This Week's Citation Classic ®

Balch W E & Wolfe R S. New approach to cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. *Appl. Environ. Microbiol.* 32:781-91, 1976.
[Department of Microbiology, University of Illinois, Urbana, IL]

The negative pressure developed during cultivation of hydrogen-oxidizing anaerobes such as methanogens made it difficult to isolate and tame these organisms in the laboratory. Each time the vessel was opened to replenish the substrate, oxygen and bacterial contamination could occur readily. When these difficulties were overcome by use of a pressurized atmosphere of hydrogen and carbon dioxide, contamination was no longer a problem. [The *SCI*® indicates that this paper has been cited in more than 270 publications.]

The Taming of Hydrogen-Oxidizing Anaerobes

Ralph S. Wolfe
Department of Microbiology
University of Illinois
Urbana, IL 61801

With the discovery that coenzyme M (CoM) was a growth factor for *Methanobrevibacter ruminantium*, we had a classical microbial growth assay for the new vitamin-coenzyme. Here was an opportunity to define the distribution of this new vitamin in the biological world. The state-of-the-art technique at that time for cultivation of fastidious non-spore-forming anaerobes was the Hungate technique,[1] which involved the use of sterile rubber stoppers to seal test tubes that contained sterile pre-reduced medium. Considerable training and expertise were required to successfully inoculate or transfer cultures, for each time a tube with a negative pressure was opened there was an opportunity for contamination by oxygen or bacteria. To follow growth of a hydrogen-oxidizing methanogen, it was necessary to open each tube and replenish the gas atmosphere by use of a gassing probe several times a day for a period of five days. Only a few investigators, such as Hungate and his students, were able to do this aseptically and without contamination.

I suggested to a new graduate student, W.E. Balch, that an exciting problem would be to determine the distribution and role of this new vitamin in the biological world. Since the growth assay by the Hungate technique was poorly reproducible and posed many problems, I suggested that growth of the methanogen in a pressurized atmosphere might provide a solution. Balch devised a method for pressurizing the hydrogen and carbon dioxide atmosphere to two atmospheres so that a smooth growth curve resulted. He used the tube developed by Miller and Wolin,[2] but replaced the serum vial seal with a solid rubber stopper. After firmly being inserted, the stopper was cut off with a razor blade. An aluminum seal was then crimped in place. After many hundreds of rubber stoppers had been cut off by razor blade, he constructed an apparatus in which the tube or vial could be held while a knife blade cut off the stopper in the manner of a guillotine. But even this was tedious; so after several thousand stoppers had been cut by guillotine, I suggested that he design a stopper with a lip that could simply be inserted and crimped in place. The injection mold for the stoppers was purchased on research grant funds, and Bellco Glass agreed to market the stopper. The "Balch stopper" became popular and was widely adopted. The number of new species of methanogens increased dramatically after the introduction of this method. The success of the method was largely due to the elimination of variability among culture tubes of media. The medium was dispensed into each tube in an anaerobic Frêter chamber,[3] and the stopper was added to each tube before removal from the chamber. In this manner, the starting atmosphere in each tube was identical, and the desired gas atmosphere was added by means of a gassing manifold. This method was widely adopted for growth of methanogens as well as many other anaerobes,[4,5] and in the hands of Karl Stetter became the method of choice for the isolation of sulfur-dependent extremely thermophilic archaebacteria.[6]

In Balch's hands, the assay for CoM could routinely detect 10 pmol. After an exhaustive search of animal, plant, and microbial tissues, the answer was very clear: CoM was present only in methanogens and not elsewhere in living organisms that we examined. We were disappointed, but the pressurized atmosphere technique and the uniqueness of CoM in methanogens later played important roles in the discovery of the archaebacteria.

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2. **Miller T L & Wolin M J.** A serum bottle modification of the Hungate technique for cultivating obligate anaerobes. *Appl. Microbiol.* 27:985-7, 1974. (Cited 205 times.)
3. **Aranki A & Frêter R.** Use of anaerobic glove boxes for the cultivation of strictly anaerobic bacteria. *Amer. J. Clin. Nutr.* 25:1329-34, 1972. (Cited 145 times.)
4. **Balch W E, Fox G E, Magrum L J, Woese C R & Wolfe R S.** Methanogens: reevaluation of a unique biological group. *Microbiol. Rev.* 43:260-96, 1979. (Cited 800 times.)
5. **Balch W E, Schoberth S, Tanner R S & Wolfe R S.** *Acetobacterium*, a new genus of hydrogen-oxidizing, carbon dioxide-reducing, anaerobic bacteria. *Int. J. Syst. Bacteriol.* 27:355-61, 1977. (Cited 125 times.)
6. **Stetter K O & Zillig W.** *Thermoplasma* and the thermophilic sulfur-dependent archaebacteria. (Woese C R & Wolfe R S, eds.) *The bacteria. Volume VIII.* San Diego, CA: Academic Press, 1985. p. 85-170.

Received September 27, 1991