of organics and exposed to sunlight (12, 15). Therefore, in addition to oxidative cellular damage due to chlorine exposure, water samples containing H_2O_2 may have afforded a secondary stress to injured coliforms. Hydrogen peroxide would be lost on M-Endo medium but persist on mT7 medium lacking peroxide-degrading compounds.

The existence of injured coliform bacteria in chlorinated waters presents an ongoing challenge to microbiologists attempting to detect these indicator organisms with currently accepted methodologies. We introduce a technique by which enhanced recovery of both coliform and HPC bacteria from chlorinated sewage can be attained by incorporation of catalase, sodium pyruvate, or both into standard recovery media. This is one of the first reports on utilization of these compounds in conjunction with the membrane filtration procedure for detection of injured indicator bacteria from aquatic environments.

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LITERATURE CITED

1. **American Public Health Association.** 1985. Standard methods for the examination of water and wastewater, 16th ed. American Public Health Association, Washington, D.C.
2. **Baird-Parker, A. C., and E. Davenport.** 1965. The effect of recovery medium on isolation of *Staphylococcus aureus* after heat treatment and after storage of frozen dried cells. J. Appl. Bacteriol. **28**:390–402.
3. **Barlow, G. H., and E. Margoliash.** 1969. Sedimentation studies on catalase complex II using photoelectric scanning optics. Biochim. Biophys. Acta **188**:159–161.
4. **Beers, R. F., and I. W. Sizer.** 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. **195**:133–140.
5. **Berg, J. D., P. V. Roberts, and A. Matin.** 1986. Effect of chlorine dioxide on selected membrane functions of *Escherichia coli*. J. Appl. Bacteriol. **60**:213–220.
6. **Bissonnette, G. K., J. J. Jezeski, G. A. McFeters, and D. G. Stuart.** 1975. Influence of environmental stress on enumeration of indicator bacteria from natural waters. Appl. Microbiol. **29**:186–194.
7. **Braswell, J. R., and R. A. McCleod.** 1974. Recovery of *Escherichia coli* from chlorinated secondary sewage. Appl. Microbiol. **28**:328–329.
8. **Brewer, D. G., S. E. Martin, and Z. J. Ordal.** 1977. Beneficial effects of catalase or pyruvate in a most-probable-number technique for the detection of *Staphylococcus aureus*. Appl. Environ. Microbiol. **34**:797–800.

8a. **Calabrese, J. P., and G. K. Bissonnette.** 1990. Improved detection of acid mine water stressed coliform bacteria on media containing catalase and sodium pyruvate. Can. J. Microbiol. **36**:544–550.

9. **Calcott, P. H., and R. A. McCleod.** 1975. The survival of *Escherichia coli* from freeze-thaw damage: the relative importance of wall and membrane damage. Can. J. Microbiol. **21**: 1960–1968.
10. **Camper, A. K., and G. A. McFeters.** 1979. Chlorine injury and the enumeration of waterborne coliform bacteria. Appl. Environ. Microbiol. **37**:633–641.
11. **Collins-Thompson, D. L., A. Hurst, and B. Aris.** 1974. Comparison of selective media for the enumeration of sublethally heated food poisoning strains of *Staphylococcus aureus*. Can. J. Microbiol. **20**:1072–1075.
12. **Cooper, W. J., and G. Zika.** 1983. Photochemical formation of hydrogen peroxide in surface and ground waters exposed to sunlight. Science **220**:711–712.
13. **D'Mello, A., and W. M. Yotis.** 1987. The action of sodium deoxycholate on *Escherichia coli*. Appl. Environ. Microbiol. **53**:1944–1946.
14. **Dowdy, S., and S. Wearden.** 1983. Statistics for research. John Wiley & Sons, Inc., New York.
15. **Drapper, W. M., and D. G. Crosby.** 1983. The photochemical generation of hydrogen peroxide in natural waters. Arch. Environ. Contam. Toxicol. **12**:121–126.
16. **Elstner, E. F., and A. Heupel.** 1973. On the decarboxylation of α-keto acids by isolated chloroplasts. Biochim. Biophys. Acta **325**:182–188.
17. **Elstner, E. F., and A. Heupel.** 1976. Formation of hydrogen peroxide by isolated cell walls from horseradish (*Armoracia lapathifolia* Gilib.). Planta **130**:175–180.
18. **Fair, G. M., J. C. Morris, S. L. Chang, I. Weil, and R. J. Burden.** 1948. The behavior of chlorine as a water disinfectant. J. Am. Water Works Assoc. **40**:1051–1061.
19. **Green, B. L., E. M. Clausen, and W. Litsky.** 1977. Two-temperature membrane filter method for enumerating fecal coliform bacteria from chlorinated effluents. Appl. Environ. Microbiol. **33**:1259–1264.
20. **Haas, C. N., and R. S. Engelbrecht.** 1980. Physiological alterations of vegetative microorganisms resulting from chlorination. J. Water Pollut. Control Fed. **52**:1976–1989.
21. **Hoyana, Y., V. Bacon, R. E. Summons, W. E. Pereira, B. Helpern, and A. M. Duffield.** 1973. Chlorination studies. IV. Reactions of aqueous hypochlorous acid with pyrimidine and purine bases. Biochem. Biophys. Res. Commun. **53**:1195–2001.
22. **Jones, P., and A. Suggett.** 1968. The catalase-hydrogen peroxide system. Biochem. J. **110**:621–629.
23. **LeChevallier, M. W., S. C. Cameron, and G. A. McFeters.** 1983. New medium for improved recovery of coliform bacteria from drinking water. Appl. Environ. Microbiol. **45**:484–492.
24. **LeChevallier, M. W., and G. A. McFeters.** 1985. Enumerating injured coliforms in drinking water. J. Am. Water Works Assoc. **77**:81–87.
25. **Lee, R. M., and P. A. Hartman.** 1989. Optimal pyruvate concentrations for recovery of coliforms from food and water. J.

Food Protect. **52**:119–121.
26. **Lin, S. D.** 1973. Evaluation of coliform tests for chlorinated secondary effluents. J. Water Pollut. Control Fed. **45**:498–506.
27. **Loewen, P. C., and J. Switala.** 1986. Purification and characterization of catalase HPII from *Escherichia coli* K12. Biochem. Cell Biol. **64**:638–646.
28. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. **193**:265–275.
29. **Mackey, B. M., and C. M. Derrick.** 1986. Peroxide sensitivity of cold-shocked *Salmonella typhimurium* and *Escherichia coli* and its relationship to minimal medium recovery. J. Appl. Bacteriol. **60**:501–511.
30. **Mackey, B. M., and D. A. Seymour.** 1987. The effect of catalase on recovery of heat-injured DNA-repair mutants of *Escherichia coli*. J. Gen. Microbiol. **133**:1601–1610.
31. **Martin, S. E., R. S. Flowers, and Z. J. Ordal.** 1976. Catalase: its effect on microbial enumeration. Appl. Environ. Microbiol. **32**:731–734.
32. **McDonald, L. C., C. R. Hackney, and B. Ray.** 1983. Enhanced recovery of injured *Escherichia coli* by compounds that degrade hydrogen peroxide or block its formation. Appl. Environ. Microbiol. **45**:360–365.
33. **McFeters, G. A., S. C. Cameron, and M. W. LeChevallier.** 1982. The influence of diluents, media, and membrane filters on detection of injured waterborne coliform bacteria. Appl. Environ. Microbiol. **43**:97–103.
34. **McFeters, G. A., J. S. Kippin, and M. W. LeChevallier.** 1986. Injured coliforms in drinking water. Appl. Environ. Microbiol. **51**:1–5.
35. **Mossel, D. A. A., A. Veldman, and I. Eelderink.** 1980. Comparison of the effects of liquid medium repair and the incorporation of catalase in MacConkey type media on the recovery of Enterobacteriaceae sublethally stressed by freezing. J. Appl. Microbiol. **49**:405–419.
36. **Presswood, W. G., and D. K. Strong.** 1978. Modification of M-FC medium by eliminating rosolic acid. Appl. Environ. Microbiol. **36**:90–94.
37. **Rayman, M. K., B. Aris, and H. B. Elderea.** 1978. The effect of compounds which degrade hydrogen peroxide on the enumeration of heat-stressed cells of *Salmonella senftenberg*. Can. J. Microbiol. **24**:883–885.
38. **Rice, E. W., K. R. Fox, H. D. Nash, E. J. Read, and A. P. Smith.** 1987. Comparison of media for recovery of total coliform bacteria from chemically treated water. Appl. Environ. Microbiol. **53**:1571–1573.
39. **Rose, R. E., E. E. Geldrich, and W. Litsky.** 1975. Improved membrane filter method for fecal coliform analysis. Appl. Microbiol. **29**:532–536.
40. **Sinel'niko, V. Y.** 1971. Hydrogen peroxide content of river water and a method for determining it. Hydrobiol. J. **7**:96–99.
41. **Stevens, A. P., R. J. Grasso, and J. E. Delany.** 1974. Measurement of fecal coliforms in estuarine water, p. 132–136. *In* D. S. Wilt (ed.), Proceeding of 8th National Shellfish Sanitation Workshop. Shellfish Sanitation Branch, Food and Drug Administration, U.S. Department of Health, Education, and Welfare, Washington, D.C.
42. **Van Baalen, C., and J. E. Marler.** 1966. Occurrence of hydrogen peroxide in sea water. Nature (London) **211**:951.
43. **Wang, G. I. J., and D. Y. C. Fung.** 1986. Significance of bacterial catalase in food microbiology: a review. J. Food Saf. **8**:47–67.
44. **Watters, S. K., B. H. Pyle, M. W. LeChevallier, and G. A. McFeters.** 1989. Enumeration of *Enterobacter cloacae* after chloramine exposure. Appl. Environ. Microbiol. **55**:3226–3228.
45. **Zika, R., E. Saltzman, W. L. Chameides, and D. D. Davis.** 1982. H_2O_2 levels in rainwater collected in South Florida and the Bahama Islands. J. Geophys. Res. **87**:5015–5017.