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Isolating "Uncultivable" Microorganisms in Pure Culture in a Simulated Natural Environment

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The majority (>99%) of microorganisms from the environment resist cultivation in the laboratory. Ribosomal RNA analysis suggests that uncultivated organisms are found in nearly every prokaryotic group, and several divisions have no known cultivable representatives. We designed a diffusion chamber that allowed the growth of previously uncultivated microorganisms in a simulated natural environment. Colonies of representative marine organisms were isolated in pure culture. These isolates did not grow on artificial media alone but formed colonies in the presence of other microorganisms. This observation may help explain the nature of microbial uncultivability.

The number of existing microbial species is estimated at 10^5 to 10^6 (*1*, *2*), but only several thousand have been isolated in pure culture (*3*), because few microorganisms from environmental samples grow on nutrient media in petri dishes (*4*–*16*). Attempts to improve the recovery of microorganisms from environmental samples by manipulating growth media have met with limited success (*6*, *15*, *17*–*19*), and the problem of uncultivability remains a major challenge (*4*).

We reasoned that uncultivable microorganisms might grow in pure culture if provided with the chemical components of their natural environment. To allow access to these components, we placed microorganisms in diffusion chambers and incubated the chambers in an aquarium that simulated these organisms' natural setting.

Intertidal marine sediment was used as a source of microorganisms (*20*). The upper layer of the sandy sediment harbors a rich community of microorganisms, primarily aerobic organoheterotrophs, which reach densities of $>10^9$ cells/g (*21*) and are mostly uncultivated (*22*, *23*). These microorganisms were separated from sediment particles, serially diluted, mixed with warm agar made with seawater, and placed in the diffusion chamber (*20*) (Fig. 1). The membranes allow exchange of chemicals between the chamber and the environment but restrict movement of cells. After the first membrane was affixed to the base of the chamber, the agar with microorganisms was poured in, and the top was sealed with another membrane (Fig. 1A). The sealed chambers were placed on the surface of the sediment collected from the tidal flat and kept in a marine aquarium (Fig. 1B). A thin layer of air was left between the agar and the top membrane. In the aquarium, this space was filled with seawater. This design allowed us to observe the undisturbed agar surface after peeling off the top membrane.

A large number of colonies of varying morphologies were observed after 1 week of incubation in the chambers (Fig. 2A). Most of these (>99%) were microcolonies invisible to the naked eye. Addition of 0.01% casein increased the number of colonies in the chamber, and this supplement appeared superior to starch or marine broth tested at a variety of concentrations (*20*).

In a series of microbial recovery experiments (*20*), we determined the fraction of cells that formed colonies inside the chambers compared with the standard petri dish method (Fig. 2B). The greatest microbial colony recovery in the chambers represented 40 ± 13% of the cells inoculated and came from a sample obtained in June 2001. The number of microcolonies obtained in different months ranged from 2 to 40% of the cells inoculated, with an overall average of 22 ± 13%. This is likely an underestimate, because the total direct microbial count included dead cells, our colony-counting technique produced conservative estimates (*20*), and the fairly dormant March sample skewed the recovery results. Representative microorganisms from the chambers were successfully isolated in pure culture by passage to new chambers. Of the 33 colonies passaged, 23 produced sustainable growth in the chambers at the first attempt.

Unexpectedly, a significant number of microcolonies appeared on the petri dishes (6 ± 4% of the number of cells inoculated). We investigated their ability to produce sustainable growth in three independent trials. Each time, 27 to 30 microcolonies were passaged to a new petri dish. Most of the transfers (86 ± 7%) did not result in microbial growth. It seems that the majority of microorganisms from the sediment could only undergo a limited number of divisions on a petri dish. The microcolonies that did grow after passage to petri dishes (14%) appeared to represent mixed cultures, and only those that produced rapidly growing macrocolonies, visible to an unaided eye, seemed capable of sustained growth on petri dishes. Counting visible colonies is the conventional method of performing microbial plate counts (*24*). Such petri-dish macrocolonies made up 0.054 ± 0.051% of the inoculum, consistent with previous reports (*15*–*17*). Finally, ~300-fold as many microorganisms produced sustainable growth in the growth chambers as in standard petri dishes.

We attempted to isolate into pure culture some of the microorganisms grown in the diffusion chambers (*20*). The isolates were considered pure if no contaminants could be detected microscopically or by polymerase chain reaction amplification of 16*S* ribosomal RNA (rRNA) gene (*20*). Several passages were required to achieve purity. Passages typically produced hundreds of microcolonies per chamber, which was more than sufficient for the purposes of the present study.

To date, two isolates, MSC1 and MSC2 (Fig. 3), have been obtained; nine others are at different stages of isolation into pure culture. A 1400–base pair sequence of 16*S* rDNA from MSC1 indicates that it is a previously undescribed bacterium, with 93% sequence similarity (*20*) to its closest relative, *Lewinella persica* [*Herpetosiphon persicus* (*25*); Class Sphingobacteria, Phylum Bacte-

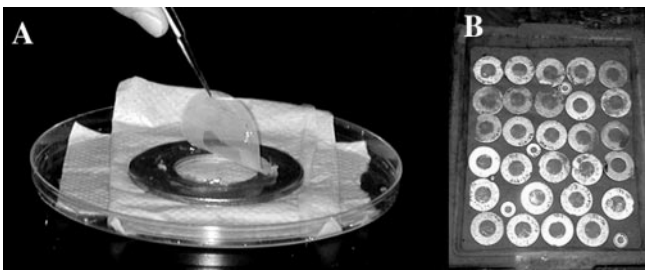

Fig. 1. Diffusion growth chamber for in situ cultivation of environmental microorganisms. (**A**) The chamber is formed by a washer sandwiched between two 0.03-μm pore-size polycarbonate membranes. (**B**) Growth chambers incubated on the surface of marine sediment.

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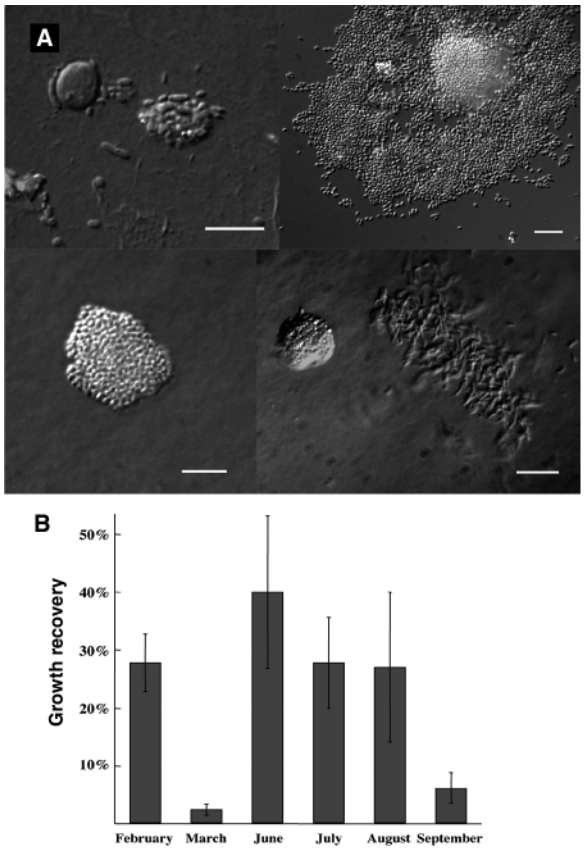

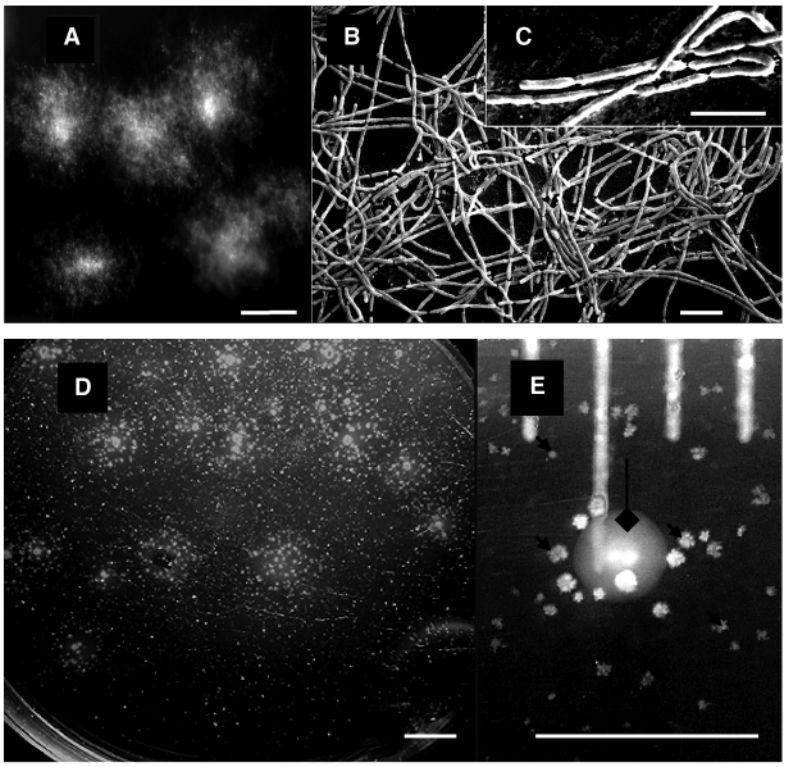

Fig. 2 (left). (**A**) Representative colonies of marine-sediment microorganisms [compound microscope view, differential interference contrast (DIC)] grown in diffusion chambers. Bars, 10 μm. (**B**) Growth recovery (±SD) of microorganisms from environmental samples in diffusion chambers. **Fig. 3 (right).** Growth of MSC1 in diffusion chambers (A to C) and in Petri dishes (D and E). (**A**) Dissecting microscope view of colonies; dark field. Bar, 50 μm. (**B**) Scanning electron microscopy (SEM) (*20*) view of a single colony. Bar, 5 μm. (**C**) SEM view of a portion of a colony. Bar, 3 μm. (**D**) Synergistic growth of MSC1 and MSC2 on petri dishes; general view of a petri dish with several MSC1/MSC2 clusters. (**E**) Magnified view of a MSC1/MSC2 cluster. Images were taken after 1 week of incubation in 0.7% casein-supplemented agar at 16°C. Bars, 3 mm.

roidetes (*26*)]. This degree of similarity is less than the convention of 98% identity, adopted for the classification of strains into a single species (*27*). *L. persica* form long, multicellular, unbranched filaments of a peach color. MSC1 differs from *L. persica* and other *Lewinella* spp. and *Herpetosiphon* spp. in details of general colony morphology (*25*). In general, these and other bacteria from the Cytophaga-Flexibacter-Bacterioides (CFB) group are thought to be primarily aerobic organoheterotrophs capable of extracellular digestion of complex biopolymers. Since the introduction of the 16*S* rRNA approach to study microbial diversity, numerous CFB sequences have been recovered from various marine environments, especially those associated with surfaces (*6*, *23*). Most of the known CFB species remain uncultivated (*28*).

MSC1 occasionally produced growth on artificial media in petri dishes (*29*), but no colonies were formed upon passage to another petri dish (*30*). Apparently, only diffusion chambers provided a suitable environment for sustainable growth (*31*).

However, MSC1 grew well in petri dishes contaminated with certain other microorganisms, and one of them (MSC2) was subsequently isolated into pure culture. The closest relative of MSC2 is probably *Arcobacter nitrofigilis* (*20*, *32*). *Arcobacter* spp. are motile, spiral curved, rod-shaped bacteria capable of nitrogen fixation and nitrate respiration but incapable of metabolizing carbohydrates (*33*). MSC2 are curved, rod-shaped bacteria and motile. The genus *Arcobacter* is commonly found in marine sediments (*22*), and related 16*S* rRNA sequences have recently been recovered from this environment (*23*).

Although the growth of MSC1 and MSC2 could be easily maintained in the chambers, their growth in petri dishes could only be achieved in coculture (Fig. 3, D and E). The pattern of colonies on the Petri dish appears to show codependence. Denser colonies of MSC1 form a gradient of increasing size converging on diffuse colonies of MSC2.

Similarly, MSC1 could be cultured in Petri dishes in coculture with either one of the other two isolates, MSC4 and MSC5. It is possible that the observed growth synergy is based on cross-feeding. However, coculture was observed on rich media (technical-grade casein, marine broth), an unlikely environment for cross-feeding. Microorganisms use pheromones to communicate both within and across species (*34*). It seems possible that microorganisms require specific signals originating from their neighbors that indicate the presence of a familiar environment. Implicit in this signaling hypothesis is that microorganisms will not grow in an unfamiliar environment even in the presence of appropriate nutrients, and this may explain why so many microorganisms cannot be isolated in pure culture on artificial media in vitro. Our diffusion chamber method bypasses this limitation.

References and Notes

1. J. M. Tiedje, *ASM News* **60**, 524 (1994).
2. D. Allsopp, R. R. Colwell, D. L., Hawksworth, *Microbial Diversity and Ecosystem Function* (CAB International, Wallingford, UK, 1995).
3. J. T. Staley, M. P. Bryant, N. Pfennig, J. G. Holt, Eds., *Bergey's Manual of Systematic Bacteriology* (Williams & Wilkins, Baltimore, MD, 1989).
4. P. Young, *ASM News* **63**, 417 (1997).
5. M. R. Barer, C. R. Harwood, *Adv. Microb. Physiol.* **41**, 93 (1999).
6. S. G. Giovannoni, M. Rappé, in *Microbial Ecology of the Oceans*, D. L. Kirchman, Ed. (Wiley-Liss, New York, 2000), pp. 47–84.

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7. S. J. Giovannoni, T. B. Britschgi, C. L. Moyer, K. G. Field, *Nature* **345**, 60 (1990).
8. V. Torsvik, J. Goksoyr, F. L. Daae, *Appl. Environ. Microbiol.* **56**, 782 (1990).
9. D. M. Ward, R. Weller, M. M. Bateson, *Nature* **345**, 63 (1990).
10. E. F. DeLong, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5685 (1992).
11. J. A. Fuhrman, K. McCallum, A. A. Davis, *Nature* **356**, 148 (1992).
12. S. M. Barns, R. E. Fundyga, M. W. Jeffries, N. R. Pace, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 1609 (1994).
13. M. A. Dojka, J. K. Harris, N. R. Pace, *Appl. Environ. Microbiol.* **66**, 1617 (2000).
14. V. S. Butkevich, *Trans. Oceanogr. Inst. Moscow* **2**, 5 (1932).
15. H. W. Jannasch, G. E. Jones, *Limnol. Oceanogr.* **4**, 128 (1959).
16. C. E. ZoBell, *Marine Microbiology: A Monograph on Hydrobacteriology* (Chronica Botanica, Waltham, MA, 1946).
17. L. A. Meyer-Reil, *Appl. Environ. Microbiol.* **36**, 506 (1978).
18. R. R. Colwell, D. J. Grimes, in *Nonculturable Microorganisms in the Environment*, R. R. Colwell, D. J. Grimes, Eds. (American Society for Microbiology, Washington, DC, 2000), pp. 1–6.
19. J. T. Staley, A. Konopka, *Annu. Rev. Microbiol.* **39**, 321 (1985).
20. Details of experimental procedures are available on *Science* Online at www.sciencemag.org/cgi/content/full/296/5570/1127/DC1.
21. S. S. Epstein, *Microb. Ecol.* **34**, 199 (1997).
22. E. Llobet-Brossa, R. Roselló-Mora, R. I. Amann, *Appl. Environ. Microbiol.* **64**, 2691 (1998).
23. A. Cifuentes *et al.*, *Appl. Environ. Microbiol.* **66**, 1715 (2000).
24. L. S. Clesceri, A. E. Greenberg, A. D. Eaton, *Standard Methods for the Examination of Water and Wastewater* (American Public Health Association, Washington, DC, ed. 20, 1998).
25. R. A. Lewin, *Can. J. Microbiol.* **16**, 517 (1970).
26. D. R. Boone, R. W. Castenholz, G. Garrity, Eds., *Bergey's Manual of Systematic Bacteriology* (Springer-Verlag, Berlin, ed. 2, 2001).
27. E. Stackebrandt, B. M. Goebel, *Int. J. Syst. Bacteriol.* **44**, 846 (1994).
28. D. J. Grimes, A. L. Mills, K. H. Nealson, in *Nonculturable Microorganisms in the Environment*, R. R. Colwell, D. J. Grimes, Eds. (American Society for Microbiology, Washington, DC, 2000), pp. 209–227.
29. Out of 60 chamber–to–petri dish passages, 19 were successful.
30. Out of 34 petri dish–to–petri dish passages, 33 were negative and one resulted in the mixed culture MSC1/MSC2 (Fig. 3, D and E).
31. Out of 34 petri dish–to–chamber passages, 27 were successful.
32. The detailed sequence comparison was difficult because of the abundant unspecified sites in the *A. nitrofigilis* sequence available in GenBank. The next closest relative of MSC2 is an uncultured *Arcobacter* [96% 16*S* rRNA similarity (*20*)].
33. F. C. Tenover, C. L. Fennel, in *Prokaryotes*, A. Ballows, H. G. Trüper, M. Dworkin, W. Harder, K.-H. Schleifer, Eds. (Springer-Verlag, Berlin, 1992), pp. 3488–3511.
34. G. M. Dunny, S. C. Winans, Eds., *Cell-Cell Signaling in Bacteria* (American Society for Microbiology, Washington, DC, 1999).

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Volunteering as Red Queen Mechanism for Cooperation in Public Goods Games

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The evolution of cooperation among nonrelated individuals is one of the fundamental problems in biology and social sciences. Reciprocal altruism fails to provide a solution if interactions are not repeated often enough or groups are too large. Punishment and reward can be very effective but require that defectors can be traced and identified. Here we present a simple but effective mechanism operating under full anonymity. Optional participation can foil exploiters and overcome the social dilemma. In voluntary public goods interactions, cooperators and defectors will coexist. We show that this result holds under very diverse assumptions on population structure and adaptation mechanisms, leading usually not to an equilibrium but to an unending cycle of adjustments (a Red Queen type of evolution). Thus, voluntary participation offers an escape hatch out of some social traps. Cooperation can subsist in sizable groups even if interactions are not repeated, defectors remain anonymous, players have no memory, and assortment is purely random.

Public goods are defining elements of all societies. Collective efforts to shelter, protect, and nourish the group have formed the backbone of human evolution from prehistoric time to global civilization. They confront individuals with the temptation to defect, i.e., to take advantage of the public good without contributing to it. This is known as Tragedy of the Commons, Free Rider Problem, Social Dilemma, or Multiperson Prisoner's Dilemma—the diversity of the names underlines the ubiquity of the issue (*1*–*7*).

Theoreticians and experimental economists investigate this issue by public goods games (*8*–*11*), which are characterized by groups of cooperators doing better than groups of defectors, but defectors always outperforming the cooperators in their group. In typical examples, the individual contributions are multiplied by a factor r and then divided equally among all players (*12*). With r smaller than the group size, this is an example of a social dilemma (*13*, *14*): Every individual player is better off defecting than cooperating, no matter what the other players do. Groups would therefore consist of defectors only and forego the public good. For two-player groups, this is the prisoner's dilemma game. In this case, cooperation based on direct or indirect reciprocation can get established, provided the probability of another round is sufficiently high (*15*, *16*). But retaliation does not work if many players are engaged in the game (*17*), because players intending to punish a defector can do so only by refraining from cooperation in subsequent rounds, thereby also punishing the cooperators in the group.

If players are offered, after each round, the option of fining specific coplayers, cooperation gets firmly established. This happens even if punishment is costly to the punisher (*18*, *19*) and if players believe that they will never meet again (*20*). But such fining, or alternatively rewarding (*21*), requires that players can discriminate individual defectors. Although reward and punishment must be major factors in human cooperation, we draw attention to a simpler mechanism. It consists in allowing the players not to participate, and to fall back on a safe "side income" that does not depend on others. Such risk-averse optional participation can foil exploiters and relax the social dilemma, even if players have no way of discriminating against defectors (*22*).

We consider three strategic types: cooperators and defectors, both willing to engage in the public goods game and speculate (though with different intentions) on the success of a joint enterprise; and "loners," who rely on some autarkic way of life. Cooperators will not stably dominate the population in such a voluntary public goods game, but neither will exploiters. Their frequencies oscillate, because the public good becomes unattractive if free riders abound.

To model this scenario with evolutionary game theory, we assume a large population consisting of cooperators, defectors, and loners. From time to time, a random sample of N individuals is offered the option to engage in a public goods game. The loners will refuse. They

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