Journal of Bacteriology

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J. Bacteriol. **2012, 194(16):4151. DOI: 10.1128/JB.00345-12.**
Published Ahead of Print 1 June 2012.

JB Journals.ASM.org

MINIREVIEW

Growing Unculturable Bacteria

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The bacteria that can be grown in the laboratory are only a small fraction of the total diversity that exists in nature. At all levels of bacterial phylogeny, uncultured clades that do not grow on standard media are playing critical roles in cycling carbon, nitrogen, and other elements, synthesizing novel natural products, and impacting the surrounding organisms and environment. While molecular techniques, such as metagenomic sequencing, can provide some information independent of our ability to culture these organisms, it is essentially impossible to learn new gene and pathway functions from pure sequence data. A true understanding of the physiology of these bacteria and their roles in ecology, host health, and natural product production requires their cultivation in the laboratory. Recent advances in growing these species include coculture with other bacteria, recreating the environment in the laboratory, and combining these approaches with microcultivation technology to increase throughput and access rare species. These studies are unraveling the molecular mechanisms of unculturability and are identifying growth factors that promote the growth of previously unculturable organisms. This minireview summarizes the recent discoveries in this area and discusses the potential future of the field.

What is an unculturable bacterium? While at first glance, there appears to be a contradiction in the title of this review, in this context, "unculturable" indicates that current laboratory culturing techniques are unable to grow a given bacterium in the laboratory. That all organisms must be growing in their natural environment is axiomatic; that many we cannot currently grow will be cultured in the future is certain. Therefore, "unculturable" does not mean "can never be cultured" but, rather, signifies that we lack critical information on their biology, and this presents both challenges and opportunities. These opportunities are the chance to learn the molecular principles behind this recalcitrant growth, allowing us to add that information to our repertoire of microbiological techniques and gaining access to previously hidden metabolic diversity that will provide new natural products and reveal factors that can contribute to both ecological balance and host health. This review examines the recent approaches that microbiologists are employing to convert currently unculturable bacteria into cultured isolates in the laboratory while concurrently beginning to discover the mechanisms behind their apparent unculturability.

HOW DO WE KNOW THERE ARE UNCULTURABLE BACTERIA?

The first evidence that not all bacteria from a given environment will grow on laboratory media came from microscopy; the number of cells that were observed microscopically far outweighed the number of colonies that grow on a petri plate (2). Given the name "The Great Plate Count Anomaly," the magnitude of the anomaly varied by environment but could reach several orders of magnitude (77). While stimulating culturing efforts, this observation also raised the question of the phylogenetic identity of these bacteria that do not grow in the laboratory. It was proposed that these are dead cells and therefore would never grow, potentially explaining the anomaly without introducing novel taxa of unculturable bacteria (76). In fact, many of these cells were shown to be metabolically active, even though they could not replicate on laboratory media (68). Additional evidence for the presence of bacterial taxa that cannot be grown in the laboratory came from molecular tools. The ability to obtain DNA sequence information from an environmental sample (by PCR amplification followed by cloning or direct sequencing) allowed characterization of phylogenetically relevant markers, such as 16S rRNA gene sequences, regardless of the viability of the organism that harbored the DNA (3). Such analyses revealed a hidden ocean of diversity that had never been seen by cultivation. Starting from 11 bacterial phyla (the highest-level division within the bacterial kingdom) described by Woese in 1987, the number of divisions of bacteria has grown to at least 85, the majority of which have no cultured representatives (1, 38, 63, 86). Given that these diverse groups must be growing somewhere in the environment in order for their DNA to be present to be sequenced, the point was driven home that the culturing efforts of the last 2 centuries had managed to replicate permissive growth conditions for only a small subset of the total bacterial diversity. While DNA sequencing from mixed populations is known to be subject to artifacts that can inflate the apparent diversity, careful controls have minimized this phenomenon (34). Furthermore, the repeated appearance of members of the missing phyla indicates a very real presence in nature. For example, the candidate phylum TM7 has been found repeatedly in many different environments. A sequence corresponding to the 16S rRNA gene of TM7 was first found in peat bogs (65), and it has since been reported to be present in a multitude of diverse environments, including soil, water, waste treatment sludge, marine sponges, the human microbiome, and many others (10, 20, 31, 32). TM7 is just one broadly distributed phylum that has resisted substantial cultivation efforts; as indicated above, most bacterial taxa have never been studied in the lab, representing enormous genetic and biochemical diversity.

WHAT IS THE SIGNIFICANCE OF THESE UNCULTURABLE BACTERIA?

One way of measuring biological diversity is counting the number of validly described species that a given branch of the tree of life possesses. For example, it is estimated that between 800,000 and 1.2 million insect species have been described to date (39, 64). This

Published ahead of print 1 June 2012

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doi:10.1128/JB.00345-12

large number is partly due to the attention they have received from investigators, but it is also due to the ease of access to the subject. Most insects require no or modest magnification to observe, and they are ubiquitous. However, it is also partly due to the high level of diversity that exists in nature, as insects occupy myriad niches in the ecosphere. Expected bacterial diversity is at least as high, and most likely orders of magnitude higher, as one might reasonably expect that most of those insect species harbor at least one bacterial endosymbiont that is unique to that insect in addition to the other, multitudinous niches bacteria inhabit in the environment (3). Given this potential diversity, it may come as a surprise to learn that there are just over 7,000 validly described bacterial species (1). As with the example of insects, two factors contribute to the number of named bacteria. The first is the level of effort involved in characterizing new species, and the second is the difficulty in culturing many of them (which is essential to describing bacterial species) (67). This "high bar" of domestic cultivation is essentially unique to microbes; it is certain that the majority of validly named macroscopic organisms were described in the wild and never cultivated in the laboratory. This distinction, requiring growth in pure culture to describe microbial species, is a direct consequence of the difficulty of determining physiologically relevant information about bacteria in the absence of a pure culture. The net result is that only a tiny fraction of the total bacterial diversity has been cultured, let alone described as a species (58). The missing bacteria, represented both by the phyla described above and by smaller phylogenetic subdivisions, probably harbor the majority of the metabolic diversity among not only the bacteria but also among all domains of life.

Natural products from bacteria (and their derivatives) account for half of all commercially available pharmaceuticals (19), and it has been estimated that the total diversity of natural small molecules (under 1 kDa) from bacteria is in excess of 10^9 unique compounds (16). Consequently, one fundamental result of having limited bacterial diversity available for study and characterization has been the collapse of the antibiotic discovery pipeline. The last new class of antibiotics that was successfully developed into a clinical therapeutic was discovered in 1987; since then, only derivatives and variations of previously discovered classes have been brought to market. This has been called the "discovery void," and it is still ongoing (72). One important reason for this is the repeated isolation of the same culturable bacteria, producing a limited diversity of natural products (47). It is estimated that at the current rate of culturing novel potential antibiotic-producing bacteria, more than 10^7 isolates will have to be screened to find the next new class of molecules (6). In addition, the screening of synthetic compound libraries has not been productive beyond modifying natural product core structures (7). The key to overcoming these imposing challenges is changing the rate at which novel strains are isolated; in order to do this, microbiologists will need to gain access to the uncultured majority (45, 72). The potential natural products from bacteria are not limited to antibiotics, however. Microbial secondary metabolites are used in organ transplantation, cancer treatment, and cholesterol control, as well as serving as insecticides, fungicides, and antiparasitics (19). Almost every aspect of human health would benefit from a greater diversity and availability of microbial natural products.

WHY ARE THEY NOT GROWING IN THE LAB?

The simple explanation for why these bacteria are not growing in the laboratory is that microbiologists are failing to replicate essential aspects of their environment. This is not for lack of trying or cleverness; when it is not clear what facet of the environment is not being properly replicated (nutrients, pH, osmotic conditions, temperature, or many more), attempting to vary all of these conditions at once results in a multidimensional matrix of possibilities that cannot be exhaustively addressed with reasonable time and effort. Attempting to tailor synthetic media to the suspected environmental conditions of the organism have made up much of the classic efforts to culture bacteria and have resulted in the thousands of bacteria now considered culturable. More recently, however, much of the progress in expanding the range of bacteria that can be grown has come from two related strategies: employing the environment itself as an aid in growing microbes and coculture with other bacteria from the same environment.

APPROACHES TO CULTURING THE MISSING BACTERIAL DIVERSITY

Simulated environments. It is difficult to replicate a natural environment at an arbitrarily high level of fidelity if it is not known which of the parameters are important for the growth of a given bacterial taxon from that setting. Therefore, one alternative is to take the bacteria back to the environment to grow them, often by moving a portion of the environment into the laboratory (see Fig. 1 for an example system). The challenge with this approach is to separate the isolates of interest from the general microbial population of the environment.

The groups of Epstein and Lewis designed a diffusion chamber to accomplish this by enclosing the bacteria within a semipermeable chamber such that the cells are unable to pass through the membrane barrier but nutrients and growth factors from the environment are able to enter (37). Before these chambers were sealed, they were inoculated with dilute suspensions of cells from marine sediment and incubated in an aquarium of seawater on a bed of sand. Microscopic examination of the chambers revealed microcolonies of bacteria growing within them, the majority of which could be further isolated and propagated by reinoculation into fresh chambers. Comparison of the number of growing microcolonies with microscopic counts of cells in the initial inoculum yielded recovery rates of up to 40%. When the same inoculum was assayed for colony production on standard petri plates, however, the recovery rate was 0.05%, consistent with earlier reports. Several of the microcolonies from the chambers were isolated in pure culture and determined to be previously uncultured bacteria. These results demonstrated that the environment itself could be a powerful tool to gain access to bacteria that could not be cultured in the lab, while a follow-up study established that the increased recovery seen in the chambers enhanced not only the total number of growing isolates but the overall diversity as well (12). This technique was also subsequently shown to be effective outside the marine environment. Subsurface soil was incubated in the chambers and on petri plates, resulting in novel isolates from the chambers but not the plates (13). The chamber was further modified into a trap, such that one of the membranes enclosing it has a pore size just large enough that filamentous bacteria can grow into the chamber by hyphal growth but nonfilamentous bacteria cannot pass through (27). In a comparison by 16S rRNA gene sequencing to standard actinobacterial petri plate isolation techniques per-

FIG 1 Bringing the environment into the laboratory. Image of a 76-l (20-gallon) aquarium for the incubation of marine sand biofilm bacteria in a simulated environment. The aquarium contains natural seawater, beach sand, flora, and invertebrate fauna from Canoe Beach, Nahant, MA. Operated in the laboratory of Kim Lewis at Northeastern University, the simulated environment is maintained with a filter system, a protein skimmer, a circulator, and regular exchanges of water freshly collected from the beach. This system can be used as an incubation environment, and the sand sediment can be used as a source of environmental bacteria.

formed on the same samples, the traps gave nearly complete enrichment for filamentous actinobacteria, with greater diversity and number, including isolates of rare groups. In a similar study, Ferrari and coworkers had cultured microcolonies of rare soil bacteria by growing them on filters suspended on soil slurry in the absence of added nutrients (22). Micromanipulation was used to grow one isolate in pure culture, and a follow-up study showed that these microcolonies could be reliably micromanipulated for downstream cultivation (23).

Bringing the environment into the laboratory also served as the basis for one of the most heralded success stories of culturing a bacterium that had previously eluded microbiologists. The SAR11 clade of *Alphaproteobacteria* is a widespread group of free-living bacteria found predominantly in seawater but also reported to be present in freshwater lakes (48). Originally identified as a prime example of a ubiquitous but uncultured bacterium of importance to primary production in the ocean, this clade is one of the most abundant proteorhodopsin-containing organisms in seawater (55). Proteorhodopsin is a light-driven proton pump that was identified in the DNA sequence of an uncultured bacterium and has been proposed to have a global impact on the carbon and energy balance in the ocean (8). In order to cultivate this organism, Giovannoni and his group used the natural environment in the form of seawater as a growth medium, and rather than attempt to contain specific cells separate from other isolates in the environmental sample, the group utilized the fact that SAR11 was one of the most abundant organisms in their samples. By diluting the samples "to extinction" (a process whereby a dilution sequence is carried out until only one or a few bacteria are present in a given culture volume), they were able to separate the bacteria of this clade into the wells of microtiter plates and grow them in pure culture (62). Provisionally named "*Candidatus* Pelagibacter ubique," its cultivation allowed characterization of this clade and its role in the marine environment. Since isolation, the genome sequence has been determined, leading to insights into its nutritional requirements and improved culture conditions (28, 82). In addition, the role of proteorhodopsin is starting to become clear, which may lead to a better understanding of the carbon flux in the ocean (78). This culture technique has now led to the culture of new members of this clade from different marine regions (75) as well as other previously uncultured isolates from both marine and freshwater environments (15, 33, 36, 59, 79).

A third method for separating uncultured bacteria of interest from the rest of the organisms in their environment while culturing them in a version of their natural environment (imported into the lab) was also described in 2002. The group of Keller developed a method for encapsulating single bacterial cells in microdroplets of solidified agarose (87). A dilution to extinction series was employed to limit the number of bacteria in each gel microdroplet (GMD), and after encapsulation, the GMDs were contained in a flow column bounded by membranes that retained the encapsu-

lated bacteria. For the marine samples cultured, seawater was constantly flushed through the columns as a growth medium. After incubation, the GMDs were passed through a flow cytometer, and those that contained a microcolony were sorted into the wells of a microtiter plate, in which phylogenetic analysis and further cultivation were carried out. Interestingly, unamended seawater as a medium yielded a higher diversity than seawater with added nutrients, as shown by 16S rRNA gene sequence analysis, reinforcing the emerging view that the natural environment can be essential for growing unculturable bacteria in the laboratory. In expanded culturing efforts using solely seawater as the growth medium, very rare bacteria were isolated as microcolonies in the GMDs, including lineages of *Planctomycetales* that were previously uncultured, some of which were <84% related to any published 16S sequence, cultured or otherwise. This included the sequencing performed on the original seawater samples, possibly indicating that the culturing method was enriching for very rare isolates (88). In a related approach, Kushmaro and coworkers used agarose spheres encapsulated in polysulfone to incubate bacteria from coral mucus in their native environment—in this case, on the surface of live coral (9). While this technique was not compared with standard cultures and the bacteria were not subsequently cultured outside the spheres, sequence analysis of a clone library of 16S rRNA genes suggested that about half of the isolates had not been cultured before.

One further interesting approach to growing uncultured bacteria uses the bacteria themselves to determine the particular aspect of the environment that is important to their growth rather than adding the entire environment to the medium. Graf and coworkers used high-throughput sequencing of RNA transcripts (RNA-seq) to determine that an uncultured *Rikenella*-like bacterium in the leech gut was utilizing mucin as a carbon and energy source (14). Using this insight, they were able to culture this isolate on medium containing mucin. The authors suggest that the RNA sequence information was more useful in this regard than the genomic DNA sequence, as RNA-seq indicated what genes were actually being expressed in the growing bacterium.

Coculture. These successes at bringing the environment into the laboratory demonstrated that there are critical differences between the standard laboratory media that were traditionally being used and the natural environment of unculturable bacteria. But what were these differences? If they could be identified, it would convey a molecular understanding of unculturability and allow the synthesis of new media that did not depend on being able to reproduce the environment in the laboratory. Observations were made in the course of environmental culturing efforts that led to the identification of some of these missing factors and their source.

It was noted that some bacteria isolated from the chambers developed by Lewis and Epstein would not grow on a petri plate unless they were growing close to other bacteria from the same environment, thereby demonstrating coculture dependence for these isolates. Similarly, the widespread photosynthetic marine bacterial genus *Prochlorococcus* was being cultured in both natural and synthetic seawater in the hopes that it could be used as a model for marine microbial ecology (50). While this organism had been cultured from the ocean many times, only two variants had ever been grown in pure culture (51, 66). The other isolates were all dependent on heterotrophic bacteria for coculture, and it was nearly impossible to grow these organisms from a single cell as a colony on a petri plate (53). A number of subsequent efforts to culture other bacteria also revealed plentiful examples of coculture-de[pendence]. [...] creased [g]rowth of the previously uncultured isolate *Catellibacterium n[odo]sariphilum* fro[m] [...] presence of sper[m] medium from [...]), w[ith] Sung and cowork[ers] identified a number of anaerobic thermophiles in the family [...] dependent on cell extract from *Geobacillus toebii* (40, 41). In these latter cases, the growth-promoting factors have yet to be identified. However, the early examples of apparent coculture dependence in both *Prochlorococcus* and marine sediment bacteria led Zinser, Epstein, and Lewis to use these systems to identify the molecular mechanisms of unculturability in these strains.

In the case of *Prochlorococcus*, the group of Zinser set out to separate a dependent strain of *Prochlorococcus* (MIT9215) from its heterotrophic helpers to determine the nature of the help provided (53). They employed an elegant technique whereby they selected for streptomycin-resistant mutants among the abundant *Prochlorococcus* cells in their coculture, while the smaller population of the helpers was not large enough to contain a spontaneous resistant mutant. They were consequently able to kill the helper population by treatment with streptomycin, resulting in an apparently pure *Prochlorococcus* culture. By maintaining this culture of MIT9215 at high density (minimal dilution on subculture), they were able to propagate it in pure culture. However, if the concentration of *Prochlorococcus* cells was diluted to below about 10^5 cells/ml, the culture would fail to grow. In addition, MIT9215 could not form isolated colonies on petri plates without adding back the helper bacteria. After determining that a large number of different helpers would allow the growth of a variety of *Prochlorococcus* strains, the researchers made the intellectual leap to test whether these helper bacteria were reducing oxidative stress and thus allowing the sensitive *Prochlorococcus* to grow. Adding catalase, an enzyme that breaks down hydrogen peroxide, allowed improved growth of MIT9215 on plates. In a following study, the same group demonstrated that removal of H_2O_2 is both necessary and sufficient for the helping effect (52). Significantly, they also showed that the natural level of H_2O_2 in surface seawater may be high enough that this helping effect may be required in the natural environment, indicating a possible evolutionary dependence on a functional microbial ecology. At this stage, it appears that the case of *Prochlorococcus* is not one of growth factor contribution from the helper but rather one of environment modification.

To place this view in context, it is necessary to note that coculture dependence had been seen before; the classic example that is used in microbiology classes is the dependence of *Haemophilus influenzae* on *Staphylococcus aureus* (18). In this case, *H. influenzae* was found to need an exogenous source of both heme (24) and NAD (termed "cozymase" at the time), originally referred to as "X" and "V" factors before they were identified, for aerobic growth (49). The heme is released from blood added to the medium, and NAD is released by *S. aureus*. While blood normally contains NAD, blood from sheep and other animals commonly used as a source of blood for culture media also contains enzymes that can destroy this factor (5, 42). The dependence of *H. influenzae* on *S. aureus* can be overcome by preheating the blood used in the petri plates (creating the oddly named "chocolate" agar); this heat treatment both releases heme and inactivates the enzyme that breaks down NAD (25, 57). Perhaps because the host, rather than

S. aureus, was apparently the ultimate source of these factors, these observations did not directly lead to systematic coculturing efforts for other bacteria. Other examples of coculture dependence were found to consist of complementing auxotrophies, such as missing amino acids or other metabolites that the dependent bacterium could not synthesize for itself. As these auxotrophies could generally be overcome by using sufficiently rich medium (for example, yeast extract), they also did not cause a paradigm shift in culturing efforts. In an example that appears to be a case of a helper being necessary only for laboratory culture, the isolation of coculture-dependent *Symbiobacterium thermophilum* from compost in 1988 by Beppu and colleagues presented a mystery as to the identity of the helping factor for almost 2 decades (80). This isolate was found to be dependent on a *Bacillus* thermophile, and in 2006, the original group determined that this helper was providing carbon dioxide, a likely component of its natural environment (85). While this growth-inducing effect may be restricted to laboratory culture, the *Bacillus* species helper also appears to ameliorate the effects of toxic metabolites produced by *S. thermophilum*, leaving open the possibility of significant interactions in the environment (84).

With the success of coculturing efforts described above, however, the Lewis, Epstein, and Clardy groups undertook a study to directly isolate bacteria from intertidal sand biofilm that would grow only in the presence of helper organisms from the same environment in order to identify further molecular mechanisms of unculturability (21). The screen was based on the hypothesis that on a "crowded" isolation plate (a petri plate in which the environmental inoculum had been spread at a concentration such that a few hundred colonies would grow), some of the colonies would be growing only because they happened to be close to a helper colony. To identify these isolates, candidate colony pairs were cross-streaked and visually screened for dependent growth of one of the bacteria. Perhaps surprisingly, given the random pairing utilized, up to 10% of the screened isolates showed dependent growth. One dependent strain, *Maribacter polysiphoniae* KLE1104, was chosen as a model to identify its mechanism of codependence. In addition to being helped by other bacteria from the environment, it was also able to grow near a laboratory strain of *Escherichia coli* on a petri plate. This allowed the use of *E. coli* mutants to identify the genes involved in producing the growth factor and revealed that gene knockouts in the enterobactin synthesis pathway rendered *E. coli* unable to induce the growth of KLE1104. Enterobactin is a siderophore, a class of secreted small molecules that are able to solubilize oxidized iron (Fe^{3+}) and thereby make this essential nutrient available to cells. Spent medium from the natural environmental helper *Micrococcus luteus* KLE1011 was subjected to an iron-binding assay-guided fractionation to elucidate the structure of the siderophores produced in the sand biofilm, revealing five novel structural modifications of the siderophore desferrioxamine. Further screens revealed a number of bacteria from this environment to be dependent on siderophores (Fig. 2), with a range of specificities in the ability to use different siderophores. Importantly, the addition of reduced iron (Fe^{2+}, a form of iron that is bioavailable but essentially nonexistent in the aerobic marine environment) was able to overcome these dependencies and allowed the researchers to bypass the specificity inherent in siderophores. Using this soluble form of iron, they isolated several rare bacteria, including a member of the *Verrucomicrobia*, a member of the *Parvularculaceae*, and a bacterium distantly related to the *Gammaproteobacteria*.

Helper-dependent isolates apparently have lost the ability to perform essential [illegible] (summarized in Fig. [illegible]), and therefore, they also have lost the ability to grow in new environments [illegible] helpers are not present. While it is possible to explain such a loss as evolutionary "cheating," whereby these bacteria escape the cost of performing these functions on their own by pirating them from their neighbors, it is also possible that they are actually using the presence of specific helpers as an indication (i.e., a signal) of a conducive environment to begin growth. An alternative to these hypotheses, called the "Black Queen Hypothesis" in an analogy with the card game of the same name, was recently proposed by Morris, Lenski, and Zinser (54). According to this theory, organisms lose functions that are being complemented by their neighbors, similar to the cheater hypothesis. However, they suggest that this dependence on helper organisms is adaptive, in that it creates specialization within microbial communities that are driven by individual selection, resulting in greater fitness for the entire community. It seems likely, given the apparent ubiquity of the phenomenon, that all of these explanations play a role under the appropriate conditions.

Host-associated environments. The coculture dependence and environmental incubation requirements described above focused on non-host-associated environments. However, as increasing focus is placed on bacteria from the human microbiome that are important in health and disease, the potential role of host-associated unculturable bacteria is being investigated. In an attempt to quantify the role of the culturable and unculturable members of the gut microbiome, the group of Gordon used germfree mice as hosts for transplanted human intestinal microbial communities (29). Fecal samples from donors were either directly inoculated into germfree mice or first passed through a petri plate-based culturing step before introduction into mice. Interestingly, the petri plate cultivation step resulted in a high density of colonies on each plate (about 5,000) which may have resulted in the growth of helper-dependent bacteria which otherwise would not have grown on laboratory media. The mice were assayed for both the composition of the microbiome and the weight gain of the fat pad, a measure of health. Mice colonized with bacteria that passed through the cultivation step showed equivalent fat pad weight gain to those that were directly colonized, a finding which led the authors to conclude that the uncultured component may not be critical for at least some aspects of host health. However, their results on how the composition of the microbiome responded to dietary perturbation showed that mice that received a direct transfer of bacteria had a stronger response than those who received the cultured bacteria, indicating that some functionality of the microbiome may have been lost in the cultivation step. Therefore, the questions of how much of the gut microbiome can be cultured in the laboratory and what role the remaining fraction plays in host health remain mostly unresolved. To address these issues, studies to rigorously identify the culturable fraction and determine how to grow the unculturable remainder will need to be paired with comprehensive, long-term monitoring of host health.

There is already direct evidence that coculture relationships are at work in the human microbiome. The Wade group has grown previously unculturable cluster A *Synergistetes* isolates from subgingival plaque in coculture with other bacteria from the mouth (83). In another approach to access the oral microbiome, Epstein and coworkers developed a miniature version of the trap described above that could be carried in a volunteer's mouth (73). The diversity obtained from the trap was compared with dilution

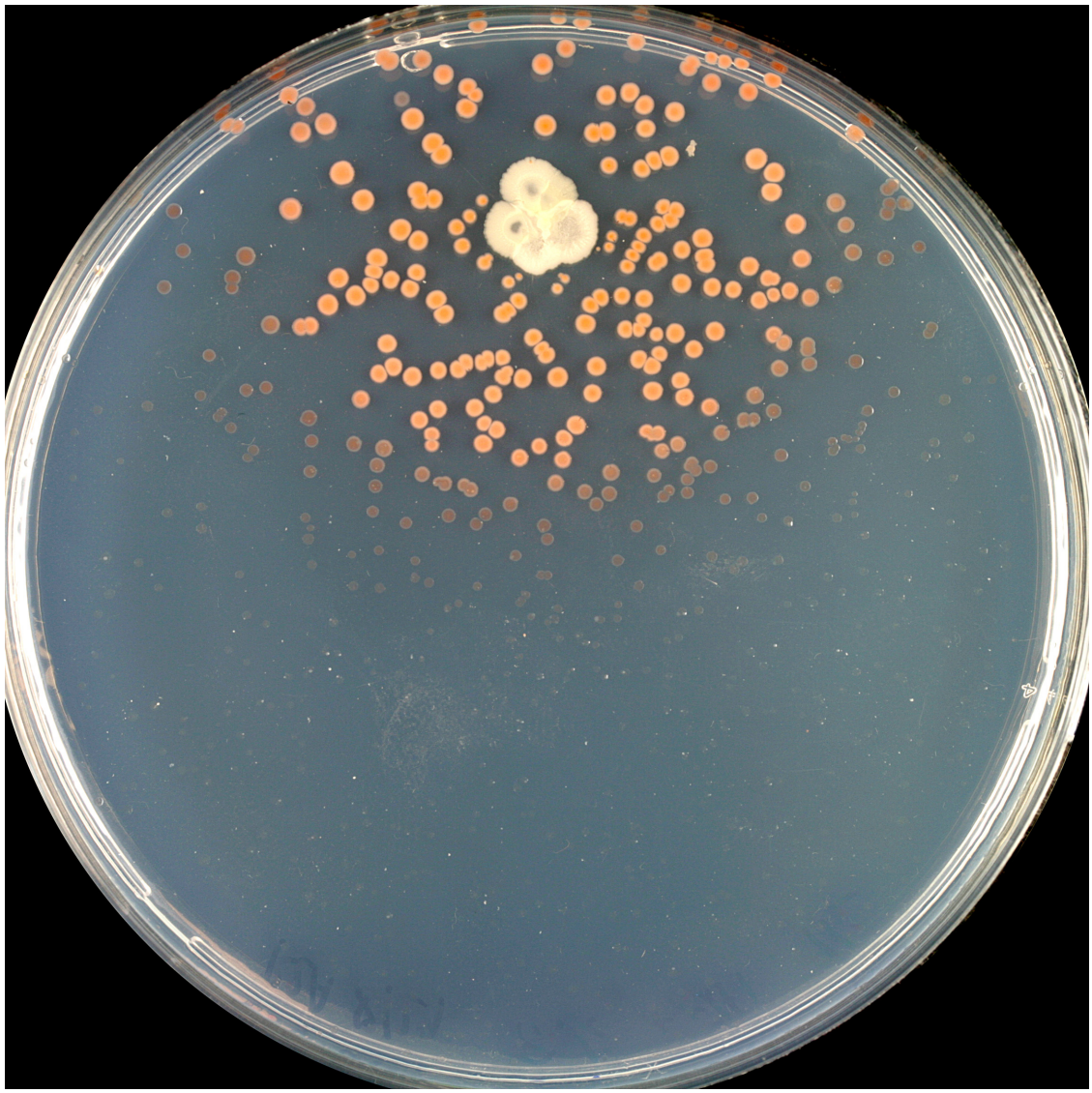

FIG 2 Coculture-dependent growth of an unculturable isolate. This petri plate shows a helper strain inducing the growth of a previously unculturable bacterium. A freshwater sediment isolate (a relative of *Bacillus marisflavi* that was originally isolated from Punderson Lake State Park, Newbury, OH) was diluted and spread evenly over the entire petri plate (R2A medium), and a culture of a helper (a relative of *Bacillus megaterium* that was isolated from the same environment) was spotted on the plate. The unculturable isolate grows only close to the helper, where a growth factor has diffused into the medium of the plate. Preliminary results indicate that the growth factor is a siderophore (A. D'Onofrio, J. M. Crawford, E. J. Stewart, K. Witt, E. Gavrish, S. Epstein, J. Clardy, and K. Lewis, unpublished data).

to extinction and direct pour plating in petri plates. Both the trap and the dilution protocols produced greater diversity than the pour plates, and 10 novel isolates were cultivated. Interestingly, there was little overlap in isolates from the different culturing methods, suggesting that multiple approaches may yield greater diversity. Given the attention to the human microbiome and the culturing advances under way, it is likely that identification of growth factors contributed by one bacterial member of the microbiome to another will happen soon.

A subset of host-associated bacteria appear to be obligate intracellular symbionts or pathogens and, as such, have not been grown outside the host or cultured cell lines. A classic example is the pathogen *Treponema pallidum* subsp. *pallidum*, the causative agent of syphilis. Identified as the cause of the disease in 1905 (69), the genome of *T. pallidum* subsp. *pallidum* was sequenced in 1998 (26), and yet even in tissue culture with host cells, this bacterium cannot be kept in continuous culture. To date, the only reliable method of propagation is live rabbits (44). More-recently identified uncultured symbionts of the gut epithelium in many animals (although apparently not humans) are the segmented filamentous bacteria (SFB; also "*Candidatus* Arthromitus"), which appear to be epicellular on host cells (17, 74). The genome sequences of mouse- and rat-associated SFB were recently completed, and like the syphilis organism, they exhibit the markedly reduced metabolic capacity characteristic of obligate, host cell-associated bacteria (43, 61, 70). SFB also have not been propagated outside host animals. The most abundant examples of bacteria closely tied to their hosts are the endosymbiotic bacteria of insects, the vast ma-

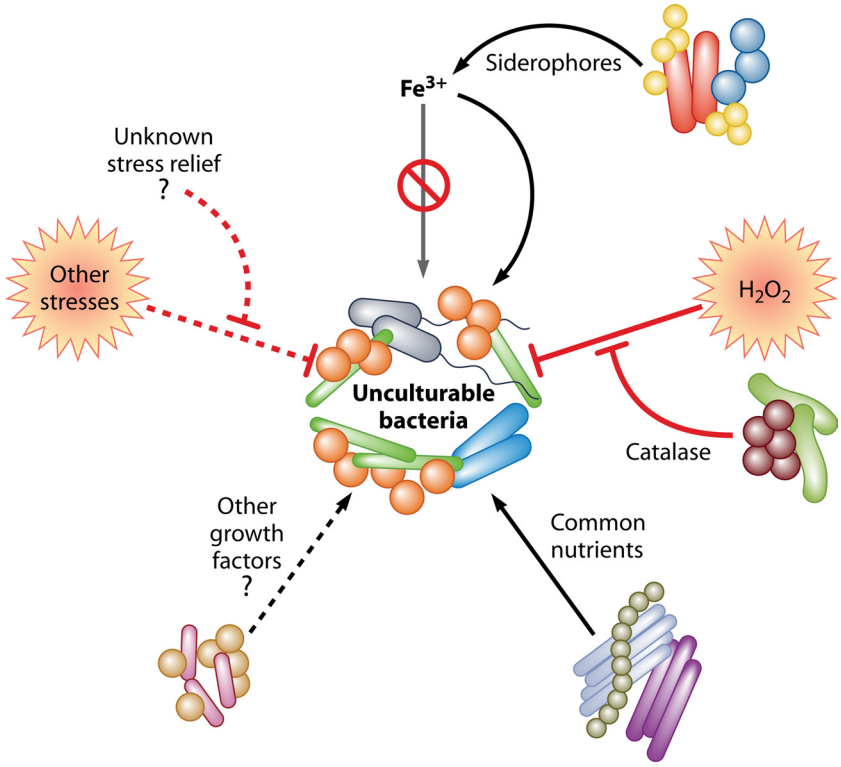

FIG 3 Model of mechanisms of coculture helping. Unculturable bacteria are schematically represented in the center of the figure, while known and potential helpers are arrayed around the periphery. Arrows indicate positive growth effects; stopped lines indicate inhibitory growth effects. Dashed arrows and inhibition lines indicate effects caused by as-yet-unidentified factors, while solid symbols indicate known factors. (Top center) In aerobic environments, molecular iron is completely oxidized (Fe^{3+}) and is unavailable to cells without specific systems for acquiring it (gray arrow with prohibition symbol). Siderophores from neighboring bacteria bind and solubilize Fe^{3+}, making it available to bacteria that cannot grow without this help. (Top right) Hydrogen peroxide (and possibly other forms of reactive oxygen species) can prevent the growth of sensitive bacteria. Helper organisms can protect against this effect by removing the oxidative stress, allowing the growth of the sensitive bacteria. (Bottom right) Helper bacteria can provide amino acids, vitamins, carbon sources, and other common nutrients that are often included in rich laboratory medium. (Bottom left and top left) Depictions of growth factors and stress-relieving effects yet to be discovered.

jority of which have never been cultured. The study of these numerous and biologically significant microbes has become its own field and has been reviewed recently (39). Examples such as these indicate, perhaps unsurprisingly, that once genome reduction occurs in a bacterium due to close host association, the difficulty of cultivation becomes significantly greater. This area may very well prove to be the greatest challenge in bacterial cultivation and therefore see the slowest progress.

Promising approaches for the future. The successes described above have, for the most part, combined traditional culturing methods (petri plates, liquid cultures) with new ways of making the medium more similar to the environment, including coculture with other environmental bacteria. The approach of Keller, however, in which cells were encapsulated in microdroplets, may foreshadow the next generation of culturing technology. One reason for this prediction is that a primary concern for the cultivation of novel bacteria is the effective throughput rate; as discussed above, the number of bacterial taxa that have never been cultured is thought to be considerable. Except for the cases in which cultivation efforts are directed at specific, preidentified strains of high biological importance, high-throughput methods will need to be developed to grow significant numbers of previously unculturable taxa. In general, efforts are under way to meet this need by combining the discoveries in growth factors and environment mimicry with highly parallel culturing, microfluidics, or both.

Highly parallel (macroscale) culture systems allow many isolates to be cultivated simultaneously, and there are a number of systems being developed. Tsuneda and coworkers developed a capillary-based culturing system based on porous hollow-fiber membranes (4). Microbial cells from an environmental sample are diluted and loaded into the fibers by syringes, and the fibers can then be lowered into a liquid environment, either simulated or natural. Dilution results in potentially single cells in many of the 96 parallel fibers, and diffusion through the porous membrane walls of the fibers allows chemical communication with the environment. After 2 months of incubation in three test environments (tidal sediment, waste treatment sludge, and a laboratory bioreactor), the fiber-based system cultivated a higher proportion of novel isolates (<97% 16S rRNA gene sequence similarity to cultured strains) than petri plates containing media designed to mimic each environment. Effectively, this created a higher-throughput system analogous to the environmental chambers described above. In a direct extension of the environmental chamber concept previously described, the Epstein and Lewis groups developed the isolation chip (ichip) for high-throughput cultivation (56). Essentially many small chambers arranged on a single sub-

strate, the ichip contains 384 holes that form the chambers, each 1 mm in diameter. The ichip was tested on soil and seawater samples and, compared to standard petri plates or a single chamber of the original design, allowed cultivation of greater total numbers of cells as well as greater total diversity of taxa.

As the sizes of the individual cultivation chambers decrease, diffusion with the surrounding environment should increase, potentially providing an additional advantage beyond that of increased throughput. Microfluidics-based cultivation miniaturizes the cell handling and incubation of microbes, potentially maximizing both of these advantages. Microfluidic devices handle liquid down to picoliter volumes, allowing the isolation and manipulation of single cells. One class of microfluidics, droplet-based microfluidics (reviewed in reference 71), has been particularly promising for cultivation systems. Early work has primarily demonstrated cell handling and isolation for cultivation. The group of Köhler developed a segmented flow chip, for which aqueous "segments" (short intervals of water separated within a stream of an alkane), flow through microchannels in a silicone wafer chip (30). Aqueous segments could be formed holding one or a few bacterial cells by dilution of a sample and subsequently incubated to allow growth of those cells in the 20- to 60-nl volume of the segment. This allowed the parallel cultivation of potentially pure cultures in a small volume, which could then be plated after multiplication. Ismagilov and coworkers used another segment-based microfluidics approach, called a "chemistrode," in which after multiplication of bacteria within the aqueous compartments, the segments could be split to allow multiple parallel processes to be carried out on a single isolate (46). This approach may allow analyses that would normally be lethal to the bacteria (such as identification of the isolate by fluorescence *in situ* hybridization, PCR amplification of the 16S rRNA gene for sequencing, or other techniques) to be carried out in parallel with cultivation. This group also subsequently showed (using a different microfluidics technique) that confinement of one or a few bacteria in a very small volume resulted in a high effective cell density per unit volume, which may trigger quorum-sensing activation in a single cell (11). It has been noted that some bacteria appear to grow only when inoculated above a certain density (such as *Prochlorococcus*, described above); this raises the possibility that confinement in small volumes by itself may allow the cultivation of previously unculturable taxa, even when only a single cell is available. Confinement in microfluidic droplets may also be used to bring together isolates for coculture. The Lin group demonstrated such a model system using auxotrophic laboratory strains in which a synthetic pair of symbiotic *Escherichia coli* mutants were diluted into 1-nl microdroplets. Growth occurred only when both variants were present in the same drop, showing coculture on the microfluidics scale (60). These are just a few examples of the many approaches to single-cell isolation, both for cultivation and for biochemical analyses (reviewed in reference 35). Combining droplet microfluidics as described above with traditional cell sorting systems may allow the isolation and cocultivation of specific unculturable bacteria of interest or the high-throughput cultivation of many novel isolates.

CONCLUSION

Although the majority of environmental bacteria are not growing in the laboratory, the last decade has seen the development of several effective approaches for growing these organisms. Coculture with other bacteria has identified the molecular mechanisms of some of these helping effects, while bringing the environment into the laboratory has allowed the cultivation of many novel isolates. In addition, microscale cultivation is increasing the rate of isolation of unculturable bacteria, and with this pool of isolates, it will be possible to identify many more of the mechanisms behind the inability of these bacteria to grow in the laboratory. With the ability to culture them, we will learn about their role in the environment, ecology, and nutrient cycling. But perhaps even more importantly, cultivating these bacteria will have profound effects for human health. For drug discovery, screening of novel isolates will reveal novel natural products that may finally end the discovery void that has plagued antibiotic development since 1987. In the human microbiome, access to the uncultured bacteria that live in and on us will improve health through an enhanced understanding of the role played by these microbes. The results of the next 30 years of cultivation efforts will likely exceed those of the last 300 years, with a similar magnitude of benefits for health, ecology, and science.

ACKNOWLEDGMENTS

I thank Anthony Bissell, Stefanie Timmermann, Kathrin Witt, and Patrick Lane for valuable assistance with the figures, Slava Epstein for references, and Kim Lewis and the members of the Antimicrobial Discovery Center for helpful comments.

REFERENCES

1. **Achtman M, Wagner M.** 2008. Microbial diversity and the genetic nature of microbial species. Nat. Rev. Microbiol. **6**:431–440.
2. **Amann J.** 1911. Die direkte Zählung der Wasserbakterien mittels des Ultramikroskops. Centralbl. Bakteriol. II Abt. **29**:381–384.
3. **Amann RI, Ludwig W, Schleifer KH.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59**:143–169.
4. **Aoi Y, et al.** 2009. Hollow-fiber membrane chamber as a device for in situ environmental cultivation. Appl. Environ. Microbiol. **75**:3826–3833.
5. **Artman M, Frankl G.** 1982. Nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate splitting enzyme(s) of sheep and rabbit erythrocytes: their effect on the growth of Haemophilus. Can. J. Microbiol. **28**:696–702.
6. **Baltz RH.** 2007. Antimicrobials from actinomycetes: back to the future. Microbe **2**:125–131.
7. **Baltz RH.** 2008. Renaissance in antibacterial discovery from actinomycetes. Curr. Opin. Pharmacol. **8**:557–563.
8. **Beja O, et al.** 2000. Bacterial rhodopsin: evidence for a new type of phototrophy in the sea. Science **289**:1902–1906.
9. **Ben-Dov E, Kramarsky-Winter E, Kushmaro A.** 2009. An in situ method for cultivating microorganisms using a double encapsulation technique. FEMS Microbiol. Ecol. **68**:363–371.
10. **Bik EM, et al.** 2010. Bacterial diversity in the oral cavity of 10 healthy individuals. ISME J. **4**:962–974.
11. **Boedicker JQ, Vincent ME, Ismagilov RF.** 2009. Microfluidic confinement of single cells of bacteria in small volumes initiates high-density behavior of quorum sensing and growth and reveals its variability. Angew Chem. Int. Ed. Engl. **48**:5908–5911.
12. **Bollmann A, Lewis K, Epstein SS.** 2007. Incubation of environmental samples in a diffusion chamber increases the diversity of recovered isolates. Appl. Environ. Microbiol. **73**:6386–6390.
13. **Bollmann A, Palumbo AV, Lewis K, Epstein SS.** 2010. Isolation and physiology of bacteria from contaminated subsurface sediments. Appl. Environ. Microbiol. **76**:7413–7419.
14. **Bomar L, Maltz M, Colston S, Graf J.** 2011. Directed culturing of microorganisms using metatranscriptomics. mBio **2**(2):e00012–11. doi: 10.1128/mBio.00012-11.
15. **Connon SA, Giovannoni SJ.** 2002. High-throughput methods for culturing microorganisms in very-low-nutrient media yield diverse new marine isolates. Appl. Environ. Microbiol. **68**:3878–3885.
16. **Davies J.** 2007. Small molecules: the lexicon of biodiversity. J. Biotechnol. **129**:3–5.

17. **Davis CP, Savage DC.** 1974. Habitat, succession, attachment, and morphology of segmented, filamentous microbes indigenous to the murine gastrointestinal tract. Infect. Immun. **10**:948–956.
18. **Davis DJ.** 1921. The accessory factors in bacterial growth. IV. The "satellite" or symbiosis phenomenon of Pfeiffer's Bacillus (B. influenzae). J. Infect. Dis. **29**:178–186.
19. **Demain AL, Sanchez S.** 2009. Microbial drug discovery: 80 years of progress. J. Antibiot. (Tokyo) **62**:5–16.
20. **Dinis JM, et al.** 2011. In search of an uncultured human-associated TM7 bacterium in the environment. PLoS One **6**:e21280. doi:10.1371/journal.pone.0021280.
21. **D'Onofrio A, et al.** 2010. Siderophores from neighboring organisms promote the growth of uncultured bacteria. Chem. Biol. **17**:254–264.
22. **Ferrari BC, Binnerup SJ, Gillings M.** 2005. Microcolony cultivation on a soil substrate membrane system selects for previously uncultured soil bacteria. Appl. Environ. Microbiol. **71**:8714–8720.
23. **Ferrari BC, Gillings MR.** 2009. Cultivation of fastidious bacteria by viability staining and micromanipulation in a soil substrate membrane system. Appl. Environ. Microbiol. **75**:3352–3354.
24. **Fildes P.** 1921. The nature of the effect of blood-pigment upon the growth of B. influenzae. Br. J. Exp. Pathol. **2**:16–25.
25. **Fleming A.** 1919. On some simply prepared culture media for B. influenzae with a note regarding the agglutination reaction of sera from patients suffering from influenza to this bacillus. Lancet **193**:138–139.
26. **Fraser CM, et al.** 1998. Complete genome sequence of Treponema pallidum, the syphilis spirochete. Science **281**:375–388.
27. **Gavrish E, Bollmann A, Epstein S, Lewis K.** 2008. A trap for in situ cultivation of filamentous actinobacteria. J. Microbiol. Methods **72**:257–262.
28. **Giovannoni SJ, et al.** 2005. Genome streamlining in a cosmopolitan oceanic bacterium. Science **309**:1242–1245.
29. **Goodman AL, et al.** 2011. Extensive personal human gut microbiota culture collections characterized and manipulated in gnotobiotic mice. Proc. Natl. Acad. Sci. U. S. A. **108**:6252–6257.
30. **Grodrian A, et al.** 2004. Segmented flow generation by chip reactors for highly parallelized cell cultivation. Biosens. Bioelectron. **19**:1421–1428.
31. **Hardoim CCP, et al.** 2009. Diversity of bacteria in the marine sponge *Aplysina fulva* in Brazilian coastal waters. Appl. Environ. Microbiol. **75**:3331–3343.
32. **Hugenholtz P, Tyson GW, Webb RI, Wagner AM, Blackall LL.** 2001. Investigation of candidate division TM7, a recently recognized major lineage of the domain bacteria with no known pure-culture representatives. Appl. Environ. Microbiol. **67**:411–419.
33. **Huggett MJ, Rappe MS.** 2012. Genome sequence of strain HIMB30, a novel member of the marine *Gammaproteobacteria*. J. Bacteriol. **194**:732–733.
34. **Huse SM, Welch DM, Morrison HG, Sogin ML.** 2010. Ironing out the wrinkles in the rare biosphere through improved OTU clustering. Environ. Microbiol. **12**:1889–1898.
35. **Ishii S, Tago K, Senoo K.** 2010. Single-cell analysis and isolation for microbiology and biotechnology: methods and applications. Appl. Microbiol. Biotechnol. **86**:1281–1292.
36. **Jang Y, Oh HM, Kim H, Kang I, Cho JC.** 2011. Genome sequence of strain IMCC1989, a novel member of the marine *Gammaproteobacteria*. J. Bacteriol. **193**:3672–3673.
37. **Kaeberlein T, Lewis K, Epstein SS.** 2002. Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment. Science **296**:1127–1129.
38. **Keller M, Zengler K.** 2004. Tapping into microbial diversity. Nat. Rev. Microbiol. **2**:141–150.
39. **Kikuchi Y.** 2009. Endosymbiotic bacteria in insects: their diversity and culturability. Microbes Environ. **24**:195–204.
40. **Kim JJ, et al.** 2008. Characterization of growth-supporting factors produced by Geobacillus toebii for the commensal thermophile Symbiobacterium toebii. J. Microbiol. Biotechnol. **18**:490–496.
41. **Kim K, Kim JJ, Masui R, Kuramitsu S, Sung MH.** 2011. A commensal symbiotic interrelationship for the growth of Symbiobacterium toebii with its partner bacterium, Geobacillus toebii. BMC Res. Notes **4**:437.
42. **Krumwiede E, Kuttner AG.** 1938. A growth inhibitory substance for the influenza group of organisms in the blood of various animal species: the use of the blood of various animals as a selective medium for the detection of hemolytic streptococci in throat cultures. J. Exp. Medicine **67**:429–441.
43. **Kuwahara T, et al.** 2011. The lifestyle of the segmented filamentous bacterium: a non-culturable gut-associated immunostimulating microbe inferred by whole-genome sequencing. DNA Res. **18**:291–303.
44. **Lafond RE, Lukehart SA.** 2006. Biological basis for syphilis. Clin. Microbiol. Rev. **19**:29–49.
45. **Lewis K, Epstein S, D'Onofrio A, Ling LL.** 2010. Uncultured microorganisms as a source of secondary metabolites. J. Antibiot. (Tokyo) **63**:468–476.
46. **Liu W, Kim HJ, Lucchetta EM, Du W, Ismagilov RF.** 2009. Isolation, incubation, and parallel functional testing and identification by FISH of rare microbial single-copy cells from multi-species mixtures using the combination of chemistrode and stochastic confinement. Lab Chip **9**:2153–2162.
47. **Livermore DM.** 2011. Discovery research: the scientific challenge of finding new antibiotics. J. Antimicrob. Chemother. **66**:1941–1944.
48. **Logares R, Brate J, Heinrich F, Shalchian-Tabrizi K, Bertilsson S.** 2010. Infrequent transitions between saline and fresh waters in one of the most abundant microbial lineages (SAR11). Mol. Biol. Evol. **27**:347–357.
49. **Lwoff A, Lwoff M.** 1937. Studies on codehydrogenases. I. Nature of growth factor "V." Proc. R. Soc. Lond. B Biol. Sci. **122**:352–359.
50. **Moore LR, et al.** 2007. Culturing the marine cyanobacterium Prochlorococcus. Limnol. Oceanogr. Methods **5**:353–362.
51. **Moore LR, Post AF, Rocap G, Chisholm SW.** 2002. Utilization of different nitrogen sources by the marine cyanobacteria Prochlorococcus and Synechococcus. Limnol. Oceanogr. **47**:989–996.
52. **Morris JJ, Johnson ZI, Szul MJ, Keller M, Zinser ER.** 2011. Dependence of the cyanobacterium Prochlorococcus on hydrogen peroxide scavenging microbes for growth at the ocean's surface. PLoS One **6**:e16805. doi:10.1371/journal.pone.0016805.
53. **Morris JJ, Kirkegaard R, Szul MJ, Johnson ZI, Zinser ER.** 2008. Facilitation of robust growth of *Prochlorococcus* colonies and dilute liquid cultures by "helper" heterotrophic bacteria. Appl. Environ. Microbiol. **74**:4530–4534.
54. **Morris JJ, Lenski RE, Zinser ER.** 2012. The Black Queen Hypothesis: evolution of dependencies through adaptive gene loss. mBio **3**(2):e00036–12. doi:10.1128/mBio.00036-12.
55. **Morris RM, et al.** 2002. SAR11 clade dominates ocean surface bacterioplankton communities. Nature **420**:806–810.
56. **Nichols D, et al.** 2010. Use of ichip for high-throughput *in situ* cultivation of "uncultivable" microbial species. Appl. Environ. Microbiol. **76**:2445–2450.
57. **Nye KJ, et al.** 1999. A comparison of blood agar supplemented with NAD with plain blood agar and chocolated blood agar in the isolation of Streptococcus pneumoniae and Haemophilus influenzae from sputum. J. Med. Microbiol. **48**:1111–1114.
58. **Pace NR.** 2009. Mapping the tree of life: progress and prospects. Microbiol. Mol. Biol. Rev. **73**:565–576.
59. **Page KA, Connon SA, Giovannoni SJ.** 2004. Representative freshwater bacterioplankton isolated from Crater Lake, Oregon. Appl. Environ. Microbiol. **70**:6542–6550.
60. **Park J, Kerner A, Burns MA, Lin XN.** 2011. Microdroplet-enabled highly parallel co-cultivation of microbial communities. PLoS One **6**:e17019. doi:10.1371/journal.pone.0017019.
61. **Prakash T, et al.** 2011. Complete genome sequences of rat and mouse segmented filamentous bacteria, a potent inducer of th17 cell differentiation. Cell Host Microbe **10**:273–284.
62. **Rappe MS, Connon SA, Vergin KL, Giovannoni SJ.** 2002. Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. Nature **418**:630–633.
63. **Rappe MS, Giovannoni SJ.** 2003. The uncultured microbial majority. Annu. Rev. Microbiol. **57**:369–394.
64. Resh VH, Cardé RT (ed). 2009. Encyclopedia of insects, 2nd ed. Academic Press, Burlington, MA.
65. **Rheims H, Rainey FA, Stackebrandt E.** 1996. A molecular approach to search for diversity among bacteria in the environment. J. Ind. Microbiol. **17**:159–169.
66. **Rippka R, et al.** 2000. Prochlorococcus marinus Chisholm et al. 1992 subsp. pastoris subsp. nov. strain PCC 9511, the first axenic chlorophyll a_2/b_2-containing cyanobacterium (Oxyphotobacteria). Int. J. Syst. Evol. Microbiol. **50**:1833–1847.
67. **Rossello-Mora R, Amann R.** 2001. The species concept for prokaryotes. FEMS Microbiol. Rev. **25**:39–67.
68. **Roszak DB, Colwell RR.** 1987. Survival strategies of bacteria in the natural environment. Microbiol. Rev. **51**:365–379.

69. **Schaudinn FN, Hoffmann E.** 1905. Vorläufiger Bericht über das Vorkommen von Spirochaeten in syphilitischen Krankheitsprodukten und bei Papillomen. Arb. K. Gesund. **22**:527–534.
70. **Sczesnak A, et al.** 2011. The genome of th17 cell-inducing segmented filamentous bacteria reveals extensive auxotrophy and adaptations to the intestinal environment. Cell Host Microbe **10**:260–272.
71. **Seemann R, Brinkmann M, Pfohl T, Herminghaus S.** 2012. Droplet based microfluidics. Rep. Prog. Phys. **75**:016601. doi:10.1088/0034-4885/75/1/016601.
72. **Silver LL.** 2011. Challenges of antibacterial discovery. Clin. Microbiol. Rev. **24**:71–109.
73. **Sizova MV, et al.** 2012. New approaches for isolation of previously uncultivated oral bacteria. Appl. Environ. Microbiol. **78**:194–203.
74. **Snel J, et al.** 1995. Comparison of 16S rRNA sequences of segmented filamentous bacteria isolated from mice, rats, and chickens and proposal of "Candidatus Arthromitus". Int. J. Syst. Bacteriol. **45**:780–782.
75. **Song J, Oh HM, Cho JC.** 2009. Improved culturability of SAR11 strains in dilution-to-extinction culturing from the East Sea, West Pacific Ocean. FEMS Microbiol. Lett. **295**:141–147.
76. **Spieckermann A.** 1912. Trink- und Gebrauchswasser. Zeitschrift für Lebensmitteluntersuchung und -Forschung A. **24**:710.
77. **Staley JT, Konopka A.** 1985. Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. Annu. Rev. Microbiol. **39**:321–346.
78. **Steindler L, Schwalbach MS, Smith DP, Chan F, Giovannoni SJ.** 2011. Energy starved *Candidatus* Pelagibacter ubique substitutes light-mediated ATP production for endogenous carbon respiration. PLoS One **6**:e19725. doi:10.1371/journal.pone.0019725.
79. **Stingl U, Desiderio RA, Cho JC, Vergin KL, Giovannoni SJ.** 2007. The SAR92 clade: an abundant coastal clade of culturable marine bacteria possessing proteorhodopsin. Appl. Environ. Microbiol. **73**:2290–2296.
80. **Suzuki S, Horinouchi S, Beppu T.** 1988. Growth of a tryptophanase-producing thermophile, Symbiobacterium thermophilum gen. nov., sp. nov., is dependent on co-culture with a Bacillus sp. J. Gen. Microbiol. **134**:2353–2362.
81. **Tanaka Y, et al.** 2004. Catellibacterium nectariphilum gen. nov., sp. nov., which requires a diffusible compound from a strain related to the genus Sphingomonas for vigorous growth. Int. J. Syst. Evol. Microbiol. **54**:955–959.
82. **Tripp HJ, et al.** 2008. SAR11 marine bacteria require exogenous reduced sulphur for growth. Nature **452**:741–744.
83. **Vartoukian SR, Palmer RM, Wade WG.** 2010. Cultivation of a Synergistetes strain representing a previously uncultivated lineage. Environ. Microbiol. **12**:916–928.
84. **Watsuji T-O, et al.** 2007. Identification of indole derivatives as self-growth inhibitors of *Symbiobacterium thermophilum*, a unique bacterium whose growth depends on coculture with a *Bacillus* sp. Appl. Environ. Microbiol. **73**:6159–6165.
85. **Watsuji TO, Kato T, Ueda K, Beppu T.** 2006. CO_2 supply induces the growth of Symbiobacterium thermophilum, a syntrophic bacterium. Biosci. Biotechnol. Biochem. **70**:753–756.
86. **Woese CR.** 1987. Bacterial evolution. Microbiol. Rev. **51**:221–271.
87. **Zengler K, et al.** 2002. Cultivating the uncultured. Proc. Natl. Acad. Sci. U. S. A. **99**:15681–15686.
88. **Zengler K, et al.** 2005. High-throughput cultivation of microorganisms using microcapsules. Methods Enzymol. **397**:124–130.