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New Medium for the Simultaneous Detection of Total Coliforms and *Escherichia coli* in Water

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A new membrane filter agar medium (MI agar) containing a chromogen, indoxyl-β-D-glucuronide, and a fluorogen, 4-methylumbelliferyl-β-D-galactopyranoside, was developed to simultaneously detect and enumerate *Escherichia coli* and total coliforms (TC) in water samples on the basis of their enzyme activities. TC produced β-galactosidase, which cleaved 4-methylumbelliferyl-β-D-galactopyranoside to form 4-methylumbelliferone, a compound that fluoresced under longwave UV light (366 nm), while *E. coli* produced β-glucuronidase, which cleaved indoxyl-β-D-glucuronide to form a blue color. The new medium TC and *E. coli* recoveries were compared with those of mEndo agar and two *E. coli* media, mTEC agar and nutrient agar supplemented with 4-methylumbelliferyl-β-D-glucuronide, using natural water samples and spiked drinking water samples. On average, the new medium recovered 1.8 times as many TC as mEndo agar, with greatly reduced background counts (≤7%). These differences were statistically significant (significance level, 0.05). Although the overall analysis revealed no statistically significant difference between the *E. coli* recoveries on MI agar and mTEC agar, the new medium recovered more *E. coli* in 16 of 23 samples (69.6%). Both MI agar and mTEC agar recovered significantly more *E. coli* than nutrient agar supplemented with 4-methylumbelliferyl-β-D-glucuronide. Specificities for *E. coli*, TC, and noncoliforms on MI agar were 95.7% (66 of 69 samples), 93.1% (161 of 173 samples), and 93.8% (61 of 65 samples), respectively. The *E. coli* false-positive and false-negative rates were both 4.3%. This selective and specific medium, which employs familiar membrane filter technology is used to analyze several types of water samples, is less expensive than the liquid chromogen and fluorogen media and may be useful for compliance monitoring of drinking water.

Chromogens and fluorogens, substrates that produce color and fluorescence, respectively, upon cleavage by a specific enzyme, have been used for many years to detect and identify coliform bacteria, including the fecal pollution indicator *Escherichia coli* (3, 22, 28). Compounds such as *o*-nitrophenyl-β-D-galactopyranoside (ONPG), *p*-nitrophenyl-β-D-galactopyranoside (PNPG), and 4-methylumbelliferyl-β-D-galactopyranoside (MUGal) have been included in a variety of media (3) to demonstrate the presence of β-galactosidase, an enzyme produced by coliforms, and 4-methylumbelliferyl-β-D-glucuronide (MUG) has been used to detect *E. coli* in milk and dairy products (14, 19), food and shellfish (14, 19, 20, 25, 31, 32, 40), water and wastewater (8, 9, 13, 19, 21, 27, 29, 31, 40), and urine and other clinical samples (7, 23, 24, 27, 35) by means of its β-glucuronidase production. Recently, other chromogens, such as indoxyl-β-D-glucuronide (IBDG) and 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc), have also been used to detect or enumerate *E. coli* in water (26, 40), urine (11), and food (20, 40). Some of the methods utilize chromogens and fluorogens in liquid media in a most-probable-number (or multiple-fermentation-tube) test, presence-absence format, or some other type of tube test, while others use agar media for direct plating or membrane filter (MF) technology.

Drinking water regulations under the Final Coliform Rule (16) state that total coliform (TC)-positive drinking water samples must be examined for the presence of *E. coli* or fecal coliforms. Currently approved MF technology (16) for detecting TC and fecal coliforms uses several different types of media and two different incubation temperatures. The MF test for *E. coli* (17, 29, 39), which uses nutrient agar supplemented with MUG, is a confirmatory test, not a primary isolation medium. The combined procedures (TC test and either fecal coliform or *E. coli* test) can take 28 to 72 h, and the standard most-probable-number fecal coliform test (1, 5) can take up to 72 h. The most recently approved *E. coli* test, (18), Colilert, is a liquid most-probable-number or presence-absence medium that can detect both coliforms and *E. coli* within 24 to 28 h.

The need for a rapid MF method to detect both types of bacteria, the increased cost of drinking water testing under the new regulations, and the potential delay in the detection of fecally contaminated drinking water with currently used methods provided the impetus for research to develop an MF method to simultaneously detect coliforms and *E. coli* within 24 h. Other potential uses, such as recreational water, monitoring source water for potable water, and groundwater, were also considered during this study. In this paper we describe the development and evaluation of a new MF medium, containing the fluorogen MUGal and the chromogen IBDG, that can be used to detect both types of organisms in a variety of water types.

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TABLE 1. Parameters tested during medium development

Parameter or type of compound	Compound used and sources(s)	Concn or levels tested
pH	Not applicable	7.0, 7.5, 8.0
Chromogen or fluorogen	ONPG; Boehringer-Mannheim GmbH, Mannheim, Germany	100, 200, 400, 800, and 1,600 μg/ml
	CPRG, sodium salt; Boehringer-Mannheim GmbH, Mannheim, Germany	10, 20, 40, 80, and 160 μg/ml
	RBDG; Research Organics, Inc., Cleveland, Ohio	10, 25, 50, 100, 200, and 400 μg/ml
	MUGal; Boehringer-Mannheim GmbH, Mannheim, Germany	50, 100, and 200 μg/ml
	IBDG, sodium salt; Marcor Development Corp., Hackensack, N.J., Arthur Ley, Saul Wolfe	50, 100, 200, 250, and 320 μg/ml
	IBDG, cyclohexylammonium salt; Sigma Chemical Co., St. Louis, Mo.	50, 100, 200, 250, 320, 400, 600, and 800 μg/ml
lac operon inducer	IPTG; Bethesda Research Laboratories, Gaithersburg, Md.	0.001 M
Antibiotic	Cefsulodin, sodium salt; Marcor Development Corp., Hackensack, N.J.	2, 3, 4, 5, 7, 10, 15, and 25 μg/ml
Buffer	Tris; Fisher Scientific Co., Fair Lawn, N.J.	0.005, 0.01, and 0.05 M (at pH 8.0)
Medium ingredients	Sodium lauryl sulfate; BDH Biochemicals, Ltd., Poole, United Kingdom	50, 100, and 200 μg/ml
	Sodium desoxycholate; Difco Laboratories, Detroit, Mich.	25, 50, and 100 μg/ml
	β-D-Lactose; Eastman Kodak Co., Rochester, N.Y.	0, 0.5, and 1 g/liter

MATERIALS AND METHODS

Medium development. (i) Bacterial cultures. The four coliform cultures used in the medium development study included *E. coli* ATCC 25922 obtained from Bactrol disks (Difco Laboratories, Detroit, Mich.) and three U. S. Environmental Protection Agency bacterial strains, *E. coli* EPA 206, *Enterobacter aerogenes* EPA 202 (also preserved as ATCC 49701), and *Klebsiella pneumoniae* EPA 207. Other cultures used in this study were *Flavobacterium multivorum*, *Flavobacterium thalophilum*, and two *Aeromonas hydrophila* isolates, all of which were obtained from Eugene Rice, Risk Reduction Engineering Laboratory, U. S. Environmental Protection Agency, Cincinnati, Ohio. All coliforms were characterized by using API 20E strips (Analytab Products, Plainview, N.Y.), and working cultures of all bacteria were maintained on tryptic soy agar (Difco) slants and stabs.

(ii) Parameters examined. A variety of ingredients, chemicals, chromogens, fluorogens, and pH values were examined during the development of the medium for TC and *E. coli*. These materials and parameters are listed in Table 1 along with the concentrations, amounts, and levels tested. The base medium consisted of modified mTEC agar (12, 38) containing a reduced amount of lactose and no pH indicators. Type GN-6 47-mm-diameter MF (pore size, 0.45 μm; Gelman Sciences, Ann Arbor, Mich.) were used for most filtrations. However, in a study of some chromogens, the effect of MF composition on color development and diffusion was examined by using cellulose mixed-ester type GN-6 and Supor polysulfone filters (pore size, 0.45 μm; Gelman), 0.4-μm-pore-size polycarbonate filters (Poretics Corp., Livermore, Calif.), and 0.2-μm-pore-size polycarbonate filters (Nuclepore Corp., Pleasanton, Calif.).

(iii) Final medium formulation. The final medium formulation (MI agar) was prepared by adding the following ingredients to 1 liter of distilled water: proteose peptone no. 3 (Difco), 5.0 g; yeast extract (Difco), 3.0 g; β-D-lactose (Eastman Kodak Co., Rochester, N.Y.), 1.0 g; MUGal (Boehringer-Mannheim Corp., Indianapolis, Ind.), 0.1 g (final concentration, 100 μg/ml); NaCl, 7.5 g; K_2HPO_4, 3.3 g; KH_2PO_4, 1.0 g; sodium lauryl sulfate (BDH Biochemicals, Ltd., Poole, United Kingdom), 0.2 g; sodium desoxycholate (Difco), 0.1 g; and agar (Difco), 15.0 g. After the ingredients were dissolved, the medium was autoclaved for 15 min at 121°C, and the agar was tempered in a 50°C water bath, the following were added to 1 liter of medium: 20 ml of a freshly prepared 16-mg/ml filter-sterilized solution of IBDG (final concentration, 320 μg/ml) and 5 ml of a freshly prepared 1-mg/ml filter-sterilized solution of cefsulodin (Marcor Development Corp., Hackensack, N.J.) (final concentration, 5 μg/ml). Some of the IBDG was obtained from Arthur Ley, Queens University, Kingston, Ontario, Canada, and from Saul Wolfe, Simon Fraser University, Burnaby, British Columbia, Canada, and some was purchased from Marcor Development Corp. The medium was then pipetted into petri dishes (9 by 50 mm; 5 ml per plate), allowed to harden, stored in a refrigerator, and used within 2 weeks. The media used in the recovery studies were freshly prepared on the day before use. Some plates from each batch were put aside and stored at 4°C for later use in a medium storage study. The slight precipitate that formed in the agar upon autoclaving and the crystals that formed upon refrigeration did not affect the performance of the medium.

The substrate MUGal was included in MI agar to detect TC. The β-galactosidase produced by these organisms cleaved the MUGal, producing 4-methylumbelliferone, a compound that fluoresced when it was exposed to longwave UV light (λ, 366 nm). Noncoliforms usually do not produce this enzyme and, hence, did not fluoresce on the medium. The IBDG was used to detect *E. coli*. The β-glucuronidase produced by this organism cleaved the substrate to form a blue color (indigo) in the colonies. Since *E. coli* is also a TC and produces β-galactosidase as well as β-glucuronidase, the blue colonies fluoresced a blue-green color under longwave UV light. Organisms other than *E. coli* rarely produce the blue color. The antibiotic cefsulodin was added to inhibit gram-positive bacteria and some noncoliform gram-negative organisms that can cause false-positive reactions.

Filtration procedure. (i) **Pure cultures.** The four bacterial cultures used for the medium development study were each inoculated into 10-ml tubes containing tryptic soy broth (TSB) (Difco), which were subsequently incubated for 24 to 48 h at 35°C. Each culture was transferred into fresh TSB the day before the experiment and incubated for 24 h at 35°C; 10-fold dilutions of each culture were made in phosphate-buffered dilution water (1, 5), and 1-ml aliquots of the 10^{-7} and 10^{-8} dilutions were filtered through 0.45-μm-pore-size cellulose ester MF. The MF funnels and bases were autoclaved before each experiment and were exposed to germicidal UV light (λ, 254 nm) between filtrations. The filters were placed on 5-ml plates containing MI agar, the standard TC medium, mEndo agar (1, 5), and an *E. coli* medium, mTEC agar (12, 38). The previously described media were incubated at their recommended temperatures for the recommended times (1, 5, 12, 38), and MI agar was incubated at 35°C for 16 to 24 h. Colonies that grew on MI agar were inspected for blue color, fluorescence under longwave UV light (366 nm), both, or neither. For optimal differentiation of fluorescent colonies with a binocular dissecting microscope (magnification, ×10 to ×15), a 4-Watt Blak Ray model UVL-21 longwave UV lamp (UVP, Inc., San Gabriel, Calif.) was placed about 6 in. (15.3 cm) from the plates. Counts were made of each of the following four different types of colonies (if they were present): fluorescent, blue (*E. coli*); nonfluorescent, blue (probable *E. coli*); fluorescent, nonblue (TC other than *E. coli*); and nonfluorescent, nonblue (background or noncoliforms). The number of TC was equal to the total number of fluorescent colonies (i.e., the sum of the number of fluorescent, blue colonies and the number of fluorescent, nonblue colonies). Nonfluorescent, blue colonies, if present, were included in the *E. coli* count because their lack of fluorescence was an artifact due to overcrowding of colonies. Background recoveries were obtained by subtracting the number of fluorescent, nonblue colonies from the total number of nonblue colonies on the same plate, counted under ordinary light, because the background organisms (nonfluorescent, nonblue) could not be counted under longwave UV light.

(ii) **Natural water samples.** After the final medium formulation was selected on the basis of the pure-culture results, a series of natural water samples and wastewater-spiked tap water samples were used to evaluate the ability of MI agar to recover a range of concentrations of both target organism groups. Several volumes or dilutions of each natural sample (determined in a preliminary analysis the day before by using mEndo agar and mTEC agar) were filtered in triplicate, and the filters were placed on MI agar. Similarly, replicate filters were placed on mEndo agar and mTEC agar. The media were incubated for the appropriate times and at the appropriate temperatures (1, 5, 12, 38), and the target and nontarget (background) colonies on the comparison media were counted and compared. After the mEndo agar target and nontarget colonies were counted, the locations of the target organisms were marked, and the MF from some samples were transferred to plates containing nutrient agar supplemented with MUG (Difco). The plates were incubated for 4 h at 35°C (17, 29, 39), and the target and nontarget colonies that fluoresced under longwave UV light were counted. The *E. coli* recoveries on nutrient agar supplemented with MUG were compared with those obtained on MI agar and mTEC agar.

(iii) **Preparation of spiked drinking water samples.** Spiked drinking water samples were prepared by adding various quantities of natural water samples containing both TC and *E. coli* to tap water and were used because naturally contaminated (i.e., coliform- and *E. coli*-positive) drinking water samples were not available. These samples simulated plumbing cross-connections, effluent-contaminated source waters, and intrusion of fecally contaminated water from the soil into broken water mains. Preliminary analyses, described below, were performed the day before the experiment to determine the amount of natural sample spike and the length of contact time necessary to achieve an *E. coli* level of ≤100 or ≤10 CFU per 100 ml.

After the tap water was allowed to run for 5 to 10 min, the preliminary analysis tap water sample was collected aseptically, and free and total chlorine residual levels were determined by using diethyl-*p*-phenylene diamine and a model CN-66 chlorine test kit (Hach, Loveland, Colo.). Various amounts or percentages of several natural water samples were added to measured volumes of the tap water. Following a suitable contact time, 1 ml of a sterile 10% sodium thiosulfate solution (1, 5) was added per liter of each sample, and replicate portions of each sample were filtered. The filters were placed on mEndo and mTEC agar plates and incubated at the recommended temperatures (1, 5, 12, 38) for 24 h. In general, smaller percentages of sample and longer contact times were used with effluent samples, while river water spikes required larger percentages of sample and shorter contact times because of the lower resistance of the microorganisms to chlorine.

On the day of the experiment, the tap water line was flushed, the chlorine residuals were determined, and a 10-liter sample of drinking water was collected and spiked with the volume or amount of natural sample determined in the preliminary analysis. After exposure for the predetermined contact time, the chlorine was neutralized, and the sample was analyzed by the procedure described above for the natural water samples.

Colony verification and identification. For each of the first 13 natural or spiked water samples analyzed, five isolates (if present) of each colony type (blue, fluorescent; nonfluorescent, blue; fluorescent, nonblue; nonfluorescent, nonblue) were picked from MI agar for verification and identification. The isolates were triple-picked to tubes containing TSB, lauryl tryptose broth (LTB) (Difco), and 2% brilliant green–lactose–bile broth (BGB) (Difco) if there was sufficient colonial growth. Whenever there was insufficient growth to do this, which frequently occurred with the nonfluorescent, nonblue background colonies, all of the growth from each colony was transferred to a tube containing TSB. After the TSB tubes were incubated for 24 to 72 h at 35°C, the cultures were transferred to tubes containing the verification media. All of the LTB and BGB tubes were incubated for up to 48 h at 35°C and inspected for growth and gas production (1, 5), and the LTB tubes with growth (with or without gas production) were transferred to EC medium (Difco). The EC medium tubes were incubated for 24 h at 44.5°C and inspected for growth and gas production (1, 5). Each TSB culture was streaked onto eosin-methylene blue agar (Difco) plates, which were then incubated for 24 to 72 h at 35°C. Isolated colonies that were ≥3 mm in diameter were picked for identification with API 20E strips. Additional biochemical tests recommended by the manufacturer were performed if necessary to identify the organisms. In addition, all of the colonies on one filter from the tap water sample spiked with 5% Sycamore Wastewater Treatment Plant nonchlorinated secondary effluent were similarly picked, verified, and identified. The specificity, selectivity, and false-positive and

false-negative rates were calculated by using the American Society for Testing and Materials procedure (2).

Relative accuracy of MI agar. The three Environmental Protection Agency stock coliform bacteria used in the medium development study were grown overnight in TSB, and 10-fold dilutions in phosphate-buffered dilution water were prepared (1, 5). Five 1-ml aliquots of the 10^{-7} and 10^{-8} dilutions of each organism were filtered, and the filters were placed on MI agar plates. A heterotrophic plate count (1, 5) was performed by using five 1-ml volumes of each of the same two dilutions of each of the cultures (1, 5), and the recovery data were used to determine the relative accuracy of MI agar compared with the reference test.

Medium storage study. Dilutions of EPA 206 *E. coli* and a natural sample (Ohio River at the public landing) were filtered in replicate, and the filters were placed on 5-ml MI agar plates that had been stored in a refrigerator at 4°C for various lengths of time (1 day and 2, 4, 7, 9, 13, and 14 weeks). After incubation, counts were made, and the recoveries of *E. coli*, TC, and background organisms were compared to determine the changes in concentration over time and the maximum length of time that the medium could be stored and still be effective.

Comparison of media containing autoclaved and filtered IBDG. To determine whether IBDG could be autoclaved along with the base medium, thereby simplifying preparation, base medium was split into two equal portions, one of which received a final concentration of 320 μg of IBDG per ml prior to autoclaving and the other received IBDG after sterilization. Cefsulodin was added to both media after autoclaving. A series of 10 natural and/or simulated contaminated tap water samples were analyzed with these two media, and the recoveries and colony specificities were compared.

Comparison of the sodium and cyclohexylammonium salts of IBDG. To determine whether the cyclohexylammonium salt of IBDG (Sigma Chemical Co., St. Louis, Mo.) performed as well as the sodium salt of IBDG, base medium was autoclaved and split into two equal volumes, one of which received 320 μg of the sodium salt of IBDG per ml and the other received 320 μg of the cyclohexylammonium salt of IBDG per ml. Cefsulodin was added to both preparations after autoclaving. A series of 10 natural and/or simulated contaminated drinking water samples were analyzed with these two formulations, and the recoveries and colony specificities were compared. In addition, media containing autoclaved and filtered cyclohexylammonium salt of IBDG were prepared and tested as described above for the sodium salt of IBDG.

Statistical analysis of the data. The recovery data in CFU per 100 ml for all media were log transformed (base *e*) and tested for normality by using the Shapiro-Wilk test (41). The recoveries of TC and *E. coli* on MI agar were compared with those obtained on mEndo agar and mTEC agar, respectively, by using analysis of variance for a randomized block design (34). In addition, MI agar *E. coli* recoveries from 12 samples were compared with *E. coli* recoveries on nutrient agar supplemented with MUG. An analysis of variance was performed on the means of the data, which were log transformed (base *e*). Recoveres of *E. coli* on media containing filtered and autoclaved IBDG and on media containing the sodium and cyclohexylammonium salts of IBDG were also compared by analysis of variance. The differences were considered to be statistically significant at a significance level of 0.05. To examine the medium performance over a range of sample types and concentrations, the samples were also grouped into categories by water type (potable water, which included the spiked tap water and groundwater samples; surface water; and wastewater effluents) and by *E. coli* counts (0 to 10, 11 to 100, 101 to 1000, and >1,000 *E. coli* cells per 100 ml) and compared by analysis of variance. Scheffé's test for homogeneity of variance (33) was used to compare the relative precision values for the log-transformed (base *e*) data, while the relative accuracy of MI agar for recovering the three coliform cultures compared with the reference test was determined by using a variance ratio test (34) with Bonferroni-adjusted comparisons (30).

RESULTS

Medium development study. When pure cultures of *E. coli*, *Enterobacter aerogenes*, and *K. pneumoniae* were used, an examination of media containing various combinations of chromogens and other ingredients listed in Table 1 showed that the medium containing MUGal and IBDG (i.e., MI agar) was the best medium for the simultaneous detection of TC and *E. coli*. On this medium, *E. coli* colonies were blue in ambient light and exhibited blue-green fluorescence when the plates were exposed to longwave UV light, while the colonies of other TC were cream colored in ambient light and exhibited blue-white fluorescence under longwave UV light. The background or noncoliform colonies, which were also cream colored in ambient light, did not fluoresce when they were exposed to UV light.

The chromogen ONPG was not useful in the agar medium because of the natural cream to pale yellow color of the bacterial colonies. TC were difficult to distinguish even when concentrations up to 1,600 μg/ml were used. In addition, extensive lateral diffusion of color further hindered target colony discrimination.

The combination of chlorophenol red-β-D-galactopyranoside (CPRG) and IBDG did not provide enough color contrast between the orchid TC colonies and the blue *E. coli* colonies, especially when enzyme production was reduced or slow. Raising the pH to 7.5 or 8.0 and adding the gratuitous *lac* operon inducer isopropyl-β-D-thiogalactopyranoside (IPTG) (10) did not improve CPRG color development, nor did changes in the concentrations of other ingredients result in any further improvements. However, addition of cefsulodin greatly reduced the background counts obtained with all of the formulations and combinations tested.

Although a medium containing a combination of resorufin-β-D-galactoside (RBDG) and IBDG showed promise initially, the large amount of lateral diffusion of pink color away from the TC colonies made the differentiation of target colonies against the pink MF difficult. Reduction of the concentration of RBDG to the point at which there was little or no color diffusion resulted in pale pink colonies that were hard to distinguish from the nontarget organisms. Attempts to decrease the diffusion by using reduced amounts of sodium lauryl sulfate and/or sodium desoxycholate, MF pretreatments, and filters having different compositions were also unsuccessful (6).

Medium storage study. The results of the medium storage study showed that the recoveries of *E. coli* from the natural sample remained constant on MI agar stored at 4°C for up to 14 weeks. Similarly, pure culture recoveries of *E. coli* did not change as the medium aged, but the sizes of the colonies decreased. TC recoveries on MI agar that was refrigerated for 2 weeks or more increased to twice the original value, but

then remained constant on medium kept up to 14 weeks. The increase was primarily due to the growth of green fluorescent (false-positive) organisms. Media stored for more than 1 month produced an increase of 1 $\log_{10}$ in background growth, but no further increase occurred on media stored up to 14 weeks. However, the elevated levels of nontarget organisms were still less than those found on freshly prepared mEndo agar.

Evaluation of the final medium formulation with natural samples and simulated contaminated drinking water samples. **(i) TC.** Recoveries of TC, *E. coli*, and background or noncoliform colonies on MI agar from natural and spiked drinking water samples were compared with those obtained with the comparison media. All 26 samples analyzed were TC positive, and 23 contained *E. coli* (Tables 2 and 3). On the average, the new medium recovered 1.8 times as many TC as mEndo agar, and this difference was statistically significant ($P = 0.0001$). When the data were analyzed by water type (surface water, wastewater effluent, or potable water, which included groundwater and spiked tapwater), MI agar recovered significantly more TC than mEndo agar with potable water samples ($P = 0.0002$) and surface water samples ($P = 0.0001$), but not with wastewater effluent samples ($P = 0.5399$).

(ii) *E. coli*. Although MI agar recovered more *E. coli* from 16 of 23 water samples (69.6%), the overall analysis revealed no significant difference between the *E. coli* recoveries on MI agar and mTEC agar, a result that might have been expected since MI agar is a modification of mTEC agar. Most of the samples with greater recovery on MI agar contained less than 1,000 *E. coli* per 100 ml, while three of the four samples with counts greater than 1,000 had decreased recovery compared with mTEC agar. When the high counts were omitted from the analysis, the new medium recovered 21% more *E. coli* than mTEC agar on the average. This difference was statistically significant ($P = 0.0307$). More samples will be needed to determine whether the difference observed with high counts with effluents is real or an artifact produced by too few samples.

Results of an analysis using the four *E. coli* count categories (0 to 10, 11 to 100, 101 to 1,000, and >1,000 *E. coli* per 100 ml) showed that MI agar recovered significantly more *E. coli* than mTEC agar only with the 11 to 100 catetory ($P = 0.0012$). When the data were analyzed by water type, no differences were found among the three types of water samples and between the two subgroups of potable water samples (i.e., spiked tap water and groundwater).

Using 12 samples, the *E. coli* recoveries on nutrient agar supplemented with MUG, a medium utilized with mEndo agar in a newly promulgated MF *E. coli* method (17, 29, 39), were compared with those on MI agar and mTEC agar. Recovery on nutrient agar supplemented with MUG was significantly lower than on either of the other two media ($P \leq 0.0004$). In addition, the precision of this medium, determined by Scheffé's test for homogeneity of variance, was significantly lower than the precision of MI agar ($P = 0.0019$), but it was not different from the precision of mTEC agar.

(iii) Background. The background counts on the new medium averaged ≤7% of the background counts on mEndo agar, and this difference was highly significant ($P < 0.0001$). The background counts on MI agar were significantly lower than those on mEndo agar with all three water types and all four count categories tested.

Precision of MI agar. The coefficients of variation for TC enumeration on MI agar and mEndo agar were 17.6 and 19.5%, respectively, while the coefficients of variation for *E. coli* enumeration on MI agar and mTEC agar were 25.1 and 33.7%, respectively. These differences were not statistically significant.

Medium specificity. Of the 265 isolates picked for identification with API 20E strips, 238 were successfully identified, 23 failed to grow after subculturing from MI agar, and 4 had repeatedly unacceptable profile numbers. The identities of the four types of colonies are shown in Table 4. A total of 57 of 59 fluorescent, blue colonies (96.6%) and 9 of 10 nonfluorescent, blue colonies (90%) were *E. coli*. The nonfluorescent, blue colonies were found only on overcrowded plates, and 8 of 9 of these organisms demonstrated fluorescence when they were retested on MI agar. Since the nonfluorescence appeared to be an artifact due to overcrowding, the two groups were combined. The specificity for the combined blue groups was 95.7% (66 of 69 colonies). Only 74.2% of the *E. coli* isolates (49 of 66 isolates) were positive for growth and gas production in EC broth. The remaining 25.8% grew, but were anaerogenic.

Three blue colonies on MI agar were not *E. coli*, giving a false-positive rate of 4.3% (3 of 69 colonies), and three organisms that demonstrated fluorescence but were not blue (i.e., TC) were actually *E. coli*, resulting in a false-negative rate of 4.3% (3 of 69 colonies). The false-negative isolates were further tested by using an *E. coli* 0157 latex test (Oxoid, Ltd., London, United Kingdom) and H7 antiserum (Difco) to determine whether enterohemorrhagic strains were present. These strains do not produce β-glucuronidase or grow at 44.5°C (22). However, they would be detected as TC on MI agar because it is incubated at 35°C. The false-negative isolates tested in this study were not *E. coli* 0157:H7.

The specificity of the medium for TC, which included all blue colonies and all fluorescent, nonblue colonies, varied depending on how TC were defined. When only the four typical genera (*Escherichia*, *Enterobacter*, *Citrobacter*, and *Klebsiella*) (5) were considered, the new medium TC specificity was 87.9% (152 of 173 colonies). However, if the organisms that verified in LTB and BGB (i.e., LTB^+ BGB^+) with genus names other than the four listed above were added, the specificity was 93.1% (161 of 173 colonies). The average coliform verification rate for typical coliforms was 77.6% (118 of 152 colonies), and the overall verification rate was 74.6% (129 of 173 colonies).

Some typical coliforms did not verify in both LTB and BGB, although they were usually able to utilize one of the lactose-containing media. This may have been due to injuries sustained from prolonged exposure to the longwave UV light used during the counting and colony-picking procedures. A longwave UV light exposure study (6) showed that verification of fluorescent, blue *E. coli* colonies was not affected by exposure times up to 60 min, but the verification rate for the fluorescent, nonblue TC colonies decreased rapidly after 15 min of exposure. The background colony verification rate was also affected, but to a lesser degree.

Only 49.2% of the background isolates (32 of 65 isolates) were members of typical noncoliform genera (i.e., genera other than *Escherichia*, *Enterobacter*, *Citrobacter*, and *Klebsiella*). However, when the background organisms that did not verify (i.e., LTB^+ BGB^-, LTB^- BGB^+, and LTB^- BGB^- organisms) were added, the specificity was 93.8% (61 of 65 isolates).

More than half of the background isolates (18 of 33 isolates) were identified as *Enterobacter agglomerans*, an organism previously classified in the noncoliform genus

TABLE 2. Comparison of the numbers of TC detected and selectivity characteristics of MI agar and mEndo agar

Sample used[a]	No. of TC per 100 ml on[b]:		No. of noncoliforms per 100 ml on[b]:		Recovery ratios[c]			
	MI agar	mEndo agar	MI agar	mEndo agar	MI_{TC}/$mEndo_{TC}$	MI_{NT}/$mEndo_{NT}$	MI_{TC}/MI_{NT}	$mEndo_{TC}$/$mEndo_{NT}$
Sycamore WTP nonchlorinated secondary effluent	3.56×10^6	1.77×10^6	3.00×10^5	1.18×10^7	2.01	0.03	11.87	0.15
Ludlow Springs	1.06×10^4	1.05×10^3	4.17×10^3	6.57×10^3	10.09	0.63	2.54	0.16
Tylersville Artesian Well	0.33	0.11	0.0	15.9	3.00	0.00	ND[d]	0.01
Ohio River (Anderson Ferry)	1.62×10^4	7.43×10^3	9.40×10^3	1.82×10^4	2.18	0.52	1.72	0.41
Clough Creek	9.19×10^2	9.67×10^2	2.50×10^2	1.67×10^3	0.95	0.15	3.68	0.58
East Fork Artesian Well	1.64×10^2	1.51×10^2	1.30×10^2	2.89×10^2	1.09	0.45	1.26	0.52
Great Miami River (Dayton, Ohio)	7.85×10^3	3.32×10^3	2.03×10^3	1.02×10^4	2.36	0.20	3.87	0.33
Clough Springs	1.67×10^2	57	2.67×10^2	3.93×10^2	2.93	0.68	0.63	0.15
Ohio River (public landing)	2.27×10^3	2.39×10^3	4.87×10^2	5.20×10^3	0.95	0.09	4.66	0.46
Winton Woods Lake	9.22×10^2	2.11×10^2	2.53×10^2	1.89×10^3	4.37	0.13	3.64	0.11
Sharon Woods Lake	6.95×10^3	5.53×10^2	4.67×10^3	9.67×10^3	12.57	0.48	1.49	0.06
Ohio River (public landing)	1.64×10^4	6.20×10^3	9.63×10^3	2.68×10^4	2.65	0.36	1.70	0.23
Tap water + 5% Muddy Creek WTP nonchlorinated secondary effluent	46.4	31.0	6.33	85.3	1.50	0.07	7.33	0.36
Tap water + 5% Sycamore WTP nonchlorinated secondary effluent	9.53	14.2	1.00	11.8	0.67	0.08	9.53	1.20
Tap Water + 10% Mill Creek WTP nonchlorinated secondary effluent	6.59×10^3	3.60×10^3	1.97×10^3	1.79×10^4	1.83	0.11	3.35	0.20
Tap water + 25% Great Miami River water	4.76×10^2	2.00×10^2	3.37×10^2	7.40×10^2	2.38	0.46	1.41	0.27
Tap water + 40% Ohio River water (Anderson Ferry)	2.81×10^2	25	1.33	84.7	11.24	0.02	211.28	0.30
Muddy Creek WTP nonchlorinated secondary effluent	5.78×10^4	7.30×10^4	1.07×10^4	1.04×10^5	0.79	0.10	5.40	0.70
Muddy Creek WTP chlorinated secondary effluent	6.35×10^3	5.70×10^3	2.87×10^3	1.88×10^4	1.11	0.15	2.21	0.30
Ohio River (Anderson Ferry)	3.96×10^5	1.43×10^5	7.00×10^4	3.27×10^5	2.77	0.21	5.66	0.44
Tap water + 10% Muddy Creek WTP primary effluent	1.31×10^4	2.23×10^3	1.19×10^4	1.81×10^4	5.87	0.66	1.10	0.12
Muddy Creek WTP primary effluent	2.72×10^6	1.74×10^6	8.67×10^5	6.13×10^6	1.56	0.14	3.14	0.28
Clough Springs	2.25×10^2	60.3	1.37×10^2	2.87×10^2	3.65	0.48	1.61	0.21
Stonelick Lake (beach)	1.42×10^3	1.17×10^2	8.23×10^3	2.28×10^4	12.14	0.36	0.17	0.01
Stonelick Lake (campground)	1.75×10^4	1.29×10^3	2.80×10^3	2.33×10^4	13.57	0.12	6.25	0.06
Great Miami River (Dayton, Ohio)	2.32×10^4	1.61×10^3	1.37×10^3	2.06×10^4	14.41	0.07	16.93	0.08

[a] WTP, Wastewater Treatment Plant.
[b] Values are the means of three replicates.
[c] Ratios of the recoveries of TC and/or nontarget organisms on two different media. The values indicate that there were significant differences in the recovery and selectivity on the different media. Abbreviations: MI_{TC}, number of TC recovered on MI agar; $mEndo_{TC}$, number of TC recovered on mEndo agar; MI_{NT}, number of background or nontarget organisms recovered on MI agar; $mEndo_{NT}$, number of background or nontarget organisms recovered on mEndo agar. The mean recovery ratios were as follows: $MI_{TC}/mEndo_{TC}$, 4.56; $MI_{NT}/mEndo_{NT}$, 0.26; MI_{TC}/MI_{NT}, 12.50; $mEndo_{TC}/mEndo_{NT}$, 0.30.
[d] ND, not determined.

Erwinia (15). Currently, these bacteria are considered members of the genus *Enterobacter* on the basis of a DNA homology of 70% (15) and, hence, are typical coliforms. However, the reactions of the isolates with conventional media (6) were those of noncoliforms; these isolates failed to ferment lactose in LTB and/or BGB and were unable to produce the typical golden green metallic sheen when growth was streaked onto mEndo agar. The lack of fluorescence of the colonies observed on MI agar may have been caused by insufficient β-galactosidase production due to injury to the organisms or by blocking or quenching of the fluorescence by the natural carotenoid pigment found in the organisms (37). Alternately, these bacteria may be *Erwinia* species that are misclassified as coliforms.

Use of various forms of IBDG in MI agar. Studies (6) of the salt form of the chromogen and the method of sterilization revealed no significant differences in chromogen color development and *E. coli* recovery. A study of the TC, *E. coli*,

TABLE 3. Comparison of *E. coli* recoveries on three media

Sample used[a]	No. of *E. coli* per 100 ml on[b]:			Recovery ratios[c]	
	MI agar	mTEC agar	Nutrient agar supplemented with MUG	MI agar/mTEC agar	MI agar/nutrient agar containing MUG
Sycamore WTP nonchlorinated secondary effluent	5.67×10^4	4.67×10^4	ND[d]	1.21	ND
Ludlow Springs	22.3	5.67	ND	3.93	ND
Ohio River (Anderson Ferry)	817	630	ND	1.30	ND
Clough Creek	35.7	11.3	ND	3.16	ND
Clough Springs	0.11	0.11	ND	1.00	ND
Ohio River (public landing)	72.7	49.3	ND	1.47	ND
Winton Woods Lake	9.07	8.00	ND	1.13	ND
Sharon Woods Lake	85.3	80.0	ND	1.07	ND
Ohio River (public landing)	477	410	ND	1.16	ND
Tap water + 5% Muddy Creek WTP nonchlorinated secondary effluent	0.87	0.53	ND	1.64	ND
Tap water + 5% Sycamore WTP nonchlorinated secondary effluent	0.40	0.60	ND	0.67	ND
Tap water + 10% Mill Creek WTP nonchlorinated secondary effluent	125	167	167	0.75	0.75
Tap water + 25% Great Miami River water (Dayton, Ohio)	7.25	3.42	6.67	2.12	1.09
Tap water + 40% Ohio River water (Anderson Ferry)	0.78	0.44	0.67	1.77	1.16
Muddy Creek WTP nonchlorinated secondary effluent	3.77×10^3	4.37×10^3	4.33×10^3 (1.00×10^4)[e]	0.86	0.87
Muddy Creek WTP chlorinated secondary effluent	180	150	100 (1.30×10^3)	1.20	1.80
Ohio River (Anderson Ferry)	9.00×10^3	9.33×10^3	9.67×10^3 (4.37×10^4)	0.96	0.93
Tap water + 10% Muddy Creek WTP primary effluent	48.3	7.22	33.3 (66.7)	6.69	1.45
Muddy Creek WTP primary effluent	1.53×10^5	1.67×10^5	1.30×10^5 (6.33×10^4)	0.92	1.18
Clough Springs	1.58	2.92	0.67 (4.33)	0.54	2.36
Stonelick Lake (beach)	144	134	167 (TNTC)[f]	1.07	0.86
Stonelick Lake (campground near sewage lagoon)	257	225	53.7 (TNTC)	1.14	4.79
Great Miami River (Dayton, Ohio)	16.3	15.7	28.0	1.04	0.58

[a] WTP, Wastewater Treatment Plant.
[b] Values are the means of three replicates.
[c] Ratios of the recoveries of *E. coli* on two different media. The mean recovery ratios were as follows: MI agar/mTEC agar, 1.60; MI agar/nutrient agar supplemented with MUG, 1.49.
[d] ND, not done.
[e] The values in parentheses are the counts for nontarget or background organisms on mEndo agar that gave positive reactions on nutrient agar supplemented with MUG.
[f] TNTC, too numerous to count.

TABLE 4. Identification of colonies picked from MI agar

Species	No. of isolates identified in each category (% of total no. of isolates in the category)				Total no. of isolates (% of total)
	Fluorescent, blue colonies (*E. coli*)	Nonfluorescent, blue colonies	Fluorescent, nonblue colonies (TC other than *E. coli*)	Nonfluorescent, nonblue colonies (background)	
Escherichia coli	57 (96.6)	9 (90.0)	3 (2.9)		69 (29.0)
Escherichia vulneris			2 (1.9)		2 (0.8)
Enterobacter species			6 (5.8)	7 (10.8)	13 (5.5)
Enterobacter amnigenus 2	1 (1.7)		2 (1.9)		3 (1.3)
Enterobacter cloacae			14 (13.5)	4 (6.2)	18 (7.6)
Enterobacter intermedium			2 (1.9)	1 (1.5)	3 (1.3)
Enterobacter agglomerans			7 (6.7)	18 (27.7)	25 (10.5)
Enterobacter aerogenes			3 (2.9)		3 (1.3)
Klebsiella species			1 (1.0)		1 (0.4)
Klebsiella pneumoniae			19 (18.3)	1 (1.5)	20 (8.4)
Klebsiella oxytoca			21 (20.2)	1 (1.5)	22 (9.2)
Klebsiella ozaenae			1 (1.0)	1 (1.5)	2 (0.8)
Citrobacter freundii			4 (3.8)		4 (1.7)
Citrobacter amalonaticus			1 (1.0)		1 (0.4)
Serratia species			4 (3.8)		4 (1.7)
Serratia liquefaciens			6 (5.8)	2 (3.1)	8 (3.4)
Serratia fonticola			3 (2.9)	2 (3.1)	5 (2.1)
Serratia plymutheca			1 (1.0)		1 (0.4)
Hafnia alvei		1 (10.0)	2 (1.9)	3 (4.6)	6 (2.5)
Kluyvera species	1 (1.7)		2 (1.9)		3 (1.3)
Cedacea lapagei				1 (1.5)	1 (0.4)
Yersinia pestis (presumptive)				1 (1.5)	1 (0.4)
Alcaligenes species				1 (1.5)	1 (0.4)
Acinetobacter calcoaceticus subsp. *lwoffi*				10 (15.4)	10 (4.2)
Salmonella species				1 (1.5)	1 (0.4)
Fluorescent *Pseudomonas* group				1 (1.5)	1 (0.4)
Centers for Disease Control group 5-E1 (*Pseudomonas luteola*)				2 (3.1)	2 (0.8)
Centers for Disease Control group 5-E2 (*Flavimonas oryzihabitans*)				1 (1.5)	1 (0.4)
Pseudomonas putrefaciens				2 (3.1)	2 (0.8)
Pseudomonas fluorescens				1 (1.5)	1 (0.4)
Pseudomonas maltophilia				2 (3.1)	2 (0.8)
Aeromonas hydrophila				1 (1.5)	1 (0.4)
Achromobacter species				1 (1.5)	1 (0.4)
Total[a]	59	10	104	65	238[b]

[a] The percentages of the total number of isolates in the four categories were as follows: fluorescent, blue colonies, 24.8%; nonfluorescent, blue colonies, 4.2%; fluorescent, nonblue colonies, 43.7%; and nonfluorescent, nonblue colonies, 27.3%.

[b] This value does not include organisms that failed to grow and organisms that repeatedly produced unacceptable profiles.

and background specificities (6) obtained for the four different media revealed few differences in the types and numbers of species found in each colony category.

Relative accuracy of the new medium. The new medium recovered 97.9% of the *E. coli* recovery of the heterotrophic plate count. The recoveries of the TC *K. pneumoniae* and *Enterobacter aerogenes* were 85.7 and 87.5%, respectively, of the reference test values. None of these differences was statistically significant.

DISCUSSION

The new medium, MI agar, is sensitive, selective, and specific and has low false-positive and false-negative rates. In addition, it is precise and accurate in recovering the two target organisms. The combined *E. coli* specificity rate of 95.7% is similar to that reported for other media (19, 22, 24), but differs from the results of Chang et al. (7). However, after subculturing and storage on tryptic soy agar slants and stabs, 29% of the MI agar *E. coli* isolates (20 of 69 isolates) restreaked on the new medium were β-glucuronidase negative (i.e., they were not able to produce the same fluorescent, blue color formed during primary isolation); 19 of these isolates were also MUG negative in the Colilert test. Similarly, subculturing and/or storage may explain the large number of β-glucuronidase-negative isolates found by Chang and coworkers (7), or their use of a lactose-containing medium for isolation (MacConkey agar) may have inhibited the MUG reaction (36).

In addition, familiar MF technology is used to simultaneously detect both TC and *E. coli* from a variety of water samples. Use of this method with potable water should simplify drinking water laboratory compliance with the Final Coliform Rule (16), eliminate the additional time, labor, and expense of repeat or serial analyses that can delay the detection of contaminated drinking water, and obviate the need for a second incubator set at the elevated temperature

needed with some media. The plates can be observed for the presence or absence of fluorescence indicative of TC and blue color indicating the presence of *E. coli*, or actual counts can be made to monitor distribution lines or treatment plant effectiveness. Furthermore, MI agar is capable of recovering *E. coli* from water samples containing high particulate concentrations and is less expensive than the liquid media containing chromogens and/or fluorogens.

This medium is not the first medium to detect the two groups of organisms simultaneously. The agar medium developed by Petzel and Hartman (31) combined a selective medium for TC identification with detection of *E. coli* with MUG. Problems encountered with this medium included the inability to use standard diluents, a high false-positive rate when high levels of flavobacteria or other oxidase-positive organisms (e.g., *Aeromonas* spp.) were present in the water samples, and difficulty in distinguishing the natural fluorescence of pseudomonads from the fluorescence produced during substrate breakdown. Furthermore, this medium could not be used for precise enumeration of the target organisms because of the large number of other gram-negative bacteria that grew on it. Some similar problems were experienced by other workers (21). Unlike the media of these investigators (21, 31), MI agar inhibited the growth of *Flavobacterium* and *Aeromonas* species, background organisms that can cause false-positive results on coliform media (21). A study (6) using two cultures of each genus showed that the recoveries of aeromonads that were able to grow on the base medium without cefsulodin were reduced by more than 4 and 5 $\log_{10}$ when the antibiotic was included in the formulation. The *Flavobacterium* species were unable to grow on the base agar alone.

Berg and Fiksdal (4) incorporated the fluorogen MUGal into liquid and agar media to detect TC in water within 15 min and fecal coliforms within 6 h, respectively. However, the TC method required expensive and complex equipment, and the level of detection was unsuitable for use with drinking water. The lack of specificity of MUGal for fecal coliforms and the lower, nonstandard incubation temperature (41.5°C) may result in an increased false-positive rate.

Several commercially available liquid presence-absence or most-probable-number media (Colilert [Environetics, Inc., Branford, Conn.] and Colisure [Millipore Corp., Bedford, Mass.]) have been developed to detect TC and *E. coli* in water samples within 24 to 28 h, but only the Colilert test has been approved for drinking water analysis (18). β-Galactosidase and β-glucuronidase reactions of the TC, *E. coli*, and background MI agar isolates identified in this study showed 92.2% (118/128), 97.1% (67/69), and 91.5% (43/47) agreement, respectively, with those of Colilert.

An agar medium (27) using two different enzyme substrates and a different base medium was developed in Germany to simultaneously detect both TC and *E. coli*. TC colonies were identified by the production of a blue color from β-galactosidase cleavage of the substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), while *E. coli* colonies were detected by the fluorescence of 4-methylumbelliferone, produced by the cleavage of MUG by β-glucuronidase. Little is known about the performance of this medium, as it has not been used or tested in the United States, and the base medium is not available in this country. However, these two enzyme substrates, as well as several other combinations of chromogens and/or fluorogens, also work in MI base agar medium (6).

The new medium is not completely without problems. For example, the cefsulodin must be added after the base agar medium is autoclaved, as this antibiotic is destroyed by excessive heat. The presence of some lateral diffusion of blue color away from the target *E. coli* colonies can make enumeration and colony picking more difficult on overcrowded plates (i.e., plates containing >200 colonies of all types). However, this problem should not affect filters containing low counts, such as those obtained with drinking water, and filtration of multiple volumes or dilutions of other water types should result in filters containing colonies that can be easily enumerated.

With a few samples, tiny flat or peaked pinpoint blue colonies (<0.5 mm in diameter on plates containing <200 colonies) were found along with the usual large fluorescent, blue *E. coli* colonies (1 to 3 mm in diameter). Ten colonies were picked and identified with API 20E strips as *Hafnia alvei* (three colonies), *Enterobacter amnigenus* 2 (one colony), *Escherichia vulneris* (four colonies), and *Citrobacter freundii* (two colonies). The reason for the production of β-glucuronidase by these organisms is not known, but other researchers (22) have also reported β-glucuronidase activity in some of these species. Alternately, the reaction may be plasmid mediated. When only the tiny pinpoint blue colonies are present on filters, water samples should not be considered *E. coli*-positive until the identity of at least one colony has been verified by another method (e.g., EC medium supplemented with MUG [17, 32] or API 20E strips). The large, very pale blue colonies occasionally observed did not appear to be a problem, as the colonies picked for identification were shown to be *E. coli*.

With some samples, a few bright green, fluorescent, nonblue colonies were observed along with the typical blue-white fluorescent TC colonies. Several of the green colonies were picked and identified as *Flavobacterium meningosepticum* (1 of 7 colonies), *Achromobacter xylosoxidans* (4 of 7 colonies), and fluorescent *Pseudomonas* species (2 of 7 colonies). Unlike the fluorescent green organisms found by Petzel and Hartman (31), those found on MI agar could be distinguished from the blue-white fluorescent TC and, when present, should be eliminated from the TC count. These organisms generally represented ≤5% of the population and were never observed in the absence of the typical blue-white fluorescent coliforms. An increase in the number of green colonies may indicate an unusual sample population or a breakdown of the cefsulodin in the medium.

Furthermore, with some samples, the ideal volume for *E. coli* enumeration may not be optimum for TC enumeration and vice versa. However, since blue *E. coli* colonies were clearly visible on a TC background that was too numerous to count, this should be of minor importance for drinking water compliance purposes, and multiple volumes or dilutions of other water types should provide accurate enumeration.

Last and most important, because of the increased recovery of the two target organism groups and the greater accuracy in detecting *E. coli* with the new medium, drinking water facilities may be out of compliance more often if this medium is used. However, use of other less sensitive or less specific methods could result in the potential use of contaminated water by the public or the rejection of acceptable water by water treatment facilities.

In conclusion, the new medium, MI agar, shows promise for use in monitoring several different types of water, including drinking water. The results of a study initiated to confirm the ability of MI agar to recover chlorine-stressed or

damaged organisms in drinking water will be reported elsewhere.

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