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Distribution of *uidA* Gene Sequences in *Escherichia coli* Isolates in Water Sources and Comparison with the Expression of β-Glucuronidase Activity in 4-Methylumbelliferyl-β-D-Glucuronide Media

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The *uidA* gene, which encodes the β-glucuronidase enzyme, was detected in 97.7% of 435 *Escherichia coli* isolates from treated and raw water sources by DNA-DNA hybridization; 92.4% of the strains expressed the translational product in 4-methylumbelliferyl-β-D-glucuronide-containing media after reinoculation. Upon initial isolation from water samples, the minimal medium *o*-nitrophenyl-β-D-galactopyranoside–4-methylumbelliferyl-β-D-glucuronide preparations failed to detect more than 50% of the *E. coli* isolates that possessed *uidA* gene. Treated water gave the lowest recovery, with Colilert producing 26% positive samples and Coliquik producing 48% positive samples. There appears to be no relationship between the intensity of the autoradiographic signals of the *uidA* gene and the expression of β-glucuronidase activity. Therefore, another variable such as physiological condition of the bacteria could be responsible for the nonexpression of the enzyme activity.

The new coliform rule released by the Environmental Protection Agency in 1992 concerning assessment of the safety of drinking water requires specific detection and identification of *Escherichia coli* (15). Consequently, new media have been proposed recently for the detection of this microorganism. These novel techniques are based on the utilization of chromogenic and fluorogenic substrates for detecting activities of specific enzymes. These methods are sensitive and allow faster detection of the microorganisms by using the primary isolation media. The most commonly used fluorogenic substrate for the detection of *E. coli* is 4-methylumbelliferyl-β-D-glucuronide (MUG) (17, 25). This substrate detects the activity of β-glucuronidase (GUR), which is the first enzyme of the hexuronide-hexuronate pathway in *E. coli* and is encoded by the *uidA* gene (4, 30). Although this methodology is promising for the easier detection of *E. coli* in water (8, 14, 19, 24–26, 32, 33), food (29), and clinical samples (9, 11), there are reports of high percentages of *E. coli* that are GUR negative when assayed in media containing MUG (5).

Also, there is controversy concerning the performance of defined substrate media containing MUG in the assessment of the bacteriological safety of drinking water. Edberg et al. (14), Covert et al. (8), and Rice et al. (33) reported high sensitivity and specificity of minimal medium *o*-nitrophenyl-β-D-galactopyranoside (MMO)-MUG, while Clark et al. (6), Hall and Moyer (20), and Schets and Havelaar (35) compared MUG preparations with current methodologies and reported poor performance of the MMO-MUG preparations in the recovery of *E. coli* from water. Further, Edberg (13) has claimed that *E. coli* isolated from water sources that produced negative results in MMO-MUG media were not *E. coli* but related *Escherichia* species that had been misidentified by commercial testing procedures. Rice et al. (32) showed that *Escherichia* strains that are not *E. coli* were negative in MMO-MUG.

Current methodologies for water testing rely only on phenotypic expression. However, recent data on the recovery of bacteria with regard to a selective media suggested that phenotype alone can be an inaccurate test procedure because of the failure of many genes to express under selective conditions upon immediate isolation from the environment (37). Also, studies for evaluation of selective media for water testing have indicated phenotypic failure (12, 16, 27). Molecular techniques offer an approach to overcome these deficiencies. A recent study comparing the polymerase chain reaction technique with methods using MMO-MUG, EC-MUG, and nutrient agar-MUG indicated that the MMO-MUG product failed to detect a statistically significant number of *E. coli* (2). The *E. coli* GUR-encoding *uidA* gene has been used as the target for *E. coli* detection (1, 2, 18, 19). However, gene probe analysis does not address the question of viability.

To determine the performance of minimal medium commercial products Colilert (CL; Access Analytical, Branford, Conn.) and Coliquik (CQ; Hach, Loveland, Colo.) and a less stringent fecal coliform isolation medium, M-FC (Difco, Detroit, Mich.), we examined 449 strains of *E. coli* isolated from raw and treated water sources for the presence of the *uidA* gene. The distribution of this gene and the intensity of the autoradiographic signals obtained in slot blotting were compared with the translational product in a commercial MMO-MUG preparation.

MATERIALS AND METHODS

***E. coli* isolates.** A total of 212 isolates were obtained from water samples collected from a treated water reservoir, and 237 isolates were obtained from untreated surface water.

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These isolates were reinoculated in the MUG-containing medium CL and analyzed by DNA-DNA hybridization to determine the presence of the *uidA* gene and the expression of the translational product in MUG-containing media. These 449 strains were isolated in a previous study designed to compare MMO-MUG media CL and CQ with the standard membrane filtration method for fecal coliforms. Only those isolates that were identified as *E. coli* in API 20E (Analytab Inc., Plainview, N.Y.) were used for this study. The isolation procedure is described elsewhere (6, 31).

***uidA* gene.** A 1.87-kb *Pst*I-*Eco*RI *uidA* fragment inserted into the polylinker site of pEMBL9 of plasmid pRAJ255 (GenBank accession number M14641) (Clontech Laboratories, Inc., Palo Alto, Calif.) was used to develop template DNA (21).

Controls. The positive control was an *E. coli* strain that produced GUR in EC-MUG, CL, and CQ media. The MUG-negative control was a strain of *Enterobacter agglomerans*.

Preparation of DNA probe. Plasmid extraction was performed by the alkaline lysis procedure of Birnboim and Doly (3), modified by the purification of nucleic acids in a Qiagen column (Qiagen, Inc., Chatsworth, Calif.). Plasmid was digested with appropriate restriction endonucleases (Bethesda Research Laboratories, Gaithersburg, Md.) under conditions specified by the manufacturer. Isolation and purification of the fragments after electrophoresis in 1% agarose were performed by using Geneclean II (Bio 101 Inc., La Jolla, Calif.). The probe DNA was radiolabeled with [α-^{32}P]dCTP (ICN Biomedicals, Inc., Costa Mesa, Calif.) by random priming to a specific activity of 9.6×10^8 cpm/μg of DNA, measured in a scintillation counter (Beckman model LS-6000 IC), and purified in Bio-Spin columns (Bio-Rad, Life Sciences Research Products, Richmond, Calif.).

Processing of cells for DNA hybridization assays. Overnight cultures grown in L broth (34) were washed twice in phosphate-buffered saline (PBS), and the cell concentration was adjusted to $\sim 5.0 \times 10^6$ CFU/200 μl of PBS. Before filtration, the nitrocellulose filters (Schleicher & Schuell, Inc., Keene, N.H.) were exposed to filter paper (Schleicher & Schuell GB 002-SB) saturated in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) until completely wet, and then a 100-μl solution of 20× SSC was filtered under gentle vacuum. Then 200 μl of a previously homogenized cell suspension was added per well of a Minifold slot blot apparatus (Schleicher & Schuell), filtered under gentle vacuum, and washed with 200 μl of PBS. The filter was then removed from the apparatus by using forceps and placed cell side up on filter paper (Whatmann 3MM) saturated with 0.5 M NaOH for 10 min to lyse the cells and denature DNA. The material was transferred to neutralizing filter paper saturated with buffer I (0.6 M NaCl, 1.0 M Tris-HCl [pH 6.8]) for 5 min and then to a filter saturated with buffer II (1.5 M NaCl, 0.5 M Tris-HCl [pH 6.8]) for 5 min (22). Filters were then air dried and baked at 80°C for 2 h under vacuum (National Appliance Co., Portland, Ore.).

Hybridization. Filters were incubated overnight at 42°C in a prehybridization solution (50% formamide, 5× Denhardt's solution, 5× SSPE, 0.1% sodium dodecyl sulfate [SDS], 100 μg of denatured salmon sperm DNA per ml). DNA template was then added, and the mixture was incubated overnight at 42°C. A high-stringency wash was performed at 68°C in 0.1× SSC–0.1% SDS for 1 h (34). For the detection of autoradiographic signals, filters were exposed overnight at −70°C to X-Omat AR X-ray film (Eastman Kodak Co., Rochester, N.Y.) with an intensifying screen (Fisher Scientific, Pittsburgh, Pa.) and developed according to the manufacturer's instructions. Filters were stripped to remove radioactive label and stained with 1% methylene green to confirm cell lysis.

Quantification of hybridization signals. The Radioanalytic Imaging System (AMBIS Systems, San Diego, Calif.) was used to quantify the radioactivity of the signals of the hybridized DNA on the membranes. The quantification method has been described elsewhere (38). The equation used for the estimation of gene copies per cell is $[(NA/M) \times (CPM^a/CPM^b)]/NC$, where NA is Avogrado's constant (per mole), M is the molecular mass of the gene probe in daltons, CPM^a is the radioactivity retained by DNA contained in test cells, CPM^b is the radioactivity retained by a known quantity of control DNA, and NC is the total number of test cells (controlled for filter saturation and linear response of the detection system). In this instance, different concentrations of the *uidA* fragment were added to determine different hybridization intensity levels. Because an independent measure of copy number such as the origin of replication within the cell was not used in these experiments, intensity is expressed in a semiquantitative manner as +, ++, or +++. Testing of all samples was repeated to ensure that intensity was similar between hybridizations.

Statistical analysis. The Statistical Package for the Social Sciences (SPSS PC*) software version 4.0 (SPSS^{XTM} Inc., Chicago, Ill.) was used to develop Pearson's correlation coefficients and *t*-test values. The significance level of each statistical test (α) was set at <0.05.

Definition of terminology. False-negative samples are defined as samples in which the CL or CQ test exhibited a negative MUG reaction when the M-FC test yielded bacteria that were identified as *E. coli* by API 20E and were positive for the presence of the *uidA* gene.

RESULTS AND DISCUSSION

Occurrence of the *uidA* gene and characterization of *E. coli* isolates. Of the 449 *E. coli* isolates assayed for the presence of the *uidA* gene, 14 (3.1%) were not lysed; thus, 435 of the *E. coli* isolates were accepted for further analysis. Of these isolates, 205 (47.1%) were obtained from treated water and 230 (52.9%) were obtained from raw water. Four hundred twenty-five (97.7%) contained the genotypic trait for the *uidA* gene (Fig. 1). This trait was not detected in 10 (2.3%) of the isolates. All of the *E. coli* isolates were identified by the API 20E system. This system was chosen because it is widely used (5, 18, 19) and previous studies (36, 38) have confirmed the accuracy of this system compared with conventional biochemical tests, ranging from 93 to 96.7% for the identification of species in the family *Enterobacteriaceae*. The results obtained by the API 20E system were grouped according to profile numbers and categories of identification (Table 1). We found that 98.4% of the isolates represented profile numbers that fit with the identification categories of excellent (73.9%), very good (18%), and acceptable (6.5%). The strains assessed for the *uidA* gene were also reinoculated in MUG-containing medium (CL), and 92.4% presented GUR activity after reinoculation (Fig. 1). These results clearly indicate that the *E. coli* isolates correctly classified according to the defined criteria (33).

Previous studies for detection of the *uidA* gene in *E. coli* from environmental sources and human isolates have been performed on smaller sample sizes. Feng et al. (18) used a probe specific for the *uidA* gene and examined 116 *E. coli* isolates, including 31 GUR-negative and 12 enterohemor-

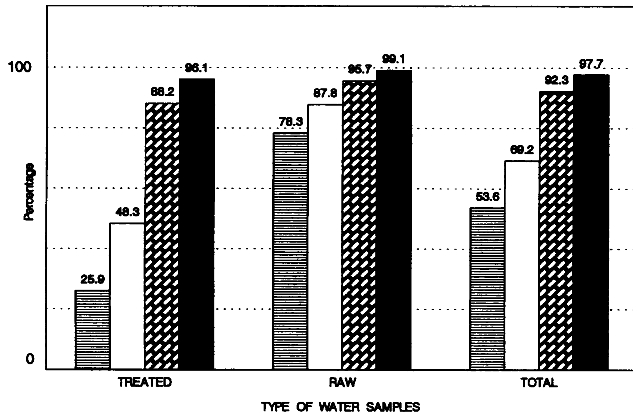

FIG. 1. Comparison among phenotypic and genotypic aspects of GUR and relationships with the recovery of *E. coli* isolates from water samples in MMO-MUG media (expressed as a percentage). Bars represent, from left to right, *E. coli* giving a positive MUG reaction in CL, *E. coli* giving a positive MUG reaction in CQ, *E. coli* giving a positive MUG reaction in CL after subculture and reinoculation, and *E. coli* hybridizing with the *uidA* gene.

rhagic *E. coli* serotype O157:H7 isolates that were previously reported to be negative for GUR activity (23). These authors detected the *uidA* gene in 112 (96.6%) of the isolates, including the GUR-negative strains. Bej et al. (1, 2) and Cleuziat and Robert-Baudouy (7) used the polymerase chain reaction amplification technique and gene probes for detecting the *uidA* gene of *E. coli*. They reported that *uidA* was found even in GUR-negative strains of this species. Green et al. (19) assayed 83 *E. coli* strains for the *uidA* gene, and all were positive, including 8.6% of the GUR-negative strains. However, other bacterial strains isolated from water, such as *Shigella* (44 to 58%) and *Salmonella* (20 to 29%) strains, some strains of the genera *Yersinia*, *Flavobacterium*, *Bacteroides*, *Corynebacterium*, *Staphylococcus*, and *Clostridium*, and streptococci of groups B and D, also can contain the *uidA* gene (25). Therefore, the presence of the *uidA* gene cannot be used for exclusive identification of *E. coli*.

On the basis of our data and reports cited above, we agree with Feng et al. (18) that "most, if not all *E. coli* carry sequences of the *uidA* gene regardless of the GUR phenotype." Thus, this genetic trait is an excellent target for use in molecular techniques to assess the safety of drinking water (1, 2, 19) if other genotypic traits unique to *E. coli* can also be identified.

It was also observed that of the 435 strains tested, only 7.6% were GUR negative after reinoculation (Fig. 1). This finding agrees with those recently presented in a review by Manafi et al. (25) indicating that GUR-positive reactions occur in 94 to 96% of *E. coli* isolates. Our data do not agree with those of Chang et al. (5), who found a mean proportion of 34% of GUR-negative *E. coli* in their studies using human *E. coli* isolates, and are also lower than the 23% of GUR-negative isolates reported by Bej et al. (1, 2). These differences may be based on differences in samples (human feces versus raw and treated drinking water [5] and reinoculation versus single inoculation [2]).

Relationship among *uidA* gene signals with initial recovery of *E. coli* in MMO-MUG media from water samples, GUR activity after reinoculation, and the identification of *E. coli* in API 20E. We scored the intensity of the autoradiographic signals of the *uidA* gene (Fig. 2). The cultures were normalized with relation to the growth phase and cell numbers applied to each well of the Minifold apparatus. Because variation in signal intensity can relate in a general manner to gene dosage (10), this analysis provides preliminary data on the relationship of the gene dosage of 5×10^6 cells per well and ability of the *uidA* gene to express upon initial isolation from water in MMO-MUG media, which implies that the copy number can affect the level of the enzyme produced.

The variables (medium type, source of water, reinoculation reaction in MMO-MUG, and API profile number) were grouped and compared with these three signal intensities, using chi-square Analysis (SPSS PC* version 4.0 program) (Fig. 3). There was no relationship between intensity of signal and the production of false-negative results upon initial isolation in MMO-MUG media (Fig. 3). However, it is important to note that only 25 isolates gave an intensity level of +++ for the *uidA* gene. Data from raw and treated water did not produce a significant correlation between the *uidA* signals and the identification profile.

Comparison of the presence of the *uidA* gene in *E. coli* isolates that yield false-negative GUR results in CL and CQ

TABLE 1. Profile numbers of 449 *E. coli* isolates recovered in water sources and classified according to identification by API 20E

Identification	No. of isolates	% of isolates	Prevalence of profile no.		
			Profile no.	No. of isolates	% of isolates
Excellent	332	73.9	5144572	124	27.6
			5044552	79	17.6
			5144552	41	9.1
			5044572	25	5.6
			1044552	12	2.7
			Other	51	11.3
Very good	81	18.0	5044553	19	4.3
			1144572	11	2.4
			1044572	10	2.2
			Other	41	9.1
Acceptable	29	6.5	5144573	8	1.8
			5044563	7	1.6
			5044543	5	1.1
			Other	9	2.0
Good likelihood but low selectivity	7	1.6	No prevalence	7	1.6

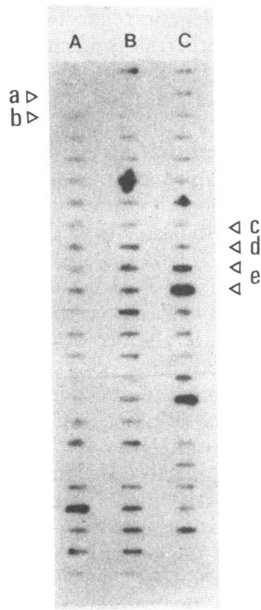

FIG. 2. Autoradiograph of *uidA* gene signals of *E. coli* isolated from raw and treated drinking waters. Negative (*Enterobacter agglomerans* [a]) and positive (*E. coli* [b]) controls in lane A are indicated on the left. Signal intensities of + (c), ++ (d), and +++ and ++++ (e) in lane C are indicated on the right.

media upon initial inoculation from water samples. When the results obtained for the presence of *uidA* gene were contrasted with the data gathered in a previous study of two commercial MUG preparations (6) compared with current methodology for the recovery of *E. coli*, it was observed that the two MMO-MUG preparations presented patterns of *E. coli* recovery that different depending on the type of water studied (Fig. 1 and 4).

In treated water, a higher percentage of *E. coli* isolates was recovered in M-FC broth but not in the two commercial MUG preparations (6). The data in Fig. 1 substantiate the findings of the previous study (6) by showing that 96.1% of the isolates possessed the *uidA* gene. However, in spite of the presence of the gene, the difference between the false-negative isolates and the percentage of strains presenting the gene shows that 70.2% of the strains in CL and 47.8% in CQ were not detected in the MMO-MUG preparations upon initial isolation. These data were based on the false-negative isolates; the eight isolates that did not present the *uidA* gene were not included in the analysis of the results. In addition, 88.8% of the isolates produced positive results upon reinoculation. It was also observed that in treated water, 24 of 205 isolates in CL and 13 of 205 of isolates in CQ presented weak fluorescence that could be misinterpreted. One possible explanation for the weak GUR reaction is low enzyme activity, which could be observed by comparing the fluorescence results with the intensity of the autoradiographic signals of the *uidA* gene. Of the 24 strains presenting weak fluorescence in CL, 62.5% have a + *uidA* gene signal, and of the 13 strains in CQ with weak fluorescence, 53.8% have a + *uidA* gene signal (Fig. 4). The results for raw water (Fig. 4) show a slightly different pattern, as the weak GUR reaction occurred in a higher percentage with a ++ *uidA* gene signal. Furthermore, the weak GUR reaction was less frequent in strains that had a +++ *uidA* gene signal. Although the number of strains is too small for a statistical analysis, this observation may suggest that gene dosage can explain the low enzyme activity under certain circumstances.

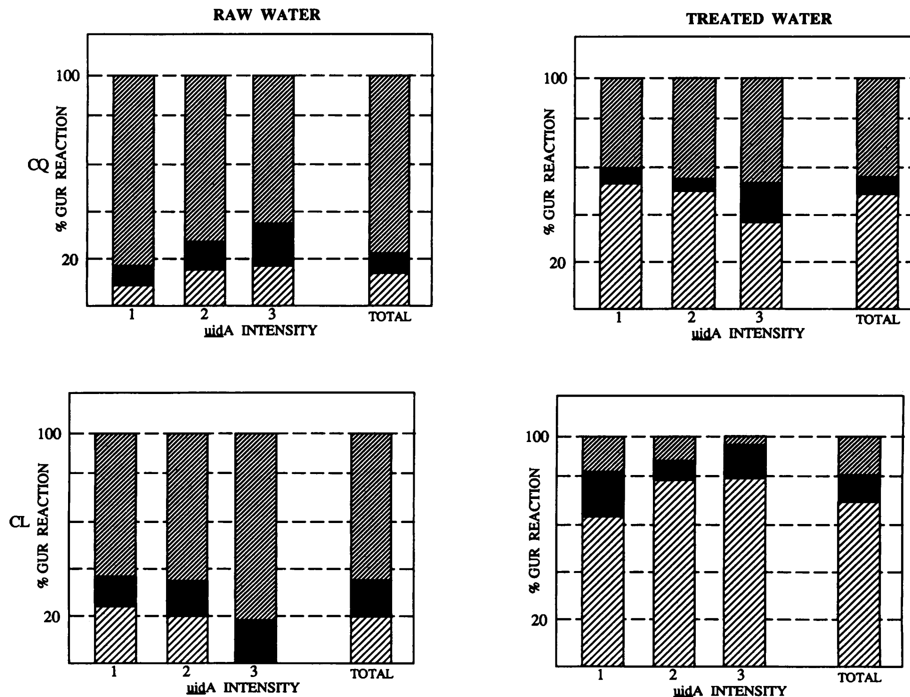

FIG. 3. Comparison among *uidA* gene signals and the performance of CL and CQ in the recovery of *E. coli* from water samples. Signals: 1, +; 2, ++; 3, +++. Isolates: ▨, false negative; ▩, weakly positive; ▨, positive.

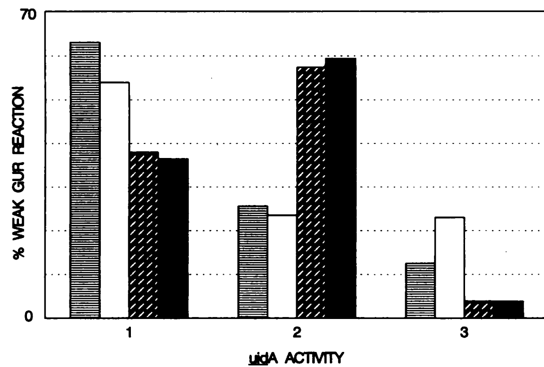

FIG. 4. Comparison among the occurrence of weak GUR reactions in CL and CQ and *uidA* intensity of *E. coli* isolates from water samples. Signals: 1, +; 2, ++; 3, +++. Bars represent from left to right, CL in treated water, CQ in treated water, CL in raw water, and CQ in raw water.

The results for the recovery of *E. coli* in CL obtained in our study disagree with those of previous studies which obtained agreement between this medium and the standard methodology (2, 8, 14, 33). This discrepancy could be because the formulation of the buffer system was changed in CL. We were unable to determine whether our product was prepared before or after the formulation change.

For raw surface water (Fig. 1), the percentage of false-negative isolates obtained in CL and CQ versus the presence of the *uidA* gene was lower, 20.8% for CL and 11.3% for CQ. These data were based on the difference between the percentage of strains positive for the *uidA* gene and the percentage of strains recovered in MMO-MUG preparations. The data obtained in CL and CQ for raw and treated water were analyzed by the *t* test. No significant difference was obtained when the results for CL and CQ were compared in raw water. In treated water, CQ gave better results than did CL. The difference between the performance of CL and CQ in treated water was significant (t value = -3.62 for 204 df and $\alpha < 0.001$).

The poor recovery of *E. coli* in treated water with MMO-MUG media and the increased recovery of this microorganism in raw water suggests that the exposure to fully treated water may cause reduced GUR activity, resulting in an inability of the organism to express the trait in MMO-MUG preparations. However, a small percentage of *E. coli* isolates from untreated (3.4%) and treated (7.3%) water that possess the *uidA* gene were unable to express it even upon reinoculation (Fig. 1). Similar results for phenotypic expression of other genes have been observed (16, 27).

Feng et al. (18), Bej et al. (1, 2), and Cleuziat and Robert-Baudouy (7) suggested that the *uidA* gene may be present in non-MUG-utilizing *E. coli* strains but is not expressed. GUR is an inducible enzyme (4, 30), and hence its expression may be affected by physiological factors or genetic differences among isolates. Our data strongly suggest that differences among the proportion of strains that possess the genetic trait and the proportion of strains that express it is based on the physiological state of the isolates and the culture medium used in isolation (Fig. 1). In this instance, the physiological state of *E. coli* is far different for bacteria isolated from treated water and untreated water, as reflected by the presence of the *uidA* gene in the isolates and their ability to express the gene under initial isolation conditions of MMO-MUG media.

Also, MMO-MUG media could be devoid of some essential nutrient that is supplied by untreated water, clinical samples, or food samples but not by treated water. It has been demonstrated, for instance, that some *E. coli* isolates from clinical samples have auxotrophy for cysteine, and for identification in MUG tests, a cysteine-supplemented inoculum should be used (28). It is possible that many environmental strains also present auxotrophy for some components not present in minimal media but because of this type of deficiency are not isolated. The data presented in this study indicate that failure cannot be attributed to misidentification or to lack of the *uidA* gene. Thus, it appears that failure is temporary, based on the physiological conditions of the organisms in most instances.

Conclusions. The *uidA* gene is an important target for the detection of *E. coli* from environmental samples by using molecular techniques, as it is present in the vast majority of *E. coli* strains, but should be used with other genotypic traits unique to *E. coli*. The performance validity of the two MMO-MUG preparations for the analysis of drinking water was poor. The failure of these media cannot be explained by the misidentification of *E. coli* isolates or by the absence of the *uidA* gene, as this gene was present in 97.7% of the isolates.

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