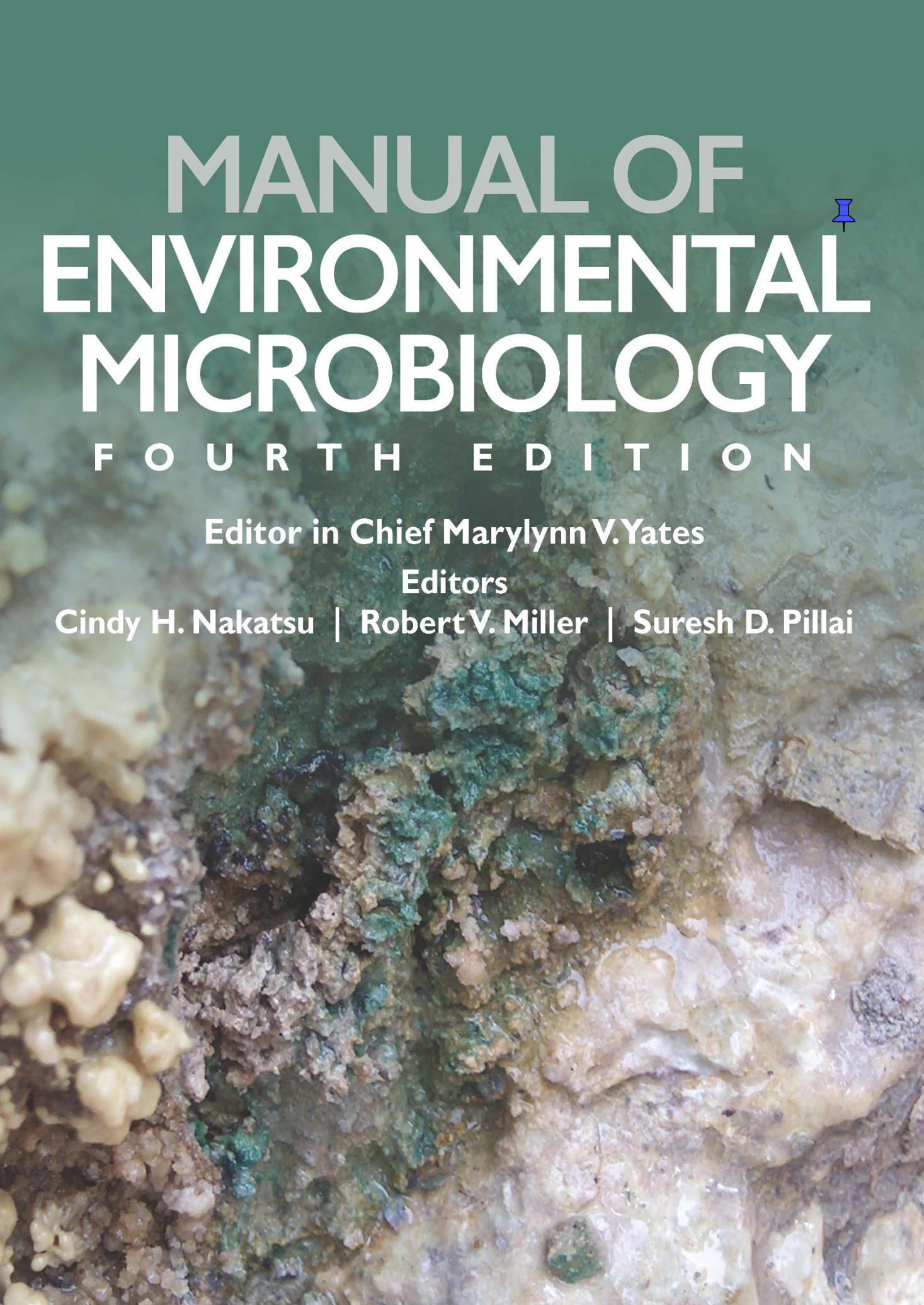
MANUAL OF ENVIRONMENTAL MICROBIOLOGY

FOURTH EDITION

Editor in Chief Marylynn V. Yates

Editors

Cindy H. Nakatsu | Robert V. Miller | Suresh D. Pillai



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Library of Congress Cataloging-in-Publication Data

Names: Yates, M. V. (Marylynn V.), editor. | Nakatsu, Cindy H., editor. | Miller, Robert V. (Robert Verne), 1945- editor. | Pillai, Suresh D., 1962- editor.
Title: Manual of environmental microbiology / editor in chief, Marylynn V. Yates; editors, Cindy H. Nakatsu, Robert V. Miller, Suresh D. Pillai.
Description: Fourth edition. | Washington, DC : ASM Press, [2016] |
Includes bibliographical references and index.
Identifiers: LCCN 2016014816 (print) | LCCN 2016016986 (ebook) |
ISBN 9781555816025 (hardcover) | ISBN 9781555818821 ()
Subjects: LCSH: Microbial ecology–Laboratory manuals. | Sanitary microbiology–Laboratory manuals.
Classification: LCC QR100 .M36 2016 (print) | LCC QR100 (ebook) | DDC 577.8–dc23
LC record available at https://lccn.loc.gov/2016014816

Printed in Canada

10 9 8 7 6 5 4 3 2 1

Address editorial correspondence to
ASM Press, 1752 N St., N.W.,
Washington, DC 20036-2904, USA

Send orders to ASM Press, P.O. Box 605, Herndon, VA 20172, USA
Phone: 800-546-2416; 703-661-1593
Fax: 703-661-1501
E-mail: books@asmusa.org
Online: http://www.asmscience.org

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INTRODUCTION

MARYLYNN V. YATES

1.1.1

There are bred certain minute creatures which cannot be seen by the eyes, which float in the air and enter the body through the mouth and nose and there cause serious diseases.

— Marcus Terentius Varro, *De Re Rustica* [On Agriculture], 37 BC

Environmental microbiology might be considered by some to be an ill-defined subject: Where does the environment begin, and where does it end? From Marcus Terentius Varro's observations regarding unseen "minute creatures" more than two millennia ago to Antonie van Leeuwenhoek's first glimpse of the "animalcula" beneath his lens, there is no place on Earth—from thermophilic, acidic springs to the air we breathe to the deepest subsurface locations we have yet been able to reach—where people have looked and *not* found microorganisms of some type. The domain of what may be considered environmental microbiology thus continues to expand beyond the textbook definition of "the study of microorganisms existing in natural and artificial environments." At the same time, our knowledge of microorganisms is increasing at an ever-more rapid rate as the result of incredible improvements in analytical methodology, especially at the molecular level. When compiling a manual of this nature, therefore, how does one determine what to include and what to exclude? In the end, the editors decided to showcase as much information as possible on some of the most important areas of environmental microbiology, to provide a clear sense of the possibilities presented by the existence of microorganisms in various environments. Further and more detailed information can be found in the wealth of expertly chosen references within each chapter.

This edition of the *Manual of Environmental Microbiology* has been reorganized, with new and updated sections to provide recent information on topics of importance to the field. The General Methodology volume, beginning with Section 2.1, is devoted to methodologies—the "how" of environmental microbial studies from analytical detection to sample collection. The methods presented in this volume are the basis of how we understand the microbial world around us and are used across environmental microbial disciplines and applications. General Methodologies begins with tried-and-true culture-based and physiological detection methods, microscopy-based methods, and molecular target–specific detection methods, before moving on to address the increasingly important study of microbes at the community level, thanks to the advent of next-generation sequencing technologies. This volume concludes with information on critical topics that are sometimes overlooked in environmental microbiology: quality assurance and quality control, which increase the usefulness and reliability of analytical data for downstream decision making.

The next volume, Environmental Public Health Microbiology, surveys the microorganisms in the water, air, or soil that can cause illness when humans are exposed to them. These potentially pathogenic microorganisms can take many avenues en route to their target host, ultimately leading to substantial health impacts and considerable negative economic and social consequences to the communities involved. The importance of understanding microbial distribution and detection for public health cannot be understated. This volume includes chapters describing the field of microbial source tracking; methods and approaches for determining predominant sources of fecal pollution of water, which has become a critical component of assessment; and determining ways to better protect water from microbial contamination. It culminates in a section on microbial risk assessment, integrating the information in the earlier sections and providing perspective for informed decision making and rational project design.

The Microbial Ecology volume addresses the various ways microorganisms can be classified and their interconnected relationships, with each other and with macroorganisms, and impacts on the environment. Some microorganisms are native to their environment and perform essential services, such as cycling nutrients or interacting with other organisms in ways that enable them to perform functions and/or inhabit environments where they would otherwise not exist. Other microorganisms may be introduced to a new environment, changing the overall ecosystem. This volume begins with an exploration of the theories behind microbial genome evolution and the evolutionary ecology of microorganisms. The subject matter then delves into more specific environments, including aquatic environments, environments with extreme conditions (extreme acidity, high salinity, low biomass, and high temperatures), and the unique environment inside the gastrointestinal tract of animals, an area of increasing study and data.

doi:10.1128/9781555818821.ch1.1.1

The final volume, Biodegradation and Biotransformation, focuses on the varied ways that microorganisms can transform or degrade nearly any natural or anthropogenic chemical compound. Since the earliest eras of the Industrial Revolution, study of microorganisms for these processes has been an active area of research. Microbial transformations of natural substances can make otherwise unavailable nutrients accessible by living organisms. Additionally, microorganisms can be used to degrade harmful contaminants, either by using the biochemical pathways naturally present or by employing genetic modification. These abilities have been exploited in numerous situations in which soil or water has been contaminated by organic or inorganic chemicals as well as for a variety of industrial and commercial uses.

Microorganisms play a critical role in the health and well-being of the planet and the human, animal, and plant life that dwells here. Our understanding of them is crucial for maintaining the environment. This fourth edition of the *Manual of Environmental Microbiology* builds on the solid foundation created by the previous three editions, and we are indebted to those who came before us. This edition is truly a collaborative triumph, and is the work of 19 editors and more than 180 contributing authors who generously gave their time, effort, and expertise. It is my hope that this reference serves as an informative and reliable source of information for current and future endeavors in environmental microbiology and inspires the next generation of researchers as they move along their path in this growing and important area.

Detection of Specific Taxa Using Chromogenic and Fluorogenic Media

MOHAMMAD MANAFI

2.1.1

OVERVIEW OF FLUOROGENIC AND CHROMOGENIC MEDIA IN ENVIRONMENTAL MICROBIOLOGY

Over the past 20 years, a number of selective chromogenic and fluorogenic media for detection and enumeration of most important bacteria and yeast in particular in food and water have been developed and marketed. Fluorogenic enzyme substrates generally consist of a specific substrate for the specific enzyme such as sugar or amino acid and a fluorogen such as 4-methylumbelliferone, being able to convert UV light (365 nm) to visible light. Chromogenic enzyme substrates are compounds that act as the substrate for specific enzymes and change color due to the action of the enzymes. Most commercially available chromogenic media have exploited indoxylic substrate. Indoxyl and its halogenated derivatives can be derivatized to form a range of esters. Halogenation of the indoxyl molecule has an effect on the color and intensity of the chromogen. 5-Bromo-4-chloro-indoxyl forms a green/blue dye, whereas 5-bromo-6-chloro-indoxyl forms a magenta dye. The incorporation of such substrates into a selective medium can eliminate the need for subculture and further biochemical tests to establish the identity of certain microorganisms. There have been some review papers dealing with the use of these substrates in environmental and clinical microbiology (1–3). A useful review describes the chemistry of chromogens and fluorogens in culture media (4). Some chromogenic or fluorogenic media (e.g., agar Listeria according to Ottaviani and Agosti [ALOA] and tryptone-bile-glucuronic [TBX] have therefore been taken up in the standardized ISO methods (ISO 11290-1/A1:2004 and ISO 16649:2001, respectively). This review describes some recent developments in chromogenic and fluorogenic culture media in microbiological diagnostic in particular in food and water microbiology.

GRAM-NEGATIVE BACTERIA

E. coli

E. coli is an important indicator of fecal contamination in samples from the food processing and water purification plants. The new enzymatic definition of *E. coli* is the possession of uidA gene coding for ß-D-glucuronidase (GUD) as an indicator for *E. coli*. GUD is an enzyme that catalyzes the hydrolysis of ß-D-glucopyranosiduronic acids into their corresponding aglycons and D-glucuronic acid (5, 6). GUD activity is measured by using different chromogenic and fluorogenic substances such as 4-methylumbelliferyl-ß-D-glucuronide (Fig. 1, MUG) or 5-bromo-4-chloro-3-indolyl-ß-D-glucuronide. MUG has been incorporated into different media, including lauryl sulfate broth, lactose broth, m-Endo broth, EC broth, violet red bile agar, ECD agar, MacConkey agar and m-FC agar, and are described earlier (7). The disadvantage of incorporating MUG into solid media is that fluorescence diffuses rapidly from the colonies into the surrounding agar. The chromogenic substrate 5-bromo-4-chloro-3-indolyl-ß-D-glucuronide (BCIG) is added into TBX agar. GUD cleaves BCIG, and the released chromosphere causes distinct blue-green-colored colonies of *E. coli* and complies with the ISO/DIS Standard 16649 for the enumeration of *E. coli* in food and animal feeding stuffs.

Coliform

The new definition of coliforms, which is not method related, is the possession of lacZ gene coding for ß-D-galactosidase which is responsible for the cleavage of lactose into glucose and galactose. The determination of ß-D-galactosidase is accomplished by using substrates such as 6-bromo-3-indolyl-ß-galactopyranoside (Salmon-Gal, red), 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (Fig. 2, blue) or fluorogenic substrate 4-methylumbelliferyl-ß-D-galactopyranoside (blue fluorescence under UV light). Attempts were made to enhance the coliform assay response by adding 1-isopropyl-ß-D-thiogalactopyronaside (IPTG) to the media (8) increasing the ß-D-galactosidase activity by improving the transfer of the substrate and/or enzyme across the outer membrane. IPTG molecule induces the lac operon but unlike the natural substrate it cannot be hydrolysed by ß-galactosidase.

E. coli and Coliforms

Commercially available media have been developed which permit rapid simultaneous detection of *E. coli* and coliforms (Table 1) such as the Colilert system (IDEXX, Branford, CT), LMX broth, and Readycult coliforms (Merck, Germany). Other representative examples are MI agar (Becton-Dickinson, USA), CHROMagar CCA (CHROMagar, France), or Chromocult coliforms (Merck). Comparative studies have been done and are reviewed elsewhere (1). Schets et al. (47) compared Colilert with Dutch standard enumeration methods for *E. coli* and coliforms in water and

doi:10.1128/9781555818821.ch2.1.1

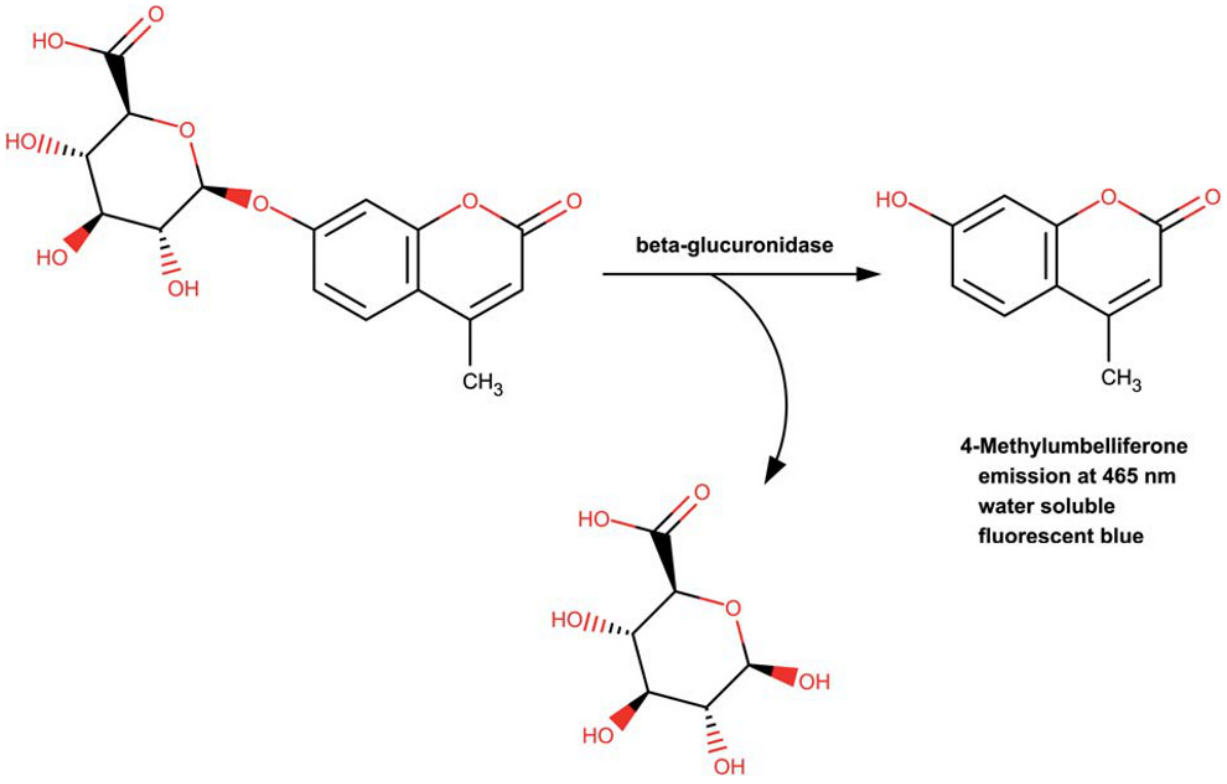

FIGURE 1 Structure of 4-Methylumbellieryl-beta-D-glucuronide (MUG) for detection of *E. coli*. doi:10.1128/9781555818821.ch2.1.1.f1

found that Colilert gave false-negative results in samples with low numbers of *E. coli* or total coliforms. An evaluation of a number of presence/absence (P/A) tests for coliforms and *E. coli*, including LMX broth (Merck) and Colilert (IDEXX) has been published under the Department of the Environment series in the United Kingdom (48). The study concludes that there is no P/A test that is best at all locations for both coliforms and *E. coli*, and as there can be marked ecological differences between sources it is important that particular P/A tests are validated in each geographical area before use. The MI agar method (10) containing indoxyl-ß-D-glucuronide and 4-methylumbelliferyl-ß-D-galactopyranoside for the simultaneous detection of *E. coli* and total coliforms, was compared with the approved method by the use of wastewater-spiked tap water samples. Overall, weighted analysis of variance (significance level 0.05) showed that the MI agar recoveries of total coliforms and *E. coli* were significantly higher than those of mEndo agar. MI agar method is approved for detecting total coliforms and *E. coli* under the total coliforms rule and for enumerating total coliforms under the surface water treatment rule in the United States. Byamukama et al. (49) described the quantification of *E. coli* contamination with Chromocult coliform agar from different polluted sites in a tropical environment. It proved to be efficient and feasible for determining fecal pollutions in the investigated area within 24 h. Blue coloration in the broth indicates the presence of total coliforms and/or *E. coli*.

E. coli O157:H7

E. coli O157:H7 is an important foodborne pathogen and can cause diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome. Several chromogenic media have been applied to

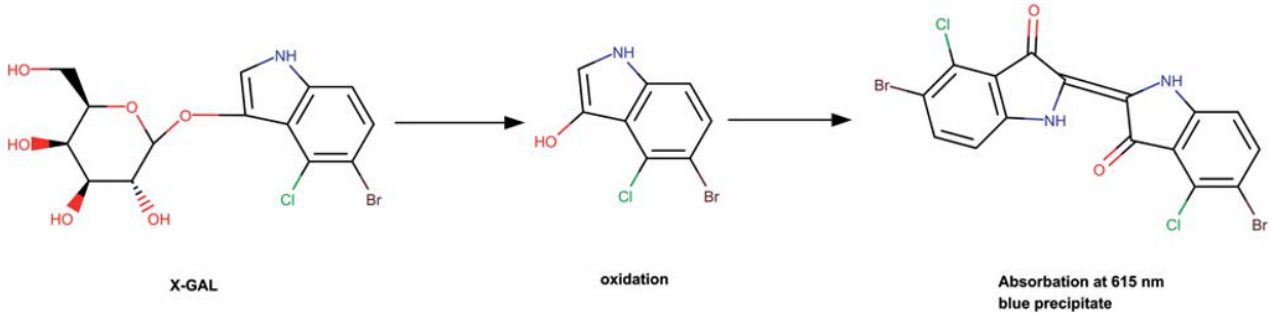

FIGURE 2 Structure of X-GAL (5-bromo-4chloro-3-indolyl-beta-D-galactopyranoside) for detection of coliforms. doi:10.1128/9781555818821.ch2.1.1.f2

TABLE 1 Example of commercial chromogenic and fluorogenic culture media

Target	Medium/colony color	Company	Reference
E. coli/coliforms	Readycult (*E. coli*: blue fluorescence /coliforms blue green coloration)	Merck (Germany)	9
	MI-agar (*E. coli*: blue colonies/coliforms blue fluorescence)	BD (USA)	10
	LMX broth (*E. coli*: blue fluorescence /coliforms blue green coloration)	Merck (Germany)	11
	Chromocult (*E. coli*: blue colonies/coliforms red colonies)	Merck (Germany)	12
	Coli Complete (*E. coli*: blue fluorescence /coliforms blue green coloration)	Biocontrol (USA)	13
	m-Coliblue (*E. coli*: blue colonies/coliforms red colonies)	Hach (USA)	14
E. coli O157:H7	CHROMagar O157 (red)	CHROMagar (France)	15
	Rainbow agar O157 (black)	Biolog (USA)	16, 17
	BCM *E. coli* O157:H7 (blue-black)	Biosynth (Switzerland)	18
Salmonella spp.	SM ID medium and SM ID 2-medium (red)	BioMérieux (France)	19
	BBL CHROMagar Salmonella (mauve)	BD (USA)	20
	Harlequin Salmonella ABC (green)	lab m (England)	21
	Rambach agar (red)	Merck (Germany)	22
	Salmonella-Chromogen-agar	Oxoid (England)	23
Cronobacter sakazakii	DFI agar (blue)	Oxoid (England)	24
	ESPM (blue)	R&F Laboratories	25
Yersinia enterocolitica	CHROMagar *Yersinia*, pathogenic (mauve) and nonpathogenic (blue).	CHROMagar (France)	26
ESBL	ChromID ESBL	bioMérieux (France)	27
	Brilliance ESBL agar	Oxoid (England)	27
Vibrio sp.	CHROMagar Vibrio	CHROMagar (France)	28
	Bio-Chrome Vibrio medium	BioMedix, USA	29
Staphylococcus aureus	CHROMagar *Staph.aureus*	CHROMagar (France)	30
	S. aureus ID agar (SA ID)	BioMérieux (France)	31
MRSA	CHROMagar MRSA	CHROMagar (France)	32
	MRSASelect	Bio-Rad (USA)	32
Listeria monocytogenes	BCM *Listeria monocytogenes* plating medium	Biosynth (Switzerland)	33
	CHROMagar Listeria	CHROMagar (France)	34
	Oxoid Chromogenic Listeria agar (OCLA)	Oxoid (England)	34
	Rapid'L.mono	Sanofi (France)	34
	Agar Listeria (ALOA)	AES (France)	34
Bacillus group	BCM *Bacillus cereus* group	Biosynth (Switzerland)	35
	B. cereus/*B. thuringiensis* agar	R&F Laboratories (USA)	36
	Chromogenic *Bacillus Cereus* agar (CBC)	Oxoid (England)	37
	Bacillus anthracis agar	R&F Laboratories (USA)	38
Enterococcus spp	mEI agar (indoxyl-ß-D-glucoside)	BD (USA)	39
	Chromocult Enterococci broth and Readycult/blue	Merck (Germany)	40
	Chromocult Enterococci agar/red	Merck (Germany)	41
	Enterolert (4-methylumbelliferone -ß-D-glucoside)/ blue fluoresence	Idexx (USA)	42
VRE	ChromID VRE agar	bioMérieux (France)	43
	CHROMagar VanRE	CHROMagar (France)	44
Clostridium perfringens	Chromoselect CP/green	Sigma (Switzerland)	45
Clostridium difficile	ChromID C. *difficile* agar	Biomérieux (France)	46

the detection of *E. coli* O157:H7 from food samples. Most are based on similar principles, relying on nonfermentation of sorbitol and/or rhamnose and lack of ß-glucuronidase activity (50). A second chromogenic substrate (for α- or ß-galactosidase) may be used to highlight the presence of *E. coli* O157:H7 among nonreactive background flora. Some selective media increase the effectiveness of *E. coli* O157:H7 isolation, including Rainbow agar O157 (RB, Biolog, Hayward, USA), BCM O157:H7 (BCM, Biosynth AG, Staad, Switzerland), Fluorocult *E. coli* O157:H7 (H7, Merck), and CHROMagar O157 (Chromagar).

The performance of three chromogenic agars—Rainbow Agar O157, CHROMagar O157, and O157:H7 ID agar—with a large collection of verotoxigenic and nontoxigenic *E. coli* strains have been tested (15, 16). Another study compared Rainbow Agar O157, BCM O157:H7, and Fluorocult HC with the conventional Sorbitol MacConkey have showed the clear advantage of the chromogenic media for the isolation of *E. coli* O157:H7 from raw ground beef and raw drinking milk (51). The behavior of *E. coli* O157:H7 was studied during the manufacture and ripening of raw goat milk lactic cheeses using O157:H7 ID (bioMérieux) and CT-SMAC agar (52). In conclusion, this new chromogenic media O157:H7 ID proved to be well suited and simplified many of the inherent problems associated with plate confirmation of *E. coli* O157:H7 using SMAC as subculture media.

Salmonella

The conventional media for the detection of *Salmonella* have a very poor specificity, creating an abundance of false

positives (such as *Citrobacter*, *Proteus*) among the rare real positive *Salmonella*. On Rambach agar, *Salmonella* strains gave red colonies because of their ability to acidify neutral red by fermentation of propylene glycol (22). This medium employs X-gal for ß-D-galactosidase positive coliforms. However, the strains of *S. typhi* and *S. paratyphi* fail to produce acid from propylene glycol, resulting in colorless colonies on Rambach agar. Furthermore, the strains of *S. enterica* subspecies *S. arizona* show blue-violet colonies on this medium (53). On SM-ID agar (bioMérieux) *Salmonella* colonies are detected by their distinctive red coloration, while coliforms appear blue, violet, or colorless. A rare study in food microbiology using chromogenic media has been done by Schönenbrücher et al. (23). The draft ISO 6579:2002 was compared to the European gold standard (DIN EN 12824:1998), including the three chromogenic plating media AES *Salmonella* agar plate, Oxoid *Salmonella* chromogen media, and Miller-Mallinson agar. MUCAP-test (Biolife, Italy) is a confirmation test for *Salmonella* species based on the rapid detection of caprylate esterase, using fluorogenic 4-methylumbelliferyl-caprylate. In the presence of C_8 esterase the substrate is cleaved with the release of 4-methylumbelliferone. Strong bluish fluorescence indicates the presence of *Salmonella* spp. Since most of the false-positive strains are oxidase positive, the combination of MUCAP and the oxidase test is recommended (54). Other chromogenic media for detection of *Salmonella* spp. are listed in Table 1.

Shigella

The chromogenic *Shigella* spp. agar (R&F Laboratories, West Chicago, IL), offers intermediate selectivity using bile salts and antibiotic supplementation (55). Colony color enhancements created by the chromogenic *Shigella* spp. agar results from the lack of the *Shigella* to utilize the carbohydrates and metabolize the chromogens. Presumptive positive colonies of *Shigella* sp. appears as white/clear colonies 1.0–3.0 mm diameter with or without a clear ring after 22–28 h incubation, whereas the colonies of other members of the *Enterobacteriaceae* are variously colored. The second medium, xylose galactosidase medium contains bile salts, D-xylose and 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside and IPTG as biochemical marker (56). *S. sonnei* give green colonies (xylose negative and ß-gal positive) and colorless colonies for other species of *Shigella* (xylose and ß-gal negative).

Cronobacter (Enterobacter) *sakazakii*

C. sakazakii is a recently described genus that is composed of six genomospecies. *C. sakazakii* is an occasional contaminant of powdered infant formula that can cause rare but severe foodborne infections such as meningitis, necrotizing enterocolitis, bacteremia, and sepsis in infants. *C. sakazakii* possesses α-glucosidase activity and a number of selective, chromogenic agars for *C. sakazakii* are based on the detection of this enzyme (57, 58, 59). 5-Bromo-4-chloro-3-indolyl-α-D-glucopyranoside (X-α-Glc) is added to a basal medium to differentiate *C. sakazakii* strains from other members of the *Enterobacteriaceae*. The enzyme α-glucosidase hydrolyses X-α-Glc, giving blue-colored colonies on DFI agar (Oxoid, UK), ESIA agar (recommended by ISO/TS 22964, AES, France), Compas (Biokar, France), or Chromocult *E. sakazakii* (Merck,), which are commercially available. Hi Chrome *E. sakazakii* agar (Sigma-Aldrich) also complies with the formulation recommended in ISO/TS 22964.

A dual chromogenic medium (ESPM) is available from R&F Laboratories (25), as well as from bioMérieux (ChromID *Sakazakii*). A second substrate, 5-bromo-4-chloro-3-indolyl-ß-cellobioside, is included in this medium. A mix of carbohydrates such as D-sorbitol, D-arabitol, and adonitol and antibiotics vancomycin and cefsulodin are added. *Cronobacter* colonies appear blue-black, with most other species appearing as colorless, yellow, or green colonies. 4-Methylumbelliferyl-α-D-glucoside, the fluorogenic substrate of α-D-glucosidase, was used as a selective marker to develop a differential medium for *C. sakazakii*. This bacterium showed strong fluorogenic characteristics clearly distinguishable from other microorganisms. On the basis of reducing background noise, an optimum basal medium and nitrogen source were selected. Incubation conditions were optimized (60, 61).

Extended-Spectrum β-lactamase (ESBL)

Rapid detection of extended-spectrum β-lactamase (ESBL)–producing Gram-negative bacilli in surveillance samples of high-risk patients allows early optimization of antimicrobial therapy and timely introduction of infection control procedures. BLSE (AES Chemunex), chromID ESBL (bioMérieux), and Brilliance ESBL agar (Oxoid) for rapid detection of ESBL-producing *Enterobacteriaceae* are the few available chromogenic media (27). A comprehensive study from Germany showing a high prevalence of TEM-, CTX-M-, and SHV-type ESBLs in *Enterobacteriaceae* isolated from retail chicken meat. The high rate of coresistance to different classes of antibiotics in the ESBL producers might reflect the common veterinary usage of these and related substances (62).

Yersinia enterocolitica

Weagant (63) described an agar, so-called YeCM, for detection of potentially virulent *Y. enterocolitica*. This agar contains cellobiose as the fermentable sugar, a chromogenic substrate, and selective inhibitors for suppression of colony formation by many competing bacteria. The strains of biotypes 1B, and biotypes 2–5 formed convex, red bull's-eye colonies on YeCM. *Y. enterocolitica* biotype 1A and other related *Yersinia* formed colonies that were purple/blue on YeCM while they formed typical red bull's-eye colonies on CIN agar. Commercially available CHROMagar *Yersinia* is a new chromogenic medium for the presumptive detection of virulent *Y. enterocolitica* in stools (26).

Campylobacter

R & F *Campylobacter jejuni*/*C. coli* is a new chromogenic plating medium. Presumptively positive colonies of *C. jejuni*/*C. coli* appear as dark salmon flat to convex colonies, 1.0–2.0 mm in diameter with and without a clear ring after 48 h at 41–42°C under microaerophilic conditions. Brilliance CampyCount Agar (Oxoid) is specifically designed for accurate, specific, and easy enumeration of *C. jejuni* and *C. coli* from poultry testing. It is a transparent medium on which Campylobacter produce distinct dark red colonies, making identification and counting of Campylobacter significantly easier than on traditional charcoal or blood-containing agar.

Vibrio

Vibrio parahaemolyticus has been one of the most important foodborne pathogens in Japan since the 1960s, and a large epidemic was caused by the pandemic serotype O3:K6 from 1997 to 2001. The traditional most-probable number (MPN) method using thiosulfate-citrate-bile-salts-sucrose medium (TCBS) for detecting *V. parahaemolyticus* cannot differentiate growth of *V. parahaemolyticus* from *Vibrio vulnificus*

or *Vibrio mimicus*. Suspicious colonies grown on TCBS need to be confirmed with lengthy biochemical tests. Recently, a chromogenic medium (Bio-Chrome *Vibrio* medium, Bio-Medix, USA) was developed for differentiating *V. parahaemolyticus* from other *Vibrio* species based on the formation of purple colonies by *V. parahaemolyticus* (29, 64). To distinguish *V. vulnificus* from other pathogens that cause necrotizing fasciitis, Nakashima et al. (65) have developed a selective isolation culture agar plate (Chromochecker *Vibrio* Agar) for use in environmental monitoring and the clinical setting. There are a few other chromogenic media for detection of *Vibrio* spp., which shows different color between *V. parahaemolyticus*, *V. cholera*, and *V. vulnificus* such as VVM (66), Chromagar *Vibrio* (28, 67, 68), and *Vibrio* ID (bioMérieux) and further evaluation studies are needed.

GRAM-POSITIVE BACTERIA

Enterococci

Enterococci are used as indicators of fecal contamination of water and food. On the basis of 16S rRNA cataloging, the genus *Streptococcus* was separated during the 1980s into the three genera *Enterococcus*, *Lactococcus*, and *Streptococcus*. Consequently, bacteria previously named *S. faecalis*, *S. faecium*, *S. avium*, and *S. gallinarum* were transferred to the revised genus *Enterococcus* as *E. faecalis*, *E. faecium*, *E. avium*, and *E. gallinarum*, respectively (69). More than 100 modifications of selective media have been described in the past for isolating fecal streptococci or enterococci from various specimens. In most literature, Slanetz-Bartley (70) agar is treated as a suitable MF medium to detect fecal enterococci from a water sample.

The use of chromogenic and fluorogenic substrates for detection of enterococci has received considerable attention (39, 71, 72). Substrates such as 5-bromo-4-chloro-3-indolyl-ß-D-glucopyranoside (XGLU) and indoxyl--ß-D-glucoside were already described for detection of ß-D-glucosidase activity (40). Enterococci produce an insoluble indigo blue complex, which diffuses into the surrounding media, forming a blue halo around the colony. Chromocult *Enterococci* broth (Merck) and Readycult Enterococci (Merck) utilize the ß-D-glucosidase reaction as an indicator for enterococci. XGLU is liberated and rapidly oxidized to bromochloroindigo, which produces blue color in Chromocult broth. Chromocult enterococci agar (EKA, Merck) uses a chromogenic mix in a selective agar; enterococci cleave chromogenic substrates in this medium and show red colonies on the plate. Nonenterococci produce colorless, blue/violet, or turquoise colonies (41).

4-Methylumbelliferyl-ß-D-glucoside (MUD) has been proven useful to detect enterococci in water. This substrate, when hydrolyzed by enterococcal ß-D-glucosidase, releases 4-methylumbelliferone, which exhibits fluorescence under a UV lamp (365 nm). Enterolert (IDEXX Laboratories), and a Microtiter plate MUST (Sanofi, France) utilize the nutrient indicator substrate MUD as a fluorogenic substrate detecting ß-D-glucosidase. The MUD method tended to give slightly lower recoveries than the agar cultivation methods with some target species at 44°C, but recoveries were better than at 41°C (73). It has to be mentioned that there may be some problems with other glucosidase-positive bacteria such as *Aerococcus* spp., in particular in natural contaminated water samples.

Membrane-*Enterococcus* indoxyl-ß-D-glucoside agar (mEI agar) is a selective culture medium used for the chromogenic detection and enumeration of enterococci in water by the single-step membrane filtration technique. It conforms to the U.S. Environmental Protection Agency Approved Method 1600: Enterococci in Water by Membrane Filtration. Rhodes and Kator (74) evaluated mEI agar with respect to specificity and recovery of enterococci from environmental waters. Extending incubation from 24 to 48 h improved enterococci recovery, but 77% of the colonies classified as nontarget were confirmed as enterococci. Messer and Dufour (75) modified the medium by reducing the triphenyltetrazolium chloride from 0.15 to 0.02 g/liter and adding 0.75 g indoxyl ß-D-glucoside per liter. The new MF medium, mEI medium, detected levels of enterococci in 24 h comparable to those detected by the original mE medium in 48 h, with the same level of statistical confidence.

Using XGLU Chromocult Enterococci broth (CEB) (Merck) and Readycult Enterococci (Merck) utilizes the ß-D-glucosidase reaction as an indicator of the presence of enterococci. XGLU is liberated and rapidly oxidized to bromochloroindigo which produces blue color in Chromocult broth as well as blue colored enterococci colonies in Chromocult Enterococci agar. Other ß-D-glucosidase-producing organisms were suppressed by the sodium azide content of the broth (40). The results obtained with pure cultures showed that 97% of the strains, which gave positive results, were identified as enterococci (*E. fecalis*, *E. faecium*, *E. durans*, *E. casseliflavus*, and *E. avium*). The false-positive strains (3%) were *Leuconostoc*, *Lactococcus lactis lactis*, and *Aerococcus* spp.

Clostridium perfringens

C. *perfringens* is an anaerobic, Gram-positive, spore-forming rod-shaped bacterium. They are widespread in the environment and also found in the digestive systems of humans and animals. C. *perfringens* is capable of surviving in soil and water for extended periods of time. Detection and enumeration of C. *perfringens* from food and environmental samples is usually based on cultivation on agar plates, but an MPN technique can also be applied. Several selective culture media for the detection and enumeration of C. *perfringens* are based on sulfite reduction as a differential characteristic and cycloserine as a selective agent. Selectivity of cultivation can be increased by using an elevated incubation temperature, since C. *perfringens* is able to grow rapidly at 45°C. The shortcoming of the selective culture and growth conditions is the poor recovery of injured vegetative cells of C. *perfringens*.

TSC-4-methylumbelliferyl phosphate (MUP) agar incorporates sodium metabisulfite and ferric ammonium citrate as an indicator for sulfite reduction and the disodium salt of MUP for detection of acid phosphatase. D-Cycloserine inhibits the accompanying bacterial flora and causes the colonies to remain smaller. All black colonies on this medium that emit light blue fluorescence when exposed to a UV lamp (365 nm) were counted as presumptive C. *perfringens*. The disadvantage of incorporating MUP into agar is that fluorescence diffuses rapidly from the colonies into the surrounding agar, and therefore the interpretation of the plates is difficult and confusing. The use of (MUP and ortho-nitrophenyl-β-d-galactopyranoside for the identification of C. perfringens was investigated (76). A liquid assay containing both compounds was a highly specific alternative method for C. perfringens confirmation, reducing incubation time from 48 to only 4 h. The new Chromoselect CP agar is now available, which can be used in the water microbiology (45). C. *perfringens* colonies give green-colored colonies, other

strains of clostridia give violet or blue colonies (Sigma, Schwitzerland).

Clostridium dificille

C. *difficile* is the major cause of colitis and pseudomembranous colitis associated with antibiotic treatment. ChromID C. *difficile* agar (bioMérieux) is a new chromogenic medium for isolation and identification of C. *difficile* in 24 h (46).

Listeria monocytogenes

L. monocytogenes is a human and animal pathogen that is widespread in nature. The organism is a transient constituent of the intestinal flora excreted by 1–10% of healthy humans. This organism can survive for many years in the cold in naturally infected sources. *L. monocytogenes* has been isolated from a wide variety of foods, including dairy products, meats, and fish. Although most of the foodborne listeriosis outbreaks have been linked to the consumption of dairy products, recent sporadic cases have been associated with meats as well as other foods. Several virulence genes have been identified in pathogenic *Listeria* spp. The lecithinase operon contains the gene *plc*B. *Plc*B encodes a lecithinase involved in cell-to-cell spread. The gene *plc*A encodes a phosphatidylinositol-specific phospholipase C (PI-PLC) that may contribute to the lysis of the phagosomal membrane. These virulence genes (*plc*A, *plcB*) occur in pathogenic *L. monocytogenes* and *L. ivanovii* (77, 78).

The detection of *L. monocytogenes* in food and environmental samples by cultivation includes enrichment step(s) for resuscitation of injured cells and concentration of the cells, followed by plating on selective media and confirmation of the tentative identifications of suspected colonies by biochemical tests. Current conventional culture techniques take approximately 1 week to complete. The recent methodology development has focused on the optimization of enrichment steps and the development of new differential culture media to obtain faster and more reliable detection of *L. monocytogenes*. Selective chromogenic *L. monocytogenes* plating media offer rapid economic detection and enumeration of pathogenic *Listeria* spp. within 24 or 48 h of incubation at 36 ± 1°C.

Several studies have been done using chromogenic media for the detection of *L. monocytogenes* (34). Their advantages are direct detection and enumeration of pathogenic *Listeria* spp. utilizing cleavage of substrates by the virulence factor PI-PLC and by phosphatidylcholinphospholipase C (PC-PLC). There are two groups of such media: the first utilizes cleavage by PI-PLC of L-alpha-phosphatidyl-inositol, forming a white precipitation zone around the colony, combined with the chromogenic substrate 5-bromo-4-chloro-3-indoxyl-beta-D-glucopyranoside for detection of ß-D-glucosidase, which occurs in all *Listeria* spp. The typical colony morphology of *Listeria* spp. is reported to be turquoise blue (ALOA, CHROMagar *Listeria*). Pathogenic *Listeriae* are additionally surrounded by a translucent halo.

The second group of media such as BCM, Rapid' L. mono, and LIMONO-Ident-agar utilizes enzyme substrate 5-bromo-4-chloro-3-indoxyl-myo-inositol-1-phosphate (X-phos-Inositol, BIOSYNTH). Enzymatic cleavage of X-phos-Inositol leads to turquoise colonies of pathogenic *Listeria* spp. easy to enumerate (*L. monocytogenes* and *L. ivanovii*). Nonpathogenic *Listeria* spp. appears clearly distinguishable as white colonies. BCM *L. monocytogenes* Plating Medium II and LIMONO-Ident-Agar additionally combine the cleavage of X-phos-Inositol in forming turquoise colonies of pathogenic *Listeria* spp. with production of a white precipitate surrounding these colonies. This precipitate is formed by addition of a selected lecithin–mixture (79, 80). For that reason, both virulence genes (*plc*A and *plc*B) may be involved in forming this precipitation zone (33). Rapid L'MONO agar (Sanofi, France) is based on the detection of phosphatidylinositol phospholipase C and xylose fermentation. In this way, the colonies of *L. ivanovii* appear blue sourrounded by a yellow halo (xylose positive) whereas the colonies of *L. monocytogenes* are blue without the halo (xylose negative).

Stessel et al. (81) evaluated six chromogenic media similar to ALOA. Additionally, the ability of chromogenic agars to facilitate growth of stressed L. monocytogenes strains and mixed cultures with competitive non-*Listeria* strains was estimated. They have found that the competitive flora of food samples is able to overgrow low numbers of *L. monocytogenes*, especially in half-Fraser enrichment. This might lead to the underestimation of *L. monocytogenes* positive samples.

CHROMagar *Listeria* agar (CHROMagar), OCLA (Oxoid), Compass *L. mono* (Biokar), OAA (bioMérieux), and Chromoplate *Listeria* (Merck) are some of this kind of chromogenic media.

B. cereus, *B. thuringiensis*, and *B. anthracis* Group

Bacillus species is a group of ubiquitous facultative anaerobic spore-forming Gram-positive rods commonly found in soil. The spores frequently contaminate a variety of foods, including produce, meat, eggs, and dairy products. Several selective and nonselective culture media have been developed for the detection of *B. cereus* from foods. The enumeration of *B. cereus* in food and industrial samples is commonly based on a plate-counting culture technique, except for samples with low cell numbers (<10 CFU/g), for which the MPN method is preferred. BCM *Bacillus cereus* group Plating Medium (Biosynth) utilizes the production of the enzyme PI-PLC cleaving substrate 5-bromo-4-chloro-3-indoxyl myo-inositol-1-phosphate by members of the *B. cereus* group (except *B. anthracis*). Typical dull blue-turquoise colonies appear on this plating medium, easy to recognize and count (35). *B. anthracis* grows on BCM *Bacillus cereus* group plating medium in size and shape like *B. cereus*, but with white colonies.

B. anthracis chromogenic agar (ACA) has been evaluated by Juergensmeyer et al. (36). ACA contains the chromogenic substrate 5-bromo-4-chloro-3-indoxyl-choline phosphate that on hydrolysis yields blue green colonies indicating the presence of PC-PLC activity. Examination of colony morphology in 18 pure culture strains of *B. anthracis* required 48 h at 35–37°C for significant color production, whereas only 24 h was required for *B. cereus* and *B. thuringiensis*. This differential rate of PC-PLC synthesis in *B. anthracis* (due to the truncated PlcR gene and PlcR regulator in *B. anthracis*) allowed for the rapid differentiation on ACA of presumptive colonies of *B. anthracis* from *B. cereus* and *B. thuringiensis* in both pure and mixed cultures. The performance of two chromogenic plating media (CBC and BCM) was compared with two standard selective plating media (PEMBA and MYP) recommended by food authorities for isolation, identification, and enumeration of *B. cereus* (37). The Chromogenic *Bacillus Cereus* agar (CBC; Oxoid) contains 5-bromo-4-chloro-3-indolyl-β-glucopyranoside that is cleaved by β-D-glucosidase and results in white colonies with a blue-green center. Tallent et al. (82) evaluated the use of Bacara, a new chromogenic agar, as an efficient method to identify and enumerate *B. cereus* group from food matrixes, even in the presence of background flora.

Staphylococcus aureus

S. aureus is a major pathogenic bacterium found in clinical field and in food industry. Nosocomial infections due to *S. aureus* create an increasing number of problems, so it is becoming more important to detect *S. aureus* in foods and clinical microbiology. CHROMagar *S. aureus* is a selective agar medium that employs a combination of chromogenic enzyme substrates. *S. aureus* strains grow as typical pink-colored colonies on this medium, whereas most other staphylococci produce white or occasionally blue colonies (30). *S. aureus* ID (bioMérieux) is similar, and the colonies of *S. aureus* are distinctive green colonies because of production of α-glucosidase. Other staphylococci form white or occasionally pink colonies due to the hydrolysis of a substrate for ß-glucosidase (31).

BIFIDOBACTERIA

The use of chromogenic X-α-D-galactoside for differential enumeration of *Bifidobacterium* spp. and lactic acid bacteria was described by Chevalier et al. (83). The X-α-D-galactoside-based medium is useful to identify *Bifidobacteria* among *Lactobacillus* or *Streptococcus* strains. *Bifidobacteria* show blue-colored colonies on the agar plate.

Methicillin-Resistant *S. aureus* (MRSA)

MRSA is a nosocomial pathogen of worldwide importance and an increasingly frequent cause of community-acquired infection. Rapid laboratory diagnosis is critical for treating, managing, and preventing MRSA infections. Different media are available (32) to detect MRSA such as Brilliance MRSA agar (Oxoid), ChromID (bioMérieux), MRSASelect (Bio-Rad), CHROMagar, Spectra MRSA (Remel, Lenexa, KS), and BBL-CHROMagar (BD Diagnostics).

Vancomycin-Resistant Enterococci (VRE)

Strains of enterococci are known to be nosocomial pathogens, which can be involved in infections of the urinary tract, surgical wounds, and the bloodstream. An increasing number of these infections are caused by enterococci that are resistant to vancomycin and to other antibiotics. Chromogenic media are available (43, 44) to detect VRE, such as chromID VRE agar (bioMérieux) and CHROMagar VanRE (BD Diagnostics).

CONCLUSIONS

Rapid detection and identification of microorganisms is of high importance in a diverse array of applied and research fields. Chromogenic and fluorogenic substrates have been used in diagnosstic culture media for the past two decades. The incorporation of such substrates into a selective medium can eliminate the need for subculture and further biochemical tests to establish the identity of certain microorganisms.

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Anaerobic Cultivation

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2.1.2

Anaerobes are key players in global cycles of elements and nutrition in natural and anthropologic ecosystems and are also causative agents of human and veterinary diseases. Numerous studies have sought to develop culturing techniques for anaerobes to enable the elucidation of their basic physiology, pathogenic mechanisms, and ecological functions. This chapter describes a brief history of the development of anaerobic culturing techniques from the historical Hungate technique to techniques and apparatuses commonly used in modern laboratories. In addition, recent progress in anaerobic culturing techniques (e.g., single-cell manipulation and isolation, the six-well plating method, the coculture method, bioreactor-based enrichment, and *in situ*/*in vivo* cultivation) are described, with several examples of the application of these techniques for the isolation of anaerobes from natural and artificial ecosystems.

Anaerobic microorganisms (hereafter designated anaerobes) are key players in global cycles of elements (e.g., carbon, nitrogen, sulfur, and iron) in natural and anthropogenic ecosystems, including aquifer/groundwater (1), animal/insect guts (2, 3), rice paddy fields (4), rumen (5), terrestrial/oceanic subsurfaces (6, 7), wetlands (8), biodiesel production processes (9), food production processes (10), and wastewater treatment processes (11). In these anoxic ecosystems, various metabolic groups of anaerobes play roles in specific biochemical reactions, such as fermentation (12, 13), anaerobic ammonium oxidation (anammox) (14), sulfate reduction (15), iron reduction (16), syntrophic substrate oxidation (17), and methanogenesis (18). In addition to these metabolic groups, anaerobes include human and animal pathogens, which were the main focus in the earliest era of modern microbiology. The challenges of cultivating, characterizing, and controlling these anaerobic pathogens organisms spurred the establishment of modernized microbiology (19–23).

A crucial difference between aerobic and anaerobic culturing techniques is that creating and maintaining an anoxic (O_2-free) condition is a prerequisite for the growth of anaerobes. This prerequisite long hindered the cultivation of pure cultures. However, a number of researchers have successfully isolated anaerobes using various means through a trial-and-error process. This chapter describes the early history and current status of anaerobic culturing techniques. The chapter comprises four sections: (a) a brief history of anaerobic culturing techniques, tracing a path through the development of the gold standard Hungate technique; (b) apparatuses (e.g., gassing manifold, anaerobic chamber, and jar-type container) and reducing agents currently used in common laboratories; (c) successful examples using the Hungate technique, specifically those involving the isolation of anaerobes that had long been refractory to isolation, referred to as-yet-uncultured organisms; and (d) novel techniques for culturing anaerobic microorganisms.

EARLY HISTORY OF ANAEROBIC CULTURING TECHNIQUES AND THE DEVELOPMENT OF THE HUNGATE TECHNIQUE

Anaerobic cultivation was first described by Pasteur in 1861 (24), who observed the growth of microorganisms involved in butyric fermentation under anoxic conditions produced by a vacuum pump, while no growth was observed in the presence of air. Forty years later, more than 50 primitive but creative methods for culturing anaerobes had been developed and were compiled by Hunziker in a review article in 1902 (25). In this 100-year-old seminal review, anaerobic culturing techniques were categorized on the basis of six concepts: (a) formation of a vacuum; (n) replacement of air by inert anoxic gases; (c) absorption of oxygen; (d) reduction of oxygen; (e) exclusion of atmospheric oxygen by means of various physical principles and mechanical devices; and (f) the combined application of any two or more of the foregoing principles. Surprisingly, these concepts still underlie current techniques. By the mid-20th century, anaerobic culturing techniques had become varied and disorganized: for example, there were reports of the use of pyrogallol, potassium hydroxide, phosphorous, cysteine, and thioglycollate to remove oxygen (26–28), whereas other studies used an inert gas-filled jar without reducing agents (29–31) or petri dishes combined with reducing agents (32, 33). In addition to these approaches, Esmarch (34) developed the roll tube technique in 1886; however, this technique did not become popular because it was laborious and time-consuming compared with the petri dish method (33, 35). Despite these difficulties, Wilson (36) implemented the roll tube technique for viable cell counting, and various types of mechanical devices for producing a roll tube with a uniform distribution of solidified medium were developed (35, 37–39). These enormous efforts were eventually integrated by Hungate to establish a sophisticated culturing technique for fastidious anaerobes, the so-called Hungate roll tube technique (40). The major

doi:10.1128/9781555818821.ch2.1.2

principles of the Hungate technique are (a) removal of oxygen from the medium; (b) mimicking the conditions in which the microbes were found (e.g., composition of the medium, pH buffering, and redox potential); (c) minimization of oxygen exposure during inoculation; and (d) rapid solidification of agar with cold water either in a mechanical device or manually (41). This technique was initially used for the isolation of anaerobic cellulose-degrading bacteria from ruminal environments (40, 41). By the end of the 1970s, the Hungate technique had been further improved by using prereduced anaerobically sterilized (PRAS) media (42), plugging the tube with butyl rubber stoppers (43), sealing with crimp-closure aluminum seals (44) or screw caps (45), inoculating with syringes and needles (46), and using pressurized (e.g., 2–3 atm) tubes or serum bottles (47) (Fig. 1). The improved Hungate technique has now become a mainstream methodology for the culturing of anaerobic microbes, including methanogens (48, 49), syntrophic bacteria (50, 51), fermentative bacteria (52), and anaerobic fungi (53).

BASIC APPARATUSES AND REAGENTS FOR ANAEROBIC CULTIVATION

Gassing Manifold

In terms of routine preparation for anaerobic cultivation, preparing many bottles/tubes of PRAS media is a time-consuming process. To overcome this problem, Balch and Wolfe (47) introduced a multichannel gassing manifold in 1976. This type of gassing manifold, including the Deoxygenized Gas Pressure Injector types IP-8 and GR-8 (Sanshin Industrial, Yokohama, Japan), is now commercially available from several manufacturers (Fig. 1c). In these systems, high-pressure gas cylinders are connected to a reactor containing a copper-based catalyst. The anoxic gas passes over the heated (approximately 220°C) copper reactor. The manifold is equipped with a three-way valve, which is connected to the gas injection, a vacuum pump, and eight valves connected to injection/vacuum needles. The gas pressure in the manifold is maintained using an ordinary two-stage regulator connected to the anoxic gas cylinder. Such gassing manifolds permit the preparation of large quantities of PRAS bottles/tubes with an appropriate gas composition and pressure and are compatible with the Hungate technique. More recently, Wolfe and Metcalf (54) developed a vacuum-vortex technique for the preparation of PRAS media with the gassing manifold. This simple technique is applicable for small-volume (approximately 50 ml) test tubes or serum bottles and combines the strong mixing of the vortex machine and the negative pressure produced by the gassing manifold. The liquid cultures prepared by this technique yielded a greater cell density of the methanogenic archaea *Methanosarcina barkeri* strain Fusaro Δhpt-WWM85 compared to the Hungate technique modified by Balch et al. (49).

Anaerobic Chamber (Glove Box) and Anaerobic Incubator

An anaerobic chamber (or glove box) provides sufficient workspace for traditional surface cultivation on Petri dishes under anaerobic conditions. The use of anaerobic chambers for cultivation was first described in the 1960s (55–58). In 1969, Aranki et al. (59) developed a glove box with clear flexible vinyl plastic to cover the chamber. They used this glove box for the isolation of anaerobic bacteria from human gingiva and mouse cecum and found that the cultures prepared in the glove box provided adequate recovery of the anaerobic bacterial colonies from the samples compared with anaerobic jar-based cultivation. Typical vinyl-type anaerobic chambers are now available from COY Laboratory Products (Grass Lake, MI, USA) (Fig. 1j), and the detailed configuration of the anaerobic chambers was described in a review article by Speers (60). Subsequently, Balish et al. (61) have introduced a transparent plastic incubator for culturing anaerobes within the anaerobic glove box, in effect making the anaerobic glove box a tool for incubation. In addition, Metcalf et al. (62) have demonstrated the suitability of an anaerobic intrachamber incubator outfitted with a vacuum line, two gas-mixture supply lines, and a rectangular air lock for culturing *Methanosarcina* spp.

Jar-Type Apparatuses and Compact Gas Generators

The archetype of jar-type apparatuses for culturing anaerobes was invented by Novy in 1893 (63), with the development of derivatives until the early 20th century (25, 28, 31, 64). The prototypic Novy jar was connected to a vacuum pump and hydrogen gas tank via tubes, and a reducing agent (e.g., pyrogallol) solution was placed in the bottom of the jar. Although the combination of vacuum pump, hydrogen gas, and reducing agent permitted the effective formation of anoxic conditions, the entire system was relatively complicated, and it was difficult to maintain the anaerobic condition during the incubation period. As an alternative to this complex system, several researchers have developed methods of generating anoxic gas based on a simple chemical reaction and a metallic catalyst. Brewer et al. (65) used sodium borohydride and cobalt chloride to produce hydrogen gas and also tested the usability of a mixture of magnesium metal, zinc chloride, and sodium chloride for this purpose (66). They subsequently developed an improved, safe jar-type anaerobic system that employs sodium borohydride, a palladium catalyst, and a carbon dioxide tablet (containing sodium bicarbonate and citric acid) to generate anoxic conditions, with an anaerobic indicator based on the methylene blue–hydrogen reaction (67, 68). These studies resulted in the development of commercial disposable anoxic gas generators, such as GasPak (BD, Franklin Lakes, NJ, USA) (69–71), GasKit (Don Whitley Scientific, West Yorkshire, UK) (72), AnaeroGen (Oxoid, Hampshire, UK) (73), Anaerocult (Merck, Darmstadt, Germany) (74), and AnaeroPack (Mitsubishi Gas Chemical, Tokyo, Japan) (75, 76) (some of which are no longer available). The combination of a jar-type apparatus with gas-generating envelopes is widely used to culture anaerobic microorganisms on petri dishes (77). In addition, the Anoxomat, an automatic evacuation-replacement system that provides anaerobic conditions in a jar, was developed in 1989 (78). This easy-to-operate apparatus enables the cultivation of obligate anaerobic bacterial strains belonging to the genera *Bacteroides*, *Clostridium*, *Fusobacterium*, *Prevotella*, *Porphyromonas*, and *Peptostreptococcus* (79, 80). The Anoxomat Mark II is currently available from Mart Microbiology (Drachten, the Netherlands).

Bicarbonate–Carbon Dioxide Buffering System

Bicarbonate–carbon dioxide is the most popular buffering system for anaerobic culture media. In this system, carbonic acid (H_2CO_3) is formed by the reaction of carbon dioxide (CO_2) with water (H_2O), then produced carbonic acid is immediately dissociated into proton (H^+) and bicarbonate (HCO_3^-):

$$CO_2 + H_2O \rightleftarrows H_2CO_3 \rightleftarrows HCO_3^- + H^+$$