The Modern Myth of "Unculturable" Bacteria/ Scotoma of contemporary microbiology

Dedicated to the pioneering microbiologists who isolated pure cultures of microbes responsible for (a) infectious diseases of animals and plants, and (b) the cyclic transformations of major chemical elements on Earth. Their characterization of the biological, physiological, and genetic properties of these organisms paved the way for current research. The careers and contributions of more than 300 of the early pioneers are profiled in the classic book by William Bulloch: The History of Bacteriology (Oxford University Press, 1938).

Howard Gest

Distinguished Professor Emeritus of Microbiology
Adjunct Professor, History and Philosophy of Science,
Indiana University, Bloomington, IN 47405
[gest@indiana.edu]

The activities of bacteria in recycling of elements on Earth and their effects on animals and plants were unknown before techniques for isolation of pure cultures were developed. Through sustained efforts of microbiologists over many decades collections of pure cultures were established and these provided experimental systems that led to our present encyclopedic knowledge of microbiology. The isolation of pure cultures required development of appropriate growth media and this aspect of microbiological research proved to be very difficult in many instances.

Nevertheless, our pioneering predecessors believed that all free-living bacteria in nature can eventually be grown in the laboratory. In contrast, a number of contemporary scientists insist that *most* bacterial species cannot be grown *in vitro*, i.e., they are "unculturable." To my knowledge, the logic of this notion is never discussed. Are there some still-undiscovered basic principles of microbial growth that escape us? Or, is "unculturability" simply a convenient excuse for avoiding arduous and time-consuming research on bacterial nutrition? The meaning of the word "unculturable" is perfectly clear; it means <u>not culturable</u>. It does not mean "somewhat unculturable" or "maybe unculturable". Is "unculturable" sloppy English or sloppy thinking? Is the word akin to saying a woman is "slightly pregnant"? Accurate definitions are important in the progress of science….see Gest (2001): Evolution of knowledge encapsulated in scientific definitions."

Diversity of bacteria

The word *diversity* can have several meanings, and the one in mind is frequently not specified. Molecular biologists interested in evolution have championed differences in 16S RNA sequences as the primary indicators of the diversity of genera and species of prokaryotes. This has led to the questionable view that molecular phylogenetic techniques provide methods for characterizing natural

microbial communities without the need to cultivate and study the actual phenotypes of living organisms.

In support of the myth of "unculturability," it is repeatedly claimed that "only a small fraction of less than 1% of the cells observed by microscopy (i.e., *in natural sources*) can be recovered as colonies on standard laboratory media" (see, for example, Amann 2000). This, of course, is a vague and inadequate criterion of culturability. How many well-known organisms—anaerobes, autotrophs, nutritionally fastidious bacteria etc.—described in *Bergey's Manual* can grow in so-called "standard media" (typically containing yeast extract, some peptone and a few salts)? Obviously, not many. Casual acceptance of this kind of criterion for "unculturable" has led some researchers to large scale speculations on the number of living bacterial species on Earth. E.O. Wilson (1999) posed this question to himself, and concluded: "Recent research suggests that the answer might be at least a thousand times greater [than ca. 4000], with the total number ranging into the millions." Amann (2000) added fuel to the speculation by noting: "If there are *just* [emphasis added] one million species that ultimately can be cultured and if their complete taxonomic description proceeds at a rate of 1,000 species/year it would take roughly the next millenium to get a fairly complete overview on microbial diversity." My own experience tells me that

if there are 50,000 truly distinctive bacterial species still unknown, their isolation and characterization will be a long time in coming. It should be noted that authoritative current texts give the number of "validly named" bacterial species as ca. 7000.

The notion that the great metabolic and nutritional diversity of bacteria is a recent revelation of molecular biological research is, of course, a fiction. This is abundantly clear from Marjorie Stephenson's classic book *Bacterial Metabolism* (1948), as well as from essays by A.J. Kluyver and C.B. van Niel (1956). Some of Kluyver's remarks: "It seems likely that a 'macrobiologist' who entered the microbiological scene around 1910 would have been most impressed by the great diversity in properties of the microbial species to which he was introduced by the microbiologist….Winogradsky, Beijerinck and those who followed them have made a thorough exploration of world. Besides the fact that these investigations have proved the practically ubiquitous occurrence of many microorganisms on earth, they have thrown a clear light on the surprisingly large diversity in nutritional requirements of the various microbial types….I think that we may expect that our 'macrobiologist' on being confronted with a nearly endless diversity of such physiological monstrosities would find the microbiological scenery bewildering."

A detailed, more recent, analysis of the meaning of diversity in the prokaryotic world was provided by Palleroni (1997), and his conclusions are worthy of attention: "Modern approaches based on the use of molecular techniques presumed to circumvent the need for culturing prokaryotes, fail to provide sufficient and reliable information for estimation of prokaryotic diversity. Many properties that make these organisms important members of the living world are amenable to observation only through the study of living cultures. Since current culture techniques do not always satisfy the need of providing a balanced pictures of the microflora composition, future developments in the study of bacterial diversity should include improvements in the culture methods to approach as closely as possible the conditions of natural habitats."

Some examples of nutritional problems in cultivating bacteria

The myth of "unculturable bacteria" persists because it is promoted by some scientists who have little experience in growing fastidious bacteria or knowledge of past investigations in which the nutritional idiosyncrasies of numerous types of organisms were defined by intensive studies. Following are a few examples of different kinds which can serve as historical lessons.

Case 1 During the 1890's, Sergei Winogradsky discovered the major classes of chemosynthetic autotrophs. He encountered difficulties in isolating pure cultures using classical procedures,

i.e., streaking plates of "nutrient agar" or "nutrient gelatin" to obtain single colonies. The history of this matter in connection with the nitrifying autotrophs is detailed in Marjorie Stephenson's *Bacterial Metabolism* (1948). In brief: "The repeated failure of numerous investigators to regain from the surface of nutrient gelatin the nitrifying organisms which were undoubtedly present in the soil culture from which the plates were sown, at length convinced Winogradsky that the gelatin plate method which had proved so successful for the isolation of disease germs must be unsuited for the present purpose….His own work on the sulphur and iron bacteria also suggested to him that organisms adapted to utilise the energy liberated by oxidation of ammonia might be ill-adapted to form colonies on nutrient gelatin, and so elude the pursuit of bacteriologists using this medium. He therefore tried a simple medium consisting of potassium phosphate, magnesium sulphate, potassium carbonate, ammonium chloride with 0.1% potassium tartrate as the sole source of carbon. Actively nitrifying soil was sown into this solution, but the result showed hardly any nitrification. Each item of the medium was then omitted in turn, with no result, until finally the organic matter was left out. The result was immediate and intense nitrification." Isolation in pure culture was the next step. Gelatin plates proved to be useless…nothing that would nitrify would grow on gelatin.

"Subsequently, Winogradsky employed a solid medium in which the appropriate salts in solution were solidified by silicic acid. On this so-called 'silica jelly, " colonies of nitrifying organisms alone developed, and could easily be obtained free from other bacteria."

Case 2 Isolation of the causative agent of cattle tuberculosis. In ca. 1910, F.W. Twort (who discovered the existence of bacteriophage) undertook to isolate the bacterium responsible for tuberculosis of cattle. The disease was causing great losses of cattle in Britain and Europe. In the introduction of the classic 1911 paper of Twort and G. Ingram [Proc. Roy. Soc. LXXXIV, pp. 517-542], the authors noted: "All writers on this disease state that the causative agent cannot be cultivated outside the animal body." They go on to demonstrate that the bacterium (*Mycobacterium pseudotuberculosis*) can in fact be grown in pure culture by adding extracts of dead cells of *Mycobacterium phlei.* This was one of the earliest researches showing requirements of many bacteria for "essential" growth factors. Later research showed that in this instance, the special requirement was a form of vitamin K. The Twort/Ingram paper is a model of hard work, persistence and deep thought. I think no reasonable scientist could read this paper and then make the statement that a bacterium is "unculturable" because it didn't grow on "standard lab media"

Case 3 Discovery and isolation of *Thermus aquaticus*.

Brock and Freeze (1969) isolated and characterized this extreme thermophile, which became of major importance in molecular biology and biotechnology. In the course of routine nutritional analysis, they observed no growth in "1% tryptone plus yeast extract, but good growth in "0.1 and 0.33% tryptone plus yeast extract." In other words, high concentrations of certain organic preparations inhibit, an important detail revealed only by methodical experimentation. Good growth occurred also in 0.1% vitamin-free casein hydrolysate or in 0.5% glutamic acid alone as the sole source of carbon, nitrogen and energy.

Case 4. Growth requirements of *Moraxella nonliquefaciens.*

Elliott Juni and his colleagues (1984) devised a novel technique for approaching analysis of complex requirements of nutritionally fastidious heterotrophic bacteria, using a species of *Moraxella.* The abstract of their paper gives a succinct description of the method, and is an excellent example of the kinds of complexity encountered in growing many bacterial species.

"A general procedure was devised for the determination of growth factor requirements of heterotrophic bacteria based on identification of individual nutrients as they are successively depleted from a limited quantity of complex medium. By using this approach, it was possible to develop a defined medium for *Moraxella nonliquefaciens* that contained nine amino acids and

three vitamins. Three of the amino acids, proline, serine, and cysteine, were required in unusually high concentrations to obtain optimal growth. Methionine had a sparing action on the requirements for serine and cysteine. Glycine could substitute for serine. Although a required nutrient, cysteine was inhibitory for growth, but this inhibitory action was antagonized by valine or leucine."

It would have been much easier to simply say that *M. nonliquefaciens* is "unculturable" in a defined medium.

Case 5 For a long time, *Bdellovibrios* were believed to be unculturable in the sense that they seemed able to grow only in the periplasmic space of a host bacterium. Gordon et al. (1993), however, demonstrated that simple heat shock, which presumably activated certain genes, enabled wild-type *Bdellovibrio bacteriovorus* to grow axenically in a defined artificial medium. Felbeck and Distel (1991) pointed out that "pure culture of endosymbiotic bacteria is notoriously difficult," and in discussing such bacteria, they use the sensible description "as-yet-unculturable symbionts,"

More history

A legion of microbiologists has provided numerous examples of bacteria that have complex growth requirements that are not

satisfied by simple concoctions of yeast extract and similar supplements. Knowledgeable microbiologists know better.

It is not news that the definition of nutritional requirements of bacteria and other microbes is often difficult and requires intensive laboratory studies. There were many known examples, in addition to those already given, of complex nutritional problems as early as 1938, when B.C.J.G. Knight published his classic monograph "Bacterial Nutrition" [182 pages; see especially "Bacteria with complex and unknown requirements", pp. 80-136]. Knight cites the early research of Andre Lwoff during the 1930's on the complicated nutritional requirements of ciliates. Pure cultures had not been previously obtained, but Lwoff developed synthetic media in which pure cultures could grow. He realized that growth factor requirements could be interpreted a loss of biosynthetic functions during evolution.

Remarks from knowledgeable microbiologists

From time to time, experienced microbiologists have made comments on the implausibility of "unculturable bacteria," but these have been largely ignored. A paper by John Fry (2000) entitled *Bacterial diversity and "unculturables"* gives interesting examples of nutritional problems and remarked on the prospects: "These examples indicate that culturing many of these 'unculturable' bacteria will be an enormous task. However, the

following arguments suggest that if more effort were put into growing these bacteria more of them would be culturable….When effort is put into growing novel aquatic bacteria they are sometimes grown relatively easily once suitable media are developed (e.g. *Legionella* spp.)". In 2004, an exemplary experimental investigation by Stevenson *et al.* provided a sophisticated model study for isolating pure cultures of previously uncultivated bacterial species from agricultural soil and the guts of termites. Using an integrative approach, Stevenson and colleagues were able to isolate bacteria from phylogenetic groups previously "under-represented in culture."

A synopsis

The recent literature contains many more examples of the "unculturability" claim. A particularly naïve repetition of the mantra is given by Dorit (2008):

"Why did it take so long to acknowledge our inner microbe? The answer stems, in part, from the fact that most bacteria cannot be grown in the laboratory. Consequently, until recently, microbiologists could not identify—let alone understand—microbes that refused to live in the world of Petri dishes and culture flasks. Until recently, if we had been interested in describing microbial diversity, we would have collected a sample from some well-defined habitat—a hot spring or a water-treatment

plant, for instance—then spread that sample on a variety of culture media and waited to see what grew. Yet for a long time, microbiology has known that only a tiny, biased sliver of microbial diversity could be cultured in the lab. As a result, we could guess, but we could never really know, what was out there."

In more serious literature, Donachie *et al.* (2007) have provided an important experimental study of microbial diversity and culturability of natural populations of bacteria. Some of their remarks:

"Overlooking a century of cultivation history and encouraging use only of ribosomal approaches leads to significant gaps in microbial community diversity data. We demonstrate that cultivation methods are critical in microbial diversity studies and that they detect organisms undetected by molecular techniques." Referring to statements in the literature that in soil, only 0.1 to 1% of bacteria are readily culturable on "common media under standard conditions," they note "Given that 100 years have passed since the Delft School pioneered the use of diverse media and incubation conditions to isolate specific microbes, those versed in cultivation methods must ponder 'What are common media under standard conditions.' How much can we reasonably expect one medium tell us of the phylogenetic diversity or the culturability of the bacteria in a sample?"

Deja vu

Fifteen years ago (Gest 1993), I summarized the problem under discussion as follows: “The requirements for growth and reproduction of extant species of bacteria are obviously met in environments that that provide appropriate chemical and physical conditions. Whether or not the requirements can be satisfied in the laboratory depends on many factors, which include the knowledge, skill, and patience of the investigator. The history of research on bacterial nutrition makes it clear that unraveling complex growth requirements and formulating optimal growth media is frequently very difficult and time-consuming. For fastidious organisms with multiple nutritional requirements, special approaches are usually needed….There is no doubt that studies on nucleic acid sequences of bacterial species are enlarging our understanding of species relationships and evolutionary patterns. But justification for pursuing such research hardly needs to be based on the myth that the ‘molecular approach’ is necessary *because* many species are “unculturable.” In any event, declaring that there is a category of unculturable bacteria in nature is a dogmatic and seriously flawed pronouncement. ‘Unculturable,’ of course, assumes that no one will ever be able to grow the organism in question in the laboratory, and obviously this is not a defensible scientific proposition.”

During 2000, I was engaged in correspondence with Carl Woese about the so-called "unculturability" of microorganisms and in one communication (1/5/00), he made the following remarkable comments: "I have never cultured an organism and know precious little about microbial physiology. Yet I am very proud of what I have accomplished in microbiology and consider the universal phylogenetic tree to be the single most important contribution to microbiology in the 20th century." Vanity aside, Woese's remarks add to the evidence that many contemporary molecular biologists suffer from *scotoma* of microbiology. Searches for "*Woese unculturable"* on Google evoke a large number of hits; we live in the age of hype.

The phenomenon of scotoma

The eminent neurologist Oliver Sacks (1995) describes "scotoma" as involving " the *deletion* of what was originally perceived, a loss of knowledge, a loss of insight, a forgetting of insights that once seemed clearly established, a regression to less perceptive explanations. All these not only beset neurology but are surprisingly common in all fields of science. They raise the deepest questions about why such lapses occur." In the case of "unculturability," there are several answers. This is grist for the mill of students of the sociology of science. Let us hope that the half-life of "unculturablity" will prove to be relatively short.

In sum, history clearly shows that molecular biologists face a challenge in attempting to defend the mantra "unculturability of most bacteria." They would be well advised to use the appellation "as-yet-uncultured" rather than "unculturable." As I pointed out earlier (Gest 1993), it is possible that certain "degenerate bacteria" such as chlamydia-like organisms can never be cultivated in pure culture *in vitro,* but such cases require adequate investigation rather than arbitrary assumptions. The study of pure cultures remains the most reliable source of basic information for understanding the properties and evolution of the vast majority of bacteria.

Wise words:

From 1667 [Thomas Spratt, History of the Royal Society]: "Of experiments intended to illustrate a preconceived truth and convince people of its validity: a most venomous thing in the making of sciences; for whoever has fixed on his cause, before he has Experimented, can hardly avoid fitting his Experiment to his cause, rather than the cause to the truth of the Experiment itself."

From George Santayana [The Life of Reason, vol. 1, 1905]:"Those who cannot remember the past are condemned to repeat it."

ACKNOWLEDGEMENTS

I thank R.G.E. Murray, University of Western Ontario, J.T. Beatty, University of British Columbia, and former associate Jeffrey Favinger for helpful suggestions.

REFERENCES

Amann, R. 2000. Who is out there? Microbial aspects of diversity. Syst. Appl. Microbiol. 23: 1-8.

Brock, T.D. and H. Freeze. 1969. *Thermus aquaticus* gen. n. and sp. n., a non-sporulating extreme thermophile. J. Bacteriol. 98: 289-297.

Donachie, S.P., J.S. Foster, and M.V. Brown. 2007. Culture clash: Challenging the dogma of microbial diversity. The ISME Journal: Multidisciplinary Journal of Microbial Ecology 1: 97-99.

Dorit, R. 2008. All things small and great. American Scientist 96: 284-286.

Felbeck, H. and D.L. Distel. 1991. Prokaryotic symbionts of marine invertebrates. In A. Ballows et. al. The Prokaryotes, 2nd ed. vol. IV, p. 3891-3906. Springer Verlag, Berlin.

Fry, J. 2000. Bacterial diversity and "unculturables." Microbiol. Today 27: 186-188.

Gest, H. 1993. Bacterial growth and reproduction in nature and in the laboratory. ASM News 59: 542-543.

Gest, H. 2001. Evolution of knowledge encapsulated in scientific definitions. Persp. Biol. Med. 44: 556-564.

Gordon, R.F., M.A, Stein and D.I. Diedrich. 1993. Heat shock-induced axenic growth of *Bdellovibrio bacteriovorus.* J. Bacteriol. 175: 2157-2161.

Juni, E., G.A. Heym, and R.C. Bradley. 1984. General approach to bacterial nutrition: Growth factor requirements of *Moraxella nonliquefaciens.* J. Bacteriol. 160: 958-965.

Kluyver, A. J. and C.B. van Niel. 1956. The Microbe's Contribution to Biology. Harvard University Press, Cambridge.

Knight, B.C.J.G. 1938. BACTERIAL NUTRITION/Material for Comparative Physiology of Bacteria. Medical Research Council Special Report Series, No. 210. His Majesty's Stationery Office, London.

Palleroni, N.J. 1997. Prokaryotic diversity and the importance of culturing. Ant. v. Leeuwenhoek. 72: 3-19.

Sacks, O. 1995. Scotoma: Forgetting and neglect in science. In: *Hidden Histories of Science*, Edit. by R.B. Silvers, pp. 141-179. A New York Review book.

Stephenson, M. 1948. Bacterial Metabolism. Third ed. Longmans Green, London.

Stevenson, B.S., S.A. Eichorst, J.T. Wertz, T.M. Schmidt, and J. A. Breznak. 2004. New strategies for cultivation and detection of previously uncultured microbes. Appl. Environ. Microbiol. 70: 4748-4755.

Wilson, E.O. 1999. The Diversity of Life. Norton, New York.