

9230 FECAL ENTEROCOCCUS/STREPTOCOCCUS GROUPS*

9230 A. Introduction

1. Taxonomy and Principles

In 1903, the genus name *Enterococcus* was proposed for gram-positive, catalase-negative, coccoid-shaped bacteria of intestinal origin.¹ Several years later, it was suggested² that the genus name be changed to *Streptococcus* because of the organisms' ability to form chains of coccoid-shaped cells and that the species name be *faecalis* because of their fecal origin. The genus name *Streptococcus* was adopted for the next 78 years and applied to coccoid-shaped, chain-forming, gram-positive bacteria of intestinal origin. In 1984, the genus name *Enterococcus* was revived, and it was suggested that *Streptococcus faecalis* and *Streptococcus faecium* be transferred to the genus *Enterococcus* on the basis of molecular approaches to microbial taxonomy.³ Shortly after the acceptance of the revived *Enterococcus* genus, *Streptococcus avium* and *Streptococcus gallinarum* also were transferred to the genus.

Although it is possible to differentiate the various species of enterococci and streptococci biochemically, it is not practical to carry out the tiered testing required to identify specific species of interest in water. Liquid and solid detection media have been

designed to identify enterococci and streptococci from the intestinal tracts of humans and other warm-blooded animals in water samples without identifying the species. Liquid and solid culture media eliminate non-enterococci and non-streptococci via sodium azide, cycloheximide, and 2,3,5-triphenyltetrazolium chloride (TTC). Enterococci are extremely hardy and can tolerate a wide variety of growth conditions. Enterococci are differentiated from streptococci by their ability to grow in 6.5% sodium chloride, on bile esculin agar, and at both $10 \pm 0.5^\circ\text{C}$ on BHI agar⁴ and $45 \pm 0.5^\circ\text{C}$ in BHI broth. See Table 9230:I for other characteristics of enterococci and streptococci.

2. References

1. THIERCELIN, E. & L. JOULAUD. 1903. Reproduction de l'entérocoque; taches centiales; granulations periopheriques et microblasts. *Comptes Rend. Séances Soc. Biol. Paris* 55:686.
2. ANDREWS, F.W. & J. HORDER. 1906. A study of the streptococci pathogens for man. *Lancet* 2:708.
3. SCHLEIFER, K.H. & R. KILPPER-BALZ. 1984. Transfer of *Streptococcus faecalis* and *Streptococcus faecium* to the genus *Enterococcus* nom. rev. as *Enterococcus faecalis* comb. nov. and *Enterococcus faecium* comb. nov. *Int. J. Syst. Bacteriol.* 34:31.
4. HUYCKE, M. M., D.F. SAHM & M.S. GILMORE. 1998. Multiple drug resistant enterococci: The nature of the problem and an agenda for the future. *Emerg. Infect. Dis.* 4:239.

* Approved by Standard Methods Committee, 2007.

Joint Task Group: Rachel T. Noble (chair), Gil Dichter, Sandra McLellan, Helena Solo-Gabriele, Fred Genthner.

9230 B. Multiple-Tube Technique

For an introduction to and description of the use of the multiple-tube fermentation approach to enumerating bacteria in environmental samples, see Sections 9221A and 9221C. This approach is recommended for analyzing drinking water, source water, and both fresh and marine recreational waters.

1. Materials and Culture Media

Preferably use commercially available medium. Follow manufacturer's instructions for storing and discarding after preparation. If the medium must be prepared from basic ingredients, follow directions below.

a. Azide dextrose broth:¹

| | |
|--------------------------------------|--------|
| Beef extract | 4.5 g |
| Tryptone or polypeptone | 15.0 g |
| Glucose | 7.5 g |
| Sodium chloride, NaCl | 7.5 g |
| Sodium azide, NaN ₃ | 0.2 g |
| Reagent-grade water | 1 L |

CAUTION: *Sodium azide is a dangerous chemical requiring special attention and care. It is toxic and mutagenic. Take*

precautions to avoid contact with this compound. Azide also can form explosive compounds if it contacts metal pipes.

Adjust pH so it is 7.2 ± 0.2 at 25°C after sterilization. If pH is out of range, adjust and retest pH; discard if pH remains out of range. The media described in this section are available commercially; follow manufacturer's instructions for storage and disposal after preparation.

b. Bile esculin azide agar:²

| | |
|-------------------------------|--------|
| Yeast extract..... | 5.0 g |
| Proteose peptone No. 3..... | 3.0 g |
| Tryptone | 17.0 g |
| Oxgall | 10.0 g |
| Esculin | 1.0 g |
| Ferric ammonium citrate | 0.5 g |
| Sodium chloride | 5.0 g |
| Sodium azide..... | 0.15 g |
| Agar | 15.0 g |
| Reagent-grade water | 1 L |

CAUTION: *Sodium azide is a dangerous chemical requiring special attention and care. It is toxic and mutagenic. Take precautions to avoid contact with this compound. Azide also can form explosive compounds if it contacts metal pipes.*

FECAL ENTEROCOCCUS/STREPTOCOCCUS GROUPS (9230)/Multiple-Tube Technique

TABLE 9230:I. SELECTED CHARACTERISTICS OF *ENTEROCOCCUS* AND *STREPTOCOCCUS* SPECIES ISOLATED FROM FECES

| Species | Fecal Origin | Bile Esculin Agar | Growth in | | |
|---------------------------|--------------|-------------------|-----------|--------------------|--------------------|
| | | | 6.5% NaCl | Growth At 10 ± 1°C | Growth at 45 ± 1°C |
| <i>E. gallinarum</i> | + | + | + | +/+ | + |
| <i>E. hirae</i> | + | + | + | +/+ | + |
| <i>E. columbae</i> | + | + | — | -/+ | -/+ |
| <i>E. cecorum</i> | + | + | — | -/+ | -/+ |
| <i>E. durans</i> | + | + | + | +/+ | + |
| <i>E. faecium</i> | + | + | + | +/+ | + |
| <i>E. faecalis</i> | + | + | + | +/+ | + |
| <i>E. casseliflavus</i> | + | + | + | +/+ | + |
| <i>E. avium</i> | + | + | + | +/+ | + |
| <i>E. munditii</i> | + | + | + | +/- | -/+ |
| <i>E. dispar</i> | + | + | + | +/+ | + |
| <i>E. saccharolyticus</i> | + | + | + | +/+ | -/+ |
| <i>E. asini</i> | + | + | — | +/+ | + |
| <i>S. hyointestinalis</i> | + | — | — | -/+ | + |
| <i>S. bovis</i> | + | + | — | -/+ | + |
| <i>S. intestinalis</i> | + | — | — | -/+ | + |
| <i>S. suis</i> | + | — | — | -/+ | + |
| <i>S. alactolyticus</i> | + | — | — | -/+ | + |
| <i>S. equines</i> | + | + | — | -/+ | + |

Adjust pH so it is 7.1 ± 0.2 at 25°C after sterilization. If pH is out of range, adjust and retest pH; discard if pH remains out of range. The media described in this section are available commercially; follow manufacturer’s instructions for storage and disposal after preparation.

2. Presumptive Test Procedure

Inoculate a series of tubes of azide dextrose broth with appropriate graduated quantities of sample. Use sample volumes of 10 mL or less. Use double-strength broth for 10-mL inocula. The sample portions used will vary in size and number with the sample character. Use only decimal multiples of 1 mL (see Section 9221 for suggested sample sizes).

Incubate inoculated tubes at 35 ± 0.5°C. Examine each tube for turbidity at the end of 24 ± 2 h. If no definite turbidity is present, reincubate, and read again at the end of 48 ± 4 h.

3. Confirmed Test Procedure

After 24 or 48 h incubation, subject all azide dextrose broth tubes showing turbidity to the confirmed test for enterococci.

Streak a portion of growth from each positive azide dextrose broth tube on bile esculin azide agar (BEA).* Invert and incubate the dish at 35 ± 0.5°C for 24 ± 2 h. Brownish-black colonies with brown halos confirm the presence of fecal streptococci.

Then, transfer brownish-black colonies with brown halos to two tubes of brain–heart infusion (BHI) broth: one with 6.5% NaCl and one without NaCl.

If growth is observed when tube is incubated at 35 ± 0.5°C after 48 ± 4 h (BHI broth with 6.5% NaCl) or 24 ± 2 h (BHI broth without NaCl), the colony is confirmed as a member of the *Enterococcus* genus. See Section 9230C.2e and f for preparation of BHI broth with and without 6.5% NaCl. The aforementioned procedure is expected to offer an acceptably accurate confirmation of the presence of the *Enterococcus* genus. However, more accuracy (≥ 90%) can be achieved by doing all of the following: observing gram-positive cocci, a catalase-negative reaction, growth on BHI agar at 10 ± 0.5°C, positive pyrrolidonylaryl- amidase (PYR) activity, and positive leucine aminopeptidase (LAP) reaction^{3,4} using a commercially available test kit.† (Refer to Table 9230:I.)

4. Computing and Recording MPN

Calculate the total fecal enterococci/streptococci density from the number of confirmed positive cultures on bile esculin azide agar and corresponding positive tubes of BHI broth with 6.5% NaCl at 35 ± 0.5°C after 48 ± 4 h. Compute the combination of positive and negative tubes and record as the most probable number (MPN). (Refer to Section 9221C.)

5. Quality Control

Test each lot of purchased or made medium for performance by inoculating it with three control bacteria. Use *Enterococcus faecium*‡ as a positive control. As negative controls, use *Serratia marcescens*§ (a gram-negative species) and *Aerococcus viridians*||

* Previous editions of *Standard Methods* recommended the use of Pfizer selective enterococcus (PSE) agar as a confirmatory test. Some of the ingredients for this agar are no longer commercially available. BEA agar is a modification of a similar medium;³ it is more selective and provides for rapid growth and efficient recovery of Group D streptococci.²

† BactiCard® Strep test kit, Remel, Inc., Lenexa, KS, or equivalent.

‡ American Type Culture Collection Product No. 35667.

§ American Type Culture Collection Product No. 43862.

|| American Type Culture Collection Product No. 10400.

(a gram-positive species). Avoid using a heavy inoculum. Incubate these controls at the appropriate temperatures for the appropriate time. Read and record results. In addition, include blank samples of sterile dilution water to check the sterility of the process.

6. References

1. MALLMAN, W.L. & E.B. SELIGMANN. 1950. A comparative study of media for the detection of streptococci in water and sewage. *Amer. J. Pub. Health* 40:286.

2. DIFCO LABORATORIES. 1998. Bacto bile esculin azide agar. *In* Difco Manual, 11th ed., p.67. Difco Laboratories, Sparks, Md.
 3. MOORE, D.F., M.H. ZHOWANDAI, D.M. FERGUSON, C. MCGEE, J.B. MOTT & J.C. STEWART. 2006. Comparison of 16SrRNA sequencing with conventional and commercial phenotypic techniques for identification of enterococci from the marine environment. *J. Appl. Microbiol.* 100:1272.
 4. FACKLAM, R.R. & M.D. COLLINS. 1989. Identification of *Enterococcus* species isolated from human infections by a conventional test scheme. *J. Clin. Microbiol.* 27:731.

9230 C. Membrane Filter Techniques

The membrane filtration approach for enumerating enterococci in environmental waters is recommended for analyzing drinking water, source water, wastewater, and both fresh and marine recreational waters. Membrane filter approaches are not recommended for highly turbid waters.

1. Laboratory Apparatus

See Section 9222B.1.

2. Materials and Culture Media

Preferably use a commercially available medium. Follow manufacturer’s instructions for storage and disposal after preparation. If medium must be prepared from basic ingredients, follow directions below.

a. mE agar:¹⁻⁴

| | |
|--------------------------------------|--------|
| Peptone..... | 10.0 g |
| Sodium chloride, NaCl..... | 15.0 g |
| Yeast extract..... | 30.0 g |
| Esculin..... | 1.0 g |
| Cycloheximide..... | 0.05 g |
| Sodium azide, NaN ₃ | 0.15 g |
| Agar..... | 15.0 g |
| Reagent-grade water..... | 1 L |

CAUTION: Cycloheximide is a poison that is extremely toxic if inhaled, swallowed, or absorbed through the skin. Work with this chemical requires special attention and care.

CAUTION: Sodium azide is a dangerous chemical requiring special attention and care. It is toxic and mutagenic. Take precautions to avoid contact with this compound. Azide also can form explosive compounds if it contacts metal pipes.

Heat to dissolve ingredients, autoclave at 121°C for 15 min to sterilize, and cool in a water bath at 44 to 46°C. Mix 0.24 g nalidixic acid in 5 mL reagent-grade water and add a few drops (approximately 0.2 mL) of 0.1N NaOH to dissolve the antibiotic. Pour the entire antibiotic–NaOH mixture through a sterile filter and add to the basal medium. Add 0.15 g 2,3,5-triphenyltetrazolium chloride (TTC) and mix well to dissolve. Pour the agar into 9- × 50-mm petri dishes to a depth of 4 to 5 mm (approximately 4 to 6 mL) and let solidify. The final pH should be 7.1 ± 0.2. If pH is out of range, adjust and retest pH; discard if pH

remains out of range. Store poured, solidified dishes in the dark at 2 to 10°C. Discard after 14 d.

b. EIA substrate:^{1,4}

| | |
|--------------------------|--------|
| Esculin..... | 1.0 g |
| Ferric citrate..... | 0.5 g |
| Agar..... | 15.0 g |
| Reagent-grade water..... | 1 L |

Heat to dissolve ingredients, autoclave at 121°C for 15 min to sterilize, and cool in a water bath at 44 to 46°C. The pH should be 7.1 ± 0.2 after autoclaving. If pH is out of range, adjust and retest pH; discard if pH remains out of range. Pour medium into 50-mm petri dishes to a depth of 4 to 5 mm (approximately 4 to 6 mL) and let solidify. Store poured dishes in the dark at 2 to 10°C. Discard after 14 d.

c. mEI agar:^{3,5} Basal medium ingredients are the same as mE agar (see ¶ 2a). Add 0.75 g indoxyl-β-D-glucoside to basal medium. Heat to dissolve ingredients, sterilize, and cool in a 44 to 46°C water bath. Mix 0.24 g nalidixic acid in 5 mL reagent-grade sterile water; add a few drops of 0.1N NaOH to dissolve; filter-sterilize the solution and add to the mEI medium. Add 0.02 g TTC separately to the mEI medium and mix. Or use the following larger-scale solutions in lieu of the previously mentioned smaller-scale media additions.

1) Nalidixic acid: Add 0.48 g nalidixic acid and 0.4 mL 10N NaOH to 10 mL reagent-grade water and mix. Filter-sterilize the solution and add 5.2 mL per liter of medium. Store solution for no more than 2 weeks at 2 to 10°C.

2) TTC: Add 0.1 g TTC to 10 mL reagent-grade water and warm to dissolve. Filter-sterilize the solution and add 2 mL per liter of medium. Store solution for no more than 2 weeks at 2 to 10°C. Pour the agar into 9- × 50-mm petri dishes to a depth of 4 to 5 mm (approximately 4 to 6 mL) and let solidify. Final pH should be 7.1 ± 0.2. If pH is out of range, adjust and retest pH; discard if pH remains out of range. Store dishes in the dark at 2 to 10°C. Discard after 14 d. (NOTE: This medium is recommended for culturing enterococci in fresh and marine recreational waters.)

d. mEnterococcus agar:

| | |
|--|--------|
| Tryptose..... | 20.0 g |
| Yeast extract..... | 5.0 g |
| Glucose..... | 2.0 g |
| Dipotassium phosphate, K ₂ HPO ₄ | 4.0 g |

| | |
|---|--------|
| Sodium azide, NaN ₃ | 0.4 g |
| 2,3,5-Triphenyltetrazolium chloride | 0.1 g |
| Agar | 10.0 g |
| Reagent-grade water | 1 L |

CAUTION: Sodium azide is a dangerous chemical requiring special attention and care. It is toxic and mutagenic. Take precautions to avoid contact with this compound. Azide also can form explosive compounds if it contacts metal pipes.

Heat to dissolve ingredients. Do not autoclave this solution because doing so will break down selective chemicals. Dispense into 9- × 50-mm petri dishes to a depth of 4 to 5 mm (approximately 4 to 6 mL) and let solidify. Final pH should be 7.2 ± 0.2. If pH is out of range, adjust and retest pH; discard if pH remains out of range. Prepare fresh medium for each set of samples. (NOTE: This medium is recommended for fecal enterococci/streptococci in fresh and marine waters.)

e. Brain–heart infusion (BHI) broth:

| | |
|---|--------|
| Infusion of calf brain | 200 g |
| Infusion of beef heart | 250 g |
| Proteose peptone | 10.0 g |
| Glucose | 2.0 g |
| Sodium chloride, NaCl | 5.0 g |
| Disodium hydrogen phosphate, Na ₂ HPO ₄ | 2.5 g |
| Reagent-grade water | 1 L |

This medium is used for verification tests of presumptive enterococci colonies. Heat to dissolve ingredients. Dispense 10 mL into tubes, and autoclave at 121°C for 15 min. The final pH should be 7.4 ± 0.2 after sterilization. If pH is out of range, adjust and retest pH; discard if pH remains out of range.

To make BHI broth with 6.5% NaCl, add another 60 g NaCl to the BHI broth formulation above.

f. Brain–heart infusion (BHI) agar: Add 15.0 g agar to the ingredients for BHI broth (§ e). Heat to dissolve agar. Autoclave at 121°C for 15 min. The pH should be 7.4 ± 0.2 after sterilization. If pH is out of range, adjust and retest pH; discard if pH remains out of range.

*g. Bile esculin agar:*⁶

| | |
|---------------------------|--------|
| Beef extract | 3.0 g |
| Peptone | 5.0 g |
| Oxgall | 40.0 g |
| Esculin | 1.0 g |
| Ferric citrate | 0.5 g |
| Agar | 15.0 g |
| Reagent-grade water | 1 L |

This medium is used for verification tests of presumptive enterococci colonies. Heat to dissolve ingredients. Dispense 8 to 10 mL into appropriate tubes for slants, or dispense an appropriate volume into a flask for subsequent pouring into dishes. Autoclave at 121°C for 15 min. Do not overheat because this may darken the medium. Cool to 44 to 46°C and slant the tubes or dispense 15 mL into 15- × 100-mm petri dishes. The final pH should be 6.6 ± 0.2 after sterilization. If pH is out of range, adjust and retest pH; discard if pH remains out of range. Store at 2 to 10°C.

3. Procedures

*a. mE method:*¹

1) Selection of sample size and filtration—Filter appropriate sample volumes through a 0.45-µm, gridded, sterile membrane

to give 20 to 60 colonies on the membrane surface. Transfer filter to hardened mE agar (§ 2a) in petri dish, avoiding air bubbles beneath the membrane.

2) Incubation—Invert culture dishes and incubate at 41 ± 0.5°C for 48 ± 4 h.

3) Substrate test—After 48 ± 4 h incubation, carefully transfer filter to EIA medium (§ 2b). Incubate at 41 ± 0.5°C for 20 min.

4) Counting—Count pink to red enterococci colonies that develop a black or reddish-brown precipitate on the underside of the filter.

When counting colonies, use a low-power binocular (10 to 16 magnification), wide-field dissecting microscope or other optical device, along with a cool, white fluorescent light source directed to provide optimal viewing.

b. mEI method:^{3,5}

1) Selection of sample size and filtration—See § 3a1).

2) Transfer and incubation—Transfer filter to hardened mEI agar (§ 2c) in petri dish, avoiding air bubbles beneath the membrane. Invert culture dishes and incubate at 41 ± 0.5°C for 24 ± 2 h.

3) Counting—Count as enterococci any colony (regardless of color) with a blue halo. Such colonies also must be greater than or equal to 0.5 mm in diameter.⁵ Use this criterion at your discretion. When counting colonies, use a low-power binocular (10 to 16 magnification), wide-field dissecting microscope or other optical device, along with a cool, white fluorescent light source directed to provide optimal viewing.

c. m Enterococcus method:

1) Selection of sample size and filtration—See § 3a1).

2) Transfer and incubation—Transfer filter to hardened mEnterococcus agar (§ 2d) in a petri dish, avoiding air bubbles. Let dishes stand for 30 min, then invert and incubate at 35 ± 0.5°C for 48 ± 4 h.

3) Counting—Count all light and dark red colonies as enterococci. When counting colonies, use a low-power binocular (10 to 16 magnification), wide-field dissecting microscope or other optical device, along with a cool, white fluorescent light source directed to provide optimal viewing.

4. Calculation of Enterococci Density

Compute density from sample quantities producing membrane filter counts within the desired 20- to 60- colony range. If the colonies are more dense than this, attempt to provide an estimated number or else note as “too numerous to count” (TNTC) as in Section 9222B.5 (1997) or 922B.4 (2006). Record densities as presumptive enterococci per 100 mL.

5. Verification Tests

Because all of these methods suffer periodically from false positives, verification tests are an important quality-control step. Include a routine verification procedure with each enterococci method, especially if the results will be used as evidence in a court of law. To verify enterococci colonies, pick selected typical colonies from a membrane and streak for isolation onto the surface of a BHI agar plate (§ 2f). Incubate at 35 ± 0.5°C for between 24 ± 2 and 48 ± 4 h.

Transfer a similar-looking portion of a single, well-isolated colony from the BHI agar plate into a BHI broth tube (§ 2e) and

to each of two clean glass slides using a sterile inoculating loop. Incubate the BHI broth at $35 \pm 0.5^\circ\text{C}$ for 24 ± 2 h. To the smear on one slide, add a few drops of freshly prepared 3% hydrogen peroxide. The appearance of bubbles constitutes a positive catalase test and indicates that the colony is not a member of the *Enterococcus* genus. If the catalase test is negative (i.e., no bubbles are produced) make a Gram stain of the second slide. Fecal streptococci and enterococci are gram-positive, ovoid cells, 0.5 to 1.0 μm in diameter, mostly in pairs or short chains.

Transfer, using a sterile inoculating loop, a loopful of the BHI broth to each of the following media: bile esculin agar (incubate at $35 \pm 0.5^\circ\text{C}$ for 48 ± 4 h); BHI agar (incubate at $10 \pm 0.5^\circ\text{C}$ for 48 ± 4 hrs); BHI broth (incubate at $45 \pm 0.5^\circ\text{C}$ for 48 ± 4 h); and BHI broth with 6.5% NaCl (incubate at $35 \pm 0.5^\circ\text{C}$ for 48 ± 4 h).

Growth of catalase-negative, gram-positive cocci on bile esculin agar (¶ 2g) and in 6.5% NaCl broth at 35°C and either in BHI agar at $10 \pm 0.5^\circ\text{C}$ or BHI broth at $45 \pm 0.5^\circ\text{C}$ confirms that the colony belongs to the *Enterococcus* genus.

When using mEI media, the incidence of non-*Enterococcus* species can be as high as 26% in marine environments.⁷ So, additional tests, such as growth in BHI broth at $45 \pm 1^\circ\text{C}$ and assays for pyrrolidonylarylamidase (PYR) and leucine aminopeptidase (LAP) activities⁸ using a test kit,* may be required to confirm *Enterococcus* genus with acceptable accuracy ($\geq 90\%$).

6. Selected Characteristics of *Enterococcus* and *Streptococcus* Species

Selected characteristics⁸⁻¹⁴ of *Enterococcus* and *Streptococcus* species isolated from feces are shown in Table 9230:I.

7. Quality Control

Test each lot of purchased or made medium for performance by inoculating it with three control bacteria. Use *Enterococcus faecium*† as a positive control. As negative controls, use *Serratia marcescens*‡ (a gram-negative species) and *Aerococcus viridians*§ (a gram-positive species). Avoid using a heavy inoculum. Incubate these controls at the appropriate temperatures for the appropriate time. Read and record results. In addition, include blank samples of sterile dilution water to check the sterility of the process.

* BactiCard® Strep test kit, Remel, Inc., Lenexa, KS, or equivalent.

† American Type Culture Collection Product No. 35667.

‡ American Type Culture Collection Product No. 43862.

§ American Type Culture Collection Product No. 10400.

8. References

1. LEVIN, M.A., J.R. FISCHER & V.J. CABELLI. 1975. Membrane filter technique for enumeration of enterococci in marine waters. *Appl. Microbiol.* 30:66.
2. AMERICAN SOCIETY FOR TESTING AND MATERIALS. 2006. Standard Test Method for Isolation and Enumeration of Enterococci from Water by the Membrane Filter Procedure. D5259-92(2006), American Soc. Testing & Materials, West Conshohocken, Pa.
3. MESSER, J.W. & A.P. DUFOUR. 1998. A rapid specific membrane filtration procedure for enumeration of enterococci in recreational water. *Appl. Environ. Microbiol.* 64:678.
4. U.S. ENVIRONMENTAL PROTECTION AGENCY. 2006. Method 1106.1: Enterococci in Water by Membrane Filtration Using membrane-Enterococcus-Esculin Iron Agar (mE-EIA). EPA 821-R-06-008, Off. Water, Washington, D.C.
5. U.S. ENVIRONMENTAL PROTECTION AGENCY. 2006. Method 1600: Enterococci in Water by Membrane Filtration Using membrane-Enterococcus Indoxyl- β -D-Glucoside Agar (mEI). EPA 821-R-06-009, Off. Water, Washington, D.C.
6. PHILLIPS, E. & P. NASH. 1985. Culture media. In E.H. Lennette, A. Ballows, W.J. Hausler, Jr. & H.J. Shadomy, eds. *Manual of Clinical Microbiology*, 5th ed., American Soc. Microbiology, Washington, D.C.
7. SNEATH, P.H.A., N.S. MAIR, M.E. SHARPE & J.S. HOLT, eds. 1986. *Bergey's Manual of Systematic Bacteriology*, Vol. 2, Williams and Wilkins, Baltimore, Md.
8. FERGUSON, D.M., D.F. MOORE, M.A. GETRICH & M.H. ZHOWANDAI. 2005. Enumeration and speciation of enterococci found in marine and intertidal sediments and coastal water in southern California. *J. Appl. Microbiol.* 99:589.
9. FARROW, J.A.E., J. KRUIZE, B.A. PHILLIPS, A.J. BROMLEY & M.D. COLLINS. 1984. Taxonomic studies on *Streptococcus bovis* and *Streptococcus equinus*: Description of *Streptococcus alactolyticus* sp. nov. and *Streptococcus saccharolyticus* sp. nov. *Syst. Appl. Microbiol.* 5:467.
10. KILPPER-BALZ, R. & K.H. SCHLEIFER. 1987. *Streptococcus suis* sp. nov., nom. rev. *Int. J. Syst. Bacteriol.* 37:160.
11. DEVRIESE, L.A., R. KILPPER-BALZ & K.H. SCHLEIFER. 1998. *Streptococcus hyointestinalis* sp. nov. from the gut of swine. *Int. J. Syst. Bacteriol.* 38:440.
12. ROBINSON, I.M., J.M. STROMLEY, V.H. VAREL & E.P. COTO. 1988. *Streptococcus intestinalis*, a new species from colons and feces of pigs. *Int. J. Syst. Bacteriol.* 38:245.
13. MANERO, A. & A.R. BLANCH. 1999. Identification of *Enterococcus* spp. with a biochemical key. *Appl. Environ. Microbiol.* 65:4425.
14. HARWOOD, J., M. BROWNELL, W. PERUSEK & J.E. WHITLOCK. 2001. Vancomycin-resistant *Enterococcus* spp. isolated from wastewater and chicken feces in the United States. *Appl. Environ. Microbiol.* 67:4930.

9230 D. Fluorogenic Substrate Enterococcus Test

The fluorogenic enzyme test uses a hydrolyzable substrate to detect enterococci. The matrix allows enterococci to express the enzyme β -D-glucosidase (esculinase) while preventing non-enterococci from yielding detectable fluorescence. This test can be used in a multiple tube, a multi-well, or a presence-absence format.

1. Principle

See Section 9223. In the method for enterococci, 4-methylumbelliferyl β -D-glucoside (4-MUG) (a fluorogenic substrate), is used rather than esculin. The β -D-glucosidase enzyme hydrolyzes the substrate and causes enterococci to fluoresce under long-wavelength (366-nm) UV light. Non-enterococcus bacteria that produce β -D-glucosidase, such as some species of the genera *Serratia*, *Klebsiella*, and *Aerococcus*, are suppressed; they should not produce a positive response within the incubation time unless there are more than 10^5 CFU/100 mL. The fluorogenic substrate *Enterococcus* sp. test is recommended for analysis of drinking water, source water, wastewater, and both fresh and marine recreational waters. Marine waters require a 1:10 dilution with sterile diluent, such as deionized or distilled water (not sterile seawater), to avoid microbial interference from certain *Bacillus* spp.

2. Materials and Culture Media

Formulations of the fluorogenic substrate are available commercially* for use with the multiple tube, multi-well, or presence-absence procedures. A commercial substrate medium is needed for quality assurance and to ensure uniform results. Store according to the manufacturer's directions. Avoid prolonged exposure of the substrate to direct sunlight. Use substrate media before manufacturer's expiration date, and discard any that is discolored.

3. Procedure

Proceed according to ¶ a, b, or c below.

a. *Multiple-tube procedure*: Select the appropriate number of tubes per sample. When examining freshwater samples, aseptically add a packet of substrate medium to a 100-mL sample, and mix well to dissolve. For a ten-tube MPN, dispense 10 mL of the sample/medium mixture into each of ten tubes. For a five-tube MPN, dispense 20 mL of the sample/medium mixture into each of five tubes. Dilute marine water samples 1:10 (add 10 mL of a well-mixed sample to a sterile container containing 90 mL of sterile diluent as indicated above) and then add substrate medium, mix, and dispense as above.

Cap tubes and incubate at $41 \pm 0.5^\circ\text{C}$ for 24 ± 2 h.

b. *Multi-well procedure*: The multi-well procedure is performed using sterilized, disposable multi-well trays. When examining freshwater samples, aseptically add a packet of substrate medium to a 100-mL sample, mix well to dissolve, pour into the

multi-well tray (following the manufacturer's directions), and seal using the manufacturer's tray sealer. Marine water samples must be diluted 1:10 (add 10 mL of well-mixed sample to a sterile container containing 90 mL of sterile deionized or distilled water). Next, aseptically add a packet of substrate medium to the container, mix well to dissolve the powder, and pour into the multi-well tray (following the manufacturer's directions). To streamline the process, analysts could mix the substrate medium with the sterile deionized or distilled water no more than 30 minutes before adding the sample. Once the sample, substrate medium, and diluent are well mixed, transfer the mixture to a multi-well tray, as indicated above.

Incubate at $41 \pm 0.5^\circ\text{C}$ for 24 ± 2 h.

c. *Presence-absence procedure*: Aseptically, add a packet of medium to a 100-mL sample in a transparent, nonfluorescent container. Cap and mix well. Incubate at $41 \pm 0.5^\circ\text{C}$ for 24 ± 2 h.

4. Computing and Reporting MPN

After incubation, examine the tubes, multi-well trays, or containers for fluorescence in a UV viewing cabinet or a dark environment with a long-wavelength (366-nm) ultraviolet lamp (preferably a 6-W bulb). Keep lamp within 13 cm (5 in) of the sample, facing away from the analyst's eyes and toward the sample. If performing the multi-tube procedure, calculate MPN from the number of positive and negative tubes, as described in Section 9221C. For the multi-well procedure, calculate MPN from the number of positive wells, using the tables provided by the manufacturer. For both methods, correct MPN for any dilution used. If using the presence-absence procedure, report the results as *Enterococcus* sp. presence or absence in 100 mL of sample.

5. Quality Control

Test each lot of commercial medium for performance by inoculating it with three control bacteria. Use *Enterococcus faecium*† as a positive control. As negative controls, use *Serratia marcescens*‡ (a gram-negative species) and *Aerococcus viridians*§ (a gram-positive species). Avoid using a heavy inoculum. Incubate these controls at $41 \pm 0.5^\circ\text{C}$ for 24 ± 2 h. Read and record results. In addition, include blank samples of sterile dilution water to check the sterility of the process.

6. Bibliography

- BUDNICK, G.E., H.T. HOWARD & D.R. MAYO. 1996. Evaluation of Enterolert for enumeration of enterococci in recreational waters. *Appl. Environ. Microbiol.* 62:3881.
- CHEN, C.M., K. DOHERTY, H. GU, G. DICHTER & A. NAQUI. 1996. Enterolert®—A rapid method for the detection of *Enterococcus*

* Enterolert® and Quanti-Tray®, Quanti-Tray®/2000, IDEXX Laboratories, Westbrook, ME.

† American Type Culture Collection Product No. 35667.

‡ American Type Culture Collection Product No. 43862.

§ American Type Culture Collection Product No. 10400.

FECAL ENTEROCOCCUS/STREPTOCOCCUS GROUPS (9230)/Fluorogenic Substrate Enterococcus Test

- spp. Q-488, Abstracts of the 96th General Meeting of the American Society for Microbiology, p. 464.
- FRICKER, E.J. & C.R. FRICKER. 1996. Use of defined substrate technology and a novel procedure for estimating the numbers of Enterococci in water. *J. Microbiol. Meth.* 27:207.
- ABBOT, S., B. CAUGHLEY & G. SCOTT. 1998. Evaluation of Enterolert[®] for the enumeration of enterococci in the marine environment. *New Zealand J. Marine Freshwater Res.* 32:505.
- ECKNER, K.F. 1998. Comparison of membrane filtration and multi-tube fermentation by the Colilert and Enterolert methods for detection of waterborne coliform bacteria, *Escherichia coli* and Enterococci used in drinking water and bathing water quality monitoring in southern Sweden. *Appl. Environ. Microbiol.* 64:3079.
- AMERICAN SOCIETY FOR TESTING AND MATERIALS. 1999. Standard Test Method for Enterococci in Water Using Enterolert[™]. D6503-99, Annual Book of ASTM Standards, Vol. 11.02. American Soc. Testing & Materials, West Conshohocken, Pa.