

Improved Method for Coliform Verification

JACK D. DIEHL, JR.

Biology Department, Lycoming College, Williamsport, Pennsylvania 17701

Received 24 September 1990/Accepted 14 December 1990

Modification of a method for coliform verification presented in *Standard Methods for the Examination of Water and Wastewater* is described. Modification of the method, which is based on β -galactosidase production, involves incorporation of a lactose operon inducer in medium upon which presumptive coliform isolates are cultured prior to β -galactosidase assay.

Several different methods for quantitative coliform analysis are described in the 17th edition of *Standard Methods for the Examination of Water and Wastewater*. One of these methods is the membrane filter (MF) procedure. When quantifying by the MF technique, presumptive coliform colonies must be verified. *Standard Methods* also presents several different coliform verification procedures, one of which is a rapid (4-h) assay for the enzymes cytochrome oxidase and β -galactosidase in isolates grown on nutrient agar. Isolates that are β -galactosidase positive and oxidase negative are coliforms. β -Galactosidase is encoded by the *lacZ* gene of the *lac* operon, which is an inducible operon (2). Substantial production of β -galactosidase requires induction of the lactose operon (3). Since nutrient agar does not contain inducers of the lactose operon it was reasoned that some coliforms might escape verification by this method. It was theorized that if an inducer of the lactose operon is added to the nutrient agar, all coliforms would transcribe the lactose structural genes and none should escape verification.

The purpose of this study was to test this theory by performing β -galactosidase assays with isolates grown on nutrient agar prepared with and without a known inducer of the lactose operon. Another aspect of this study was to determine whether the addition of the lactose operon inducer to nutrient agar altered the results of oxidase assays done with cells grown on the modified medium.

A total of 64 water samples from rural residential dwellings were assayed by the MF technique (1). This technique involves drawing 100 ml of water through a 0.45- μ m-pore-size cellulose ester filter (Gelman Sciences Inc., Ann Arbor, Mich.), which is subsequently placed on a cellulose fiber pad saturated with Endo-MF medium and incubated at 35°C for 20 to 24 h. Coliform colonies that emerge on this medium have a gold-green color with a characteristic metallic sheen. Presumptive coliforms were picked from well-isolated colonies with a sterile nichrome needle and inoculated into 0.5 ml of nutrient broth. The broth cultures were incubated for 5 to 6 h at 35°C. One drop from each broth culture was then inoculated onto nutrient agar slants prepared with and without the gratuitous *lac* operon inducer isopropyl- β -D-thiogalactopyranoside (IPTG). The IPTG was added to the nutrient agar before autoclaving; the concentration was 10^{-3} M. Inoculum from the broth was also streaked on tryptic soy agar and eosine-methylene blue agar plates to test for purity of the culture. The slants and plates were incubated for 20 to 24 h at 35°C.

β -Galactosidase activity was determined as described in

Standard Methods. Briefly, the procedure involved the use of an iron-free loop to harvest cells from the slant; cells were then mixed in 0.5 ml of phosphate-buffered saline (pH 7.0). One drop of toluene was added to each saline suspension, followed by the addition of 0.25 ml of *o*-nitrophenyl- β -D-galactopyranoside (ONPG), which is a substrate for β -galactosidase. The ONPG was prepared at a concentration of 4 mg/ml of H₂O. Although *Standard Methods* indicates in the MF procedure that coliforms will be ONPG positive within 4 h, the quality control section indicates that reaction mixtures should be read at 0.5, 1, and 24 h. In this study, mixtures were incubated for 24 h in a 35°C water bath. If β -galactosidase is present, the ONPG, which is colorless, will be hydrolyzed to galactose and *o*-nitrophenol, which is yellow. The assay mixtures were examined at intervals, and the time of the appearance of the yellow color was recorded.

The oxidase test, also described in *Standard Methods*, was performed with the cells that remained on the slants. This involved placing one drop of 1.0% aqueous *p*-aminodimethylaniline oxalate and one drop of 1.0% α -naphthol in 95.0% ethanol directly on the slant. Oxidase-positive cultures immediately turn a dark navy blue, while oxidase-negative cultures exhibit no immediate color change.

Control cultures included *Escherichia coli*, which is β -galactosidase positive and oxidase negative, and *Pseudomonas aeruginosa*, which is β -galactosidase negative and oxidase positive.

One hundred and eighty presumptive coliforms were isolated in this study. All of the 180 isolates were β -galactosidase positive when cultured on nutrient agar containing the IPTG inducer; the appearance of the yellow color, indicative of a positive test, occurred within 10 min. Twenty-four of the 180 isolates (13.3%) cultured on nutrient agar without the IPTG inducer did not hydrolyze the ONPG in 24 h. The time of appearance of the yellow color was consistently slower for the isolates cultured in the absence of IPTG that did test positive for β -galactosidase (86.7%) than it was for cells cultured in the presence of the inducer, with the range being from 3 to 15 h.

The presence of IPTG did not alter the results of the oxidase test for any of the 180 isolates in this study. Coliforms were consistently oxidase negative whether cultured in the presence or absence of IPTG, whereas *P. aeruginosa* from cultures with and without IPTG were consistently positive.

This study indicates that 13.3% of the coliforms would have escaped verification if β -galactosidase assays had been performed with presumptive coliforms cultured on nutrient

agar. One hundred percent of the same isolates cultured in the presence of IPTG tested positive for β -galactosidase. The presence of IPTG had no detectable influence on the oxidase test when performed as described here. It is concluded that coliform verification is improved by the addition of IPTG to nutrient agar.

I thank Lycoming College for providing facilities and resources to make possible this research and Patricia Bloss for providing valuable technical assistance.

REFERENCES

1. **American Public Health Association.** 1989. Standard methods for the examination of water and wastewater, 17th ed. American Public Health Association, Washington, D.C.
2. **Beckwith, J.** 1987. The lactose operon, p. 1444–1452. In F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 2. American Society for Microbiology, Washington, D.C.
3. **Monod, J., A. M. Peppenheimer, and G. Cohen-Bazire.** 1952. La cinétique de la biosynthèse de la β -galactosidase chez *E. coli* considérée comme fonction de la croissance. *Biochim. Biophys. Acta* **9**:648–660.