MILESTONE 1

Culturing anaerobes

Understanding the role of our microbiota in health and disease has long been hampered by the strict growth requirements of many of its constituent members. Underpinning modern day investigations into the vast complexity and functions of the human microbiota are fundamental methodologies to culture anaerobic bacteria outside their natural environment.

From the rudimentary oxygen-free culture methods in the era of Pasteur, and subsequent advances in surface culture in the early twentieth century, the mid-1900s saw a substantial expansion and refinement of anaerobic culture techniques, largely due to the pioneering work of Robert. E. Hungate. In a 1944 study of cellulose-degrading microorganisms in the bovine rumen, his revolutionary roll-tube approach enabled the successful culture of *Clostridium cellobioparus* and, in 1950, he published a complete description of his technique. The protocol used rubber-stoppered tubes of boiled culture medium with cellulose agar, through which anoxic gas was bubbled to remove any remaining oxygen. Firstly, passing this gas through a column of hot, reduced copper wire excluded any oxygen from the gas itself, and the subsequent addition of a reducing agent to the medium removed residual traces of oxygen. Rolling tubes under cold water produced a thin layer of solid agarose medium, and for the first time, anaerobiosis was maintained throughout manipulations using a constant flow of anoxic gas. The method, now known as 'the Hungate technique', is still in use to this day.

Several modifications later emerged, such as the VPI (Virginia Polytech Institute) method for larger-scale culture introduced by Moore in 1966, using prereduced medium and prehardened roll tubes. Hungate also made adaptations to culture methanogens, the strictest of anaerobes, reported in 1969. Others, such as Spears and Freter in 1967, similarly recognised the importance of continuously avoiding any exposure to oxygen, yet the Hungate technique was still more efficient and enabled a wealth of anaerobes that had not grown previously in surface cultures to be isolated for further study.

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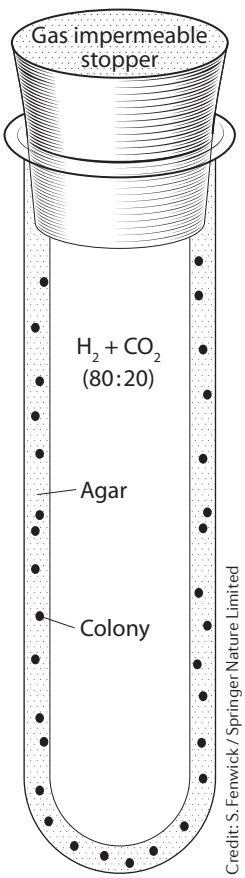
Alternative approaches used today were also launched in the mid-late 1960s, namely the GasPak and the anaerobic glove-box. The former, a self-contained combustion jar system, quickly made surface culture of anaerobic microorgansims accessible to more laboratories. The glove-box, a sealed chamber with attached gloves, filled with anoxic gases, was also a popular choice, simplifying equipment and procedures for oxygen-free culture.

As well as apparatus to create an oxygen-free environment, culturing anaerobes requires appropriate media, which must have a low oxidation-reduction potential, as well as the substrates obtained by microorganisms in their natural habitat. Many researchers working on *Bacteroides* species were instrumental in determining the requirements of specific anaerobic microorganisms, and a recent breakthrough in media composition (the inclusion of antioxidants) has since permitted the aerobic growth of anaerobic bacteria.

Moving into the twenty-first century, the advent of metagenomics revealed that the majority of environmental microbial biodiversity remained uncultured, inspiring a rebirth of culture techniques. Recent culture-dependent efforts to characterize the human microbiota (see MILESTONE 19) utilised dilution culturing and culminated in the development of culturomics; a high-throughput methodology using hundreds of different culture conditions, prolonged incubations, and matrix-assisted laser desorption/ionization–time of flight (MALDI–TOF) spectrometry, combined with 16S ribosomal RNA gene sequencing for the rapid identification of a great number of previously uncultured gut bacteria.

With a large proportion of the human microbiota requiring oxygen-free growth conditions, early breakthroughs in anaerobic culture were crucial in enabling more of our microbiota to be isolated and classified, and for their metabolism, distribution and roles within the microbiota to be studied. Initial methodologies paved the way for higher-throughput technologies that provide vital insights about the functions of the bacteria inhabiting the human body, and their effects on the human host. Now, with our understanding of the importance of the gut microbiota in human health advancing by the day, we are even more indebted to these early researchers and their innovations enabling the culture of anaerobes.

Hannah Clark, *Nature Protocols*



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ORIGINAL ARTICLE Hungate, R. E. Studies on cellulose fermentation: I. The culture and physiology of an anaerobic cellulose-digesting bacterium. *J. Bacteriol.* **48**, 499–513 (1944).
FURTHER READING Hall, I. C. Practical methods in the purification of obligate anaerobes. *J. Infect. Dis.* **27**, 576–590 (1920) | Hall, I. C. Differentiation and identification of the sporulating anaerobes. *J. Infect. Dis.* **30**, 445–504 (1922) | Hungate, R. E. The anaerobic mesophilic cellulolytic bacteria. *Bacteriol. Rev.* **14**, 1–49 (1950) | Bryant, M. P. & Doetsch, R. N. Factors necessary for the growth of *Bacteroides succinogenes* in the volatile acid fraction of rumen fluid. *Science* **120**, 944–945 (1954) | Moore W. E. C. Techniques for routine culture of fastidious anaerobes. *Intern. J. Syst. Bacteriol.* **16**, 173–190 (1966) | Brewer, J. H. & Allgeier, D. L. Safe self-contained carbon dioxide-hydrogen anaerobic system. *Appl. Microbiol.* **14**, 985–988 (1966) | Spears R. W. & Freter, R. Improved isolation of anaerobic bacteria from the mouse cecum by maintaining continuous strict anaerobiosis. *Proc. Soc. Exp. Biol. Med.* **124**, 903–909 (1967) | Drasar, B. S. Cultivation of anaerobic intestinal bacteria. *J. Pathol. Bacteriol.* **94**, 417–427 (1967) | Savage, D. C., Dubos, R. & Schaedler, R. W. The gastrointestinal epithelium and its autochthonous bacterial flora. *J. Exp. Med.* **127**, 67–76 (1968) | Aranki, A. et al. Isolation of anaerobic bacteria from human gingiva and mouse cecum by means of a simplified glove box procedure. *Appl. Microbiol.* **17**, 568–576 (1969) | Hungate R. E. Chapter IV: A roll tube method for cultivation of strict anaerobes. *Method. Microbiol.* **3**, 117–132 (1969) | Sutter, V. L. & Finegold, S. M. Antibiotic disc susceptibility tests for rapid presumptive identification of gram-negative anaerobic bacteria. *Appl. Microbiol.* **21**, 13–20 (1970) | Sonnenwirth, A. C. Evolution of anaerobic methodology. *Am. J. Clin. Nutr.* **25**, 1295–1298 (1972) | Holdeman, L. V. & Moore, W. E. C. Roll-tube techniques for anaerobic bacteria. *Am. J. Clin. Nutr.* **25**, 1314–1317 (1972) | Salyers, A. A. Energy sources of major intestinal fermentative anaerobes. *Am. J. Clin. Nutr.* **32**, 158–163 (1979) | Goodman, A. L. et al. Extensive personal human gut microbiota culture collections characterized and manipulated in gnotobiotic mice. *Proc. Natl Acad. Sci. USA* **108**, 6252–6257 (2011) | Lagier, J. C. et al. Microbial culturomics: paradigm shift in the human gut microbiome study. *Clin. Microbiol. Infect.* **18**, 1185–1193 (2012) | Dione, N. et al. A quasi-universal medium to break the aerobic/anaerobic bacterial culture dichotomy in clinical microbiology. *Clin. Microbiol. Infect.* **22**, 53–58 (2016) | Lagier, J. C. et al. Culturing the human microbiota and culturomics. *Nat. Rev. Micro.* **16**, 540–550 (2018).