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Annual Review of Microbiology

Present and Future of Culturing Bacteria

Jörg Overmann,[1,2] Birte Abt,[1,2] and Johannes Sikorski[1]

[1]Leibniz-Institute DSMZ-German Collection of Microorganisms and Cell Cultures, 38124 Braunschweig, Germany; email: joerg.overmann@dsmz.de

[2]German Center for Infection Research, Partner Site Hannover-Braunschweig, 38124 Braunschweig, Germany

Annu. Rev. Microbiol. 2017. 71:711–30

First published as a Review in Advance on July 21, 2017

The *Annual Review of Microbiology* is online at micro.annualreviews.org

https://doi.org/10.1146/annurev-micro-090816-093449

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Keywords

cultivation, bacterial cultures, uncultured, uncultivable, culture collections

Abstract

The cultivation of bacteria is highly biased toward a few phylogenetic groups. Many of the currently underexplored bacterial lineages likely have novel biosynthetic pathways and unknown biochemical features. New cultivation concepts have been developed based on an improved understanding of the ecology of previously not-cultured bacteria. Particularly successful were improved media that mimic the natural types and concentrations of substrates and nutrients, high-throughput cultivation techniques, and approaches that exploit biofilm formation and bacterial interactions. Metagenomics and single-cell genomics can reveal unknown metabolic features of not-yet-cultured bacteria and, if complemented by culture-independent physiological analyses, will help to target functional novelty more efficiently. However, numerous novel types of bacteria that were initially enriched subsequently escaped isolation. Future cultivation work will therefore need to focus on improved subcultivation, purification, and preservation techniques to recover and utilize a larger fraction of microbial diversity.

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INTRODUCTION

Laboratory cultivation of microorganisms commenced more than 150 years ago. To date, it has enabled the description of ~12,000 species (**https://www.dsmz.de/bacterial-diversity/prokaryotic-nomenclature-up-to-date.html**). Ongoing cultivation efforts continue to yield between 600 and 800 novel bacterial species per year (100). Yet, these figures appear minute when compared to estimates of global bacterial diversity, which range between 10^7 and 10^9 species (27). The total species richness of Bacteria and Archaea in the oceans exceeds 37,000 (126), and even individual soils harbor up to 54,000 species (114).

First comparisons of the 16S rRNA sequences from cultivated bacteria with those determined in their natural environment revealed that particular groups and even entire bacterial phyla had systematically escaped cultivation (7, 36, 127). These so-called uncultivable, unculturable, or nonculturable bacteria represent a current major challenge in microbiology (23, 24, 73, 113, 129, 144, 145) and are repeatedly addressed in the literature (>200 articles in the five years from 2012 through 2016; **https://www.ncbi.nlm.nih.gov/**). In only a few cases, such as the human microbiome, can the representative, dominant bacteria be readily cultured in the laboratory (39).

Because cultivation of a novel microorganism is often tedious and time consuming, progress has been rather slow over the last two decades (4, 13–15, 50, 57, 58, 69, 75, 78, 104). However, the vast unexplored bacterial diversity is expected to offer novel fundamental insights into biological principles and new applications for biotechnology and human health. Meanwhile, advanced cultivation technology in combination with information provided by culture-independent experimental approaches provides the chance to gain access to many biologically novel, not-yet-cultured bacteria.

THE RELEVANCE OF CULTURING BACTERIAL DIVERSITY

Many of the currently underexplored bacterial lineages are likely to feature novel biosynthetic pathways and unknown biochemical characteristics and therefore potentially offer new and innovative solutions for biotechnology, agriculture, and public health. For instance, novel compounds may be

produced by "Tectomicrobia" (recently proposed as a candidate phylum) (137), *Acidobacteria* (109), *Chloroflexi* (91), *Planctomycetes* (70), and *Myxobacteria* (146). Previously unknown *Chloroflexi* of the genus *Dehalococcoides* degrade anthropogenic organohalide compounds and now are employed in bioremediation (72). A better understanding of the key bacterial species of the human intestinal microbiome would provide novel opportunities for the treatment of diseases such as obesity, inflammatory bowel disease, and type 2 diabetes (17). Also, the identification of the causative agents of infectious diseases through fulfillment of Koch's postulates and the analysis of the virulence mechanisms or antibiotic susceptibilities of bacterial pathogens rely on the availability of isolated strains.

Meanwhile, the pace of sequencing bacterial genomes surpasses that of isolating novel species (100), and metagenomics or single-cell genomics can uncover unexpected physiological capacities of not-yet-cultivated microorganisms (109, 133, 137). However, omics approaches cannot substitute for cultivation-based studies of phylogenetic and functional novelty since functional predictions largely rely on the availability of well-annotated genomes from cultured representatives (23). Thus, there is no prediction regarding the function of ≥50% of the detected genes from candidate bacterial phyla (86, 145) (**Figure 1*a***), and an even larger fraction (85%) cannot be assigned to any metabolic pathway (86). Correspondingly, unknown protein families discovered in the marine bacterioplankton outnumber known families, and 65–90% of the genes in soil metagenomes cannot be annotated (23, 143). Interestingly, the fraction of unknown functional genes in the cultured bacterial phyla correlates with the fraction of cultured representatives, which emphasizes the relevance of studying biologically novel isolates (**Figure 1*b***).

More complex functional traits of bacteria, such as autotrophic growth via the oxidation of phosphite (116) or arsenite (96) or the functions of novel types of photosynthetic antennae in *Proteobacteria* (51, 106), cannot be easily deduced from bacterial genome sequences alone. In particular, unusual kinetics or enzyme characteristics like the high-affinity ammonium oxidation of the thaumarchaeon "*Candidatus* Nitrosopumilus maritimus" (89) or the unprecedented low-maintenance energy requirement of an extremely low-light-adapted anoxygenic phototroph (87) require culture-based experimentation. In some cases, the physiological role of allegedly well-known gene families remains obscure. Thus, physiological traits like glycoside hydrolysis inferred from genome analysis did not correspond with the observed phenotype (60, 134). Biochemical verification of hypothetical novel pathways is required to elucidate the enzymatic mechanism (94).

WHAT HAS BEEN MISSED BY CULTIVATION-BASED APPROACHES?

In the environment, bacterial cells of not-yet-cultured phylotypes are physiologically active, as indicated by transcription of functional genes (49), assimilation of radioactively labeled organic carbon compounds (26, 97), incorporation of the thymidine analog bromodeoxyuridine (BrdU) into DNA (107), or live-dead staining (69). Therefore, if appropriate incubation conditions are provided, these bacteria should also divide in the laboratory, rendering the terms uncultivable, unculturable, and nonculturable unsuitable.

If appropriate cultivation techniques were available and a sufficiently large number of cultivation trials conducted (e.g., by high-throughput cultivation approaches), it should be possible to culture bacteria independently of their phylogenetic affiliation. On the contrary, all described species fall into only 30 of ~80 currently recognized bacterial phyla and 3 of ~26 archaeal phyla (64, 122; **https://www.dsmz.de/bacterial-diversity/prokaryotic-nomenclature-up-to-date.html**). An entire cluster of 34 bacterial phyla (the so-called Candidate Phyla Radiation) was recently identified through metagenomics studies of a groundwater bacterial community and encompasses exclusively not-yet-cultivated bacteria (11, 64). With 90% of all validly described bacteria affiliated with the four phyla *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* (113), the cultivated fraction

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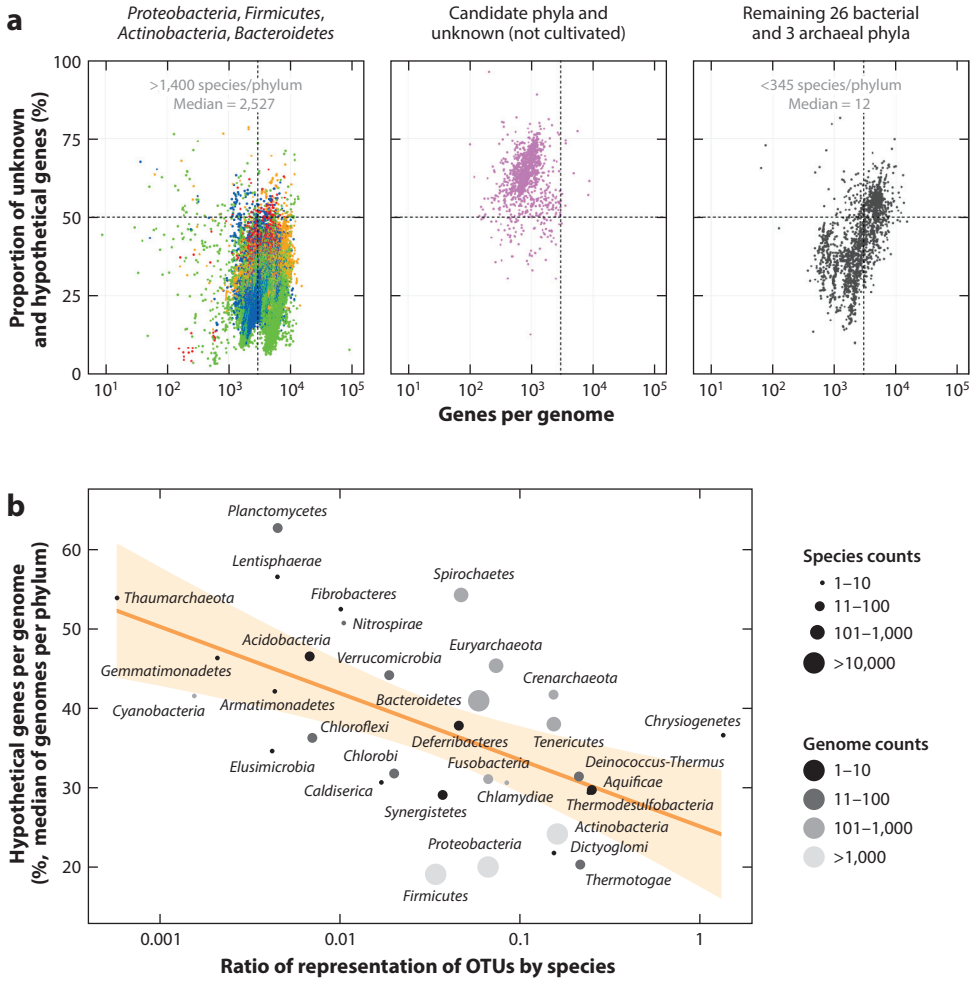

Figure 1

Genomic features of species in cultured and uncultured phyla. (*a*) Incompleteness of genome annotation. Dots represent 57,741 genomes of the well-cultured phyla *Proteobacteria* (*green*), *Actinobacteria* (*orange*), *Firmicutes* (*blue*), and *Bacteroidetes* (*red*); 1,167 genomes of uncultured candidate and unknown phyla (*pink*); and 2,131 genomes from the remaining 29 phyla (*dark gray*). (*b*) The incompleteness of genome annotation negatively correlates with the representation of phyla by cultured species. The taxonomy of 226,267 species-level operational taxonomic units (OTUs) as defined by clustering of full-length, high-quality 16S rRNA sequences was obtained from the SILVA database (122). Counts of cultured species were obtained from the Prokaryotic Nomenclature Up-to-Date database (**https://www.dsmz.de/bacterial-diversity/prokaryotic-nomenclature-up-to-date.html**). The Spearman's rank correlation is significant ($P = 0.00065$, $\rho = -0.58$). The shaded area represents the 95% confidence interval.

of prokaryotic diversity is heavily biased. Although reports of the successful initial cultivation of previously unculturable bacterial groups (e.g., *Acidobacteria*, *Verrucomicrobia*, *Gemmatimonadetes*) have started to accumulate (47, 69, 71, 115), the corresponding isolates have not become available. In fact, the timing of new species descriptions demonstrates that the bias has become even more pronounced over the last 10 years; meanwhile only 7% of newly described species fall into the remaining 29 phyla of cultured bacteria and archaea (**Figure 2**). Furthermore, most of the bacterial

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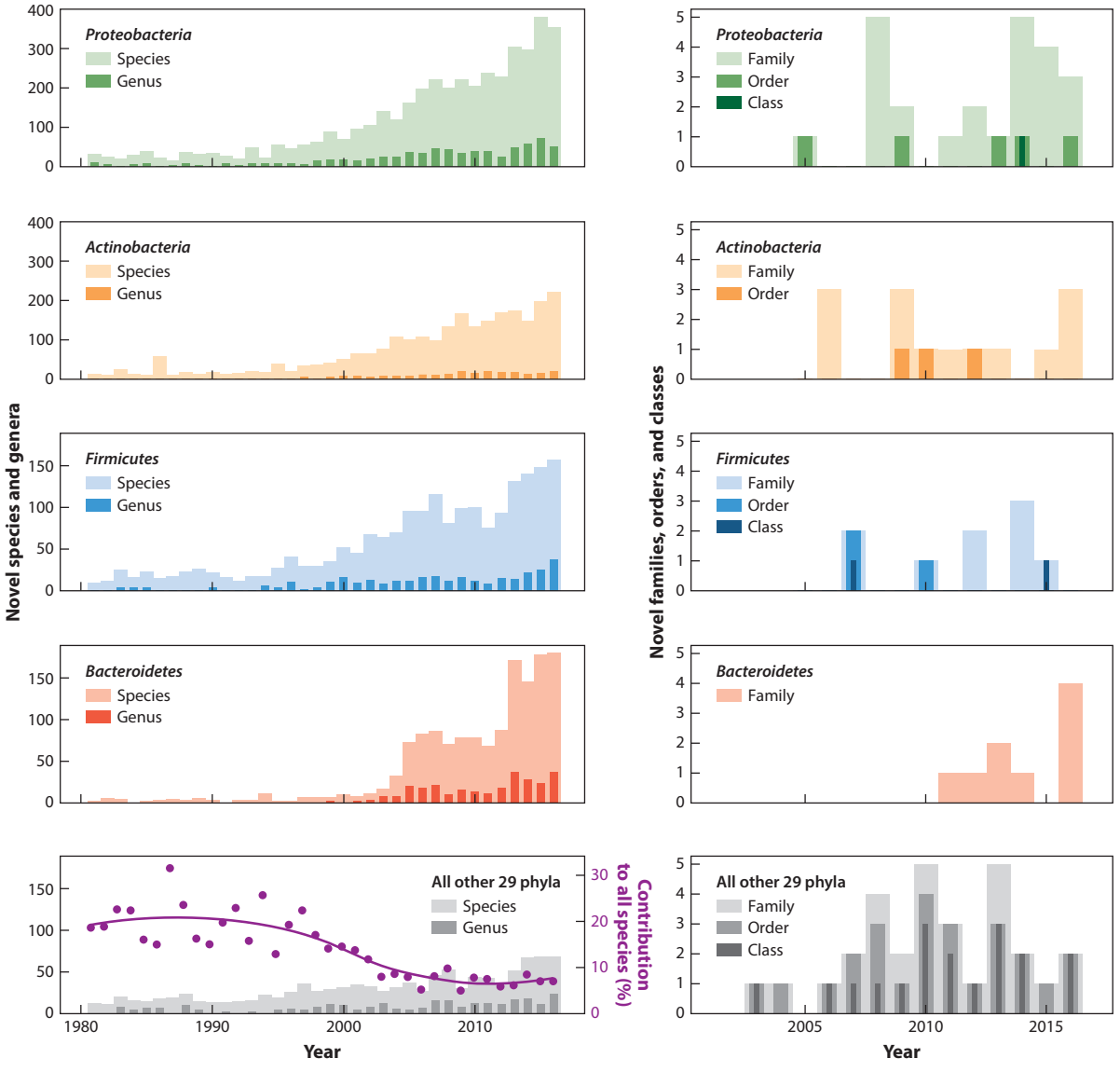

Figure 2

Novel taxa represented by newly described isolates based on an analysis of the Prokaryotic Nomenclature Up-to-Date database (**https://www.dsmz.de/bacterial-diversity/prokaryotic-nomenclature-up-to-date.html**). (*Left*) Five plots depict the annual counts of newly described species and genera of *Proteobacteria* (*green*), *Actinobacteria* (*orange*), *Firmicutes* (*blue*), *Bacteroidetes* (*red*), and all remaining 29 phyla with cultured representatives (*gray*). The fraction of novel species described for the poorly represented 29 phyla decreased with time (*purple*), reaching 7% over the last years. (*Right*) Five plots depict annual counts of novel families, orders, and classes.

and archaeal isolates represent novel species rather than higher taxa and hence are closely related to described species (**Figure 2**).

A prominent example of an underrepresented phylum is the *Acidobacteria*. *Acidobacteria* constitute a dominant group of soil bacteria with >12,000 unique phylotypes and >6,500 species-level

operational taxonomic groups discovered to date. Yet, only 51 species could be described, and these mostly fall into just one of the 26 established classes of *Acidobacteria* (134).

THE BIOLOGICAL BASIS OF UNCULTURABILITY AND SOME SOLUTIONS

Certain physiological groups of bacteria require specific growth conditions, as has already been reviewed (e.g., for anaerobes see 100). This contribution focuses on new cultivation concepts that have been developed based on an improved understanding of the ecology of previously not-cultured bacteria and have permitted the isolation of novel types of bacteria in pure culture.

Oligotrophy

Low nutrient concentrations are particularly prevalent in marine environments. Forty-two percent of the world's oceans have extremely low productivity, with nutrient concentrations <100 nM and micromolar concentrations of utilizable dissolved organic carbon (74, 108, 142). The widely applied Marine Broth 2216 (148) reproduces the average ionic composition of natural seawater but contains much higher concentrations of organic carbon and nutrients (50). Accordingly, the vast majority of previously cultivated bacteria that are cultured in Marine Broth and similar media are copiotrophs, bacteria that are adapted to utilize high concentrations of carbon and nutrients (142).

In contrast, oligotrophic bacteria grow at very low concentrations of organic carbon (1–15 mg C/L) compounds (77, 108). The oligotrophic lifestyle has been documented for phylogenetically diverse bacteria, including "*Candidatus* Pelagibacter ubique" (SAR11 clade), *Sphingopyxis alaskensis*, the SAR116 clade (all *Alphaproteobacteria*), oligotrophic marine *Gammaproteobacteria*, marine *Actinobacteria*, *Cyanobacteria* (*Prochlorococcus*), and *Planctomycetes* (22, 45, 118, 129). They are characterized by high-affinity uptake systems; low growth rates that are independent of substrate concentrations; small cells; lack of motility; and reduced genome sizes due to a lack of genes for metabolic reactions (e.g., siderophore synthesis and uptake, extracellular enzymes), signaling (chemotaxis, quorum sensing), and regulation (49, 82). The typically low GC content of oligotrophic bacteria may reflect an adaptation to nitrogen limitation and results in a strong bias toward utilization of tyrosine, phenylalanine, isoleucine, glutamate, asparagine, lysine, and serine in cellular proteins (129). To our knowledge, this bias has so far not been considered in laboratory cultivation of oligotrophs.

Corresponding to these adaptations, media successfully applied for growing oligotrophic marine bacteria have been based on 0.2-μm-filtered, autoclaved seawater amended with low concentrations of NH_4Cl and KH_2PO_4 (1 and 0.1 μM, respectively), vitamins, and sugars and organic acids (each of the latter at 50–100 μM) (22). Artificial freshwater supplemented with diverse organic carbon compounds at concentrations of 200 μM was successful for growing freshwater bacteria (4, 15). The dilution-to-extinction approach takes advantage of the often high relative abundance of oligotrophic bacteria and uses an inoculum of ≤1 growing cell per vessel to generate cultures (18). Numerous replicates are conveniently set up in polystyrene microtiter dishes. Growth needs to be detected by fluorescent staining and microscopic inspection, because the low nutrient concentrations allow only small increases in cell numbers and cells may have doubling times of only 30 h (14, 25, 110). The micro–Petri dish offers a miniaturized format for cultivation, providing up to a million wells in an acrylic polymer laminated onto porous aluminum oxide, and features individual compartments as small as 7 × 7 μm (67). The micro–Petri dish enables improved oxygen transfer and the removal of waste products yet allows few cell divisions. Furthermore, disposable microfluidic cultivation devices enabling growth of bacterial microcolonies in monolayers and under defined environmental conditions have been developed (52).

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Nutrient-limited conditions for the growth of aquatic bacteria can also be realized on solid media. Thus, the established technique for the isolation of *Planctomycetes* employs water agar covered by water from the original habitat as the source of nutrients and is supplemented with low concentrations of yeast extract and peptone, or *N*-acetylglucosamine (78, 118).

Facultatively oligotrophic bacteria, such as the marine *Sphingopyxis alaskensis*, the limnic *Sandarakinorhabdus limnophila*, and *Polynucleobacter necessarius*, multiply over a broad range of nutrient concentrations (47, 54). They can be enriched and isolated at organic carbon substrate concentrations of 0.001% w/v but also grow at much higher concentrations of 0.1% or even 1% (32, 47).

In addition, bacterial growth can be limited by organic carbon in soils. Only those media that mimic the inorganic ion concentrations in the pore water solution of soils and that contain reduced concentrations of carbon substrates have been reported to support the growth of *Acidobacteria*, *Gemmatimonadetes*, *Chloroflexi*, and *Planctomycetes* (28, 39, 69). Concentrations of 0.05% peptone, 0.025% yeast extract, and 0.1% glucose (w/v) have been applied very successfully to culture, isolate, and characterize the first representatives of the previously not-cultured *Acidobacteria* subdivisions 4 and 6 as well as the rarely isolated *Rubrobacteria* (40, 42, 62, 63, 105, 140). The required low initial supply of substrates can also be realized through the use of polymeric carbohydrates as the sole carbon source. Monomeric substrates are only slowly liberated at the beginning of the enrichments because of the low abundance of hydrolytic exoenzymes. This can lead to significantly greater success cultivating soil samples compared to media containing the corresponding monomers (115).

Starvation, Dormancy, Ultramicrobacteria, and Population Heterogeneity

The stress response to substrate depletion has been studied mostly for copiotrophic, pathogenic bacteria and comprises changes in cell morphology and composition, gene expression, and physiology (65). Marine *Vibrio* spp. form miniaturized cells, express high-affinity substrate uptake systems, and retain viability for extended periods of time. Other bacteria form dormant cells with very low metabolic activity that retain viability (90, 119). This response often also involves a pronounced decrease in ribosome content (10). The cellular ribosome content, determined in populations of soil bacteria, was found to be three orders of magnitude lower than that of exponentially growing laboratory cultures, which suggests that bacterial starvation is pronounced in the soil environment (141). The starvation response is relevant to bacterial cultivation because starved or dormant cells are often not capable of growing on complex, high-nutrient media and only grow on media with reduced organic carbon content (90, 119). This initial inhibition may be related to the observed inhibition of starved cells of many species by the very substrate for which they were previously starved (substrate-accelerated death) (19). In addition, many previously not cultured bacteria grow only slowly: Fastidious soil bacteria require one to three months of incubation for colony formation to become visible (29, 69).

Not only the starved cells of laboratory copiotrophs but also most freshwater and marine planktonic bacteria are so small as to be called ultramicrobacteria (defined as cells with a volume $<0.1\ \mu m^3$). Ultramicrobacteria include slow-growing obligate oligotrophs (110), facultative oligotrophs (32), and also nonoligotrophic bacteria from terrestrial (68) and intestinal habitats (44). *Elusimicrobium minutum* even reaches the theoretical lower size limit for the cytoplasm of living cells [$0.008\ \mu m^3$ (44)] and doubling times of >80 h. Size-selective filtration through 0.2-μm pore membrane filters proved to be the critical step to separate *E. minutum* from accompanying faster-growing bacteria and allowed the isolation of the first representative of the phylum *Endomicrobia* (44).

Based on these observations, the filtration-acclimatization method was established. It allows physical enrichment of ultramicrobacteria and accounts for their slow growth and sensitivity

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to high substrate concentrations. The method involves an initial filtration step selecting small cells, followed by increasing concentrations of complex organic carbon substrates (nutrient broth, peptone, and yeast extract; increasing from 5 mg/L to 3 g/L^{-1}), which are added to the filtrate (57). This technique was successfully applied to enrich and isolate freshwater actinobacteria that had not been cultivated for a decade (55, 56). Similarly, dominant marine flavobacteria can be subcultured at higher carbon concentrations after initial growth under oligotrophic conditions (58).

Because the signal compound cyclic AMP (cAMP) is involved in the starvation response (summarized in References 65 and 100), extracellular cAMP may keep bacterial cells in a nutrient-scavenging state and prevent their transition into a stationary phase. Indeed, addition of small concentrations of cAMP to cultivation trials in low-nutrient freshwater or marine media has been shown to significantly increase the cultivation success by one order of magnitude (13, 15).

However, even on improved growth media, and despite their often numerical dominance in the environment, only a few cells from natural populations of previously not-cultured bacteria multiply in culture (28, 71, 115, 125). This has been related to a heterogeneous physiological response among cells in natural populations. For example, a tenfold variation in substrate uptake rates and rare expression of alternative substrate metabolic pathways have been observed in clonal laboratory populations and have been associated with stochastic fluctuations in gene expression (1, 61). In a similar fashion, to survive unfavorable conditions, bacteria may enter a long-term dormant state from which only a few cells exit, in a stochastic manner, resulting in the observed low fraction of cultivable cells (16, 34).

Regardless of the underlying cause, unsuccessful cultivation of these not-yet-cultured groups necessitates high-throughput approaches. Also, since oftentimes little is known of growth requirements, a range of conditions must be tested (79), which usually requires producing and testing tens of thousands of cultures. This can be accomplished by automation of media distribution and inoculation (14). Another time-consuming and often costly step is the reliable identification of the rare target organisms among the numerous cultures produced. In the so-called microbial culturomics approach, this is accomplished with matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry—which, however, cannot be applied to unknown bacterial species (79, 120). Unknown bacterial phylotypes can be recognized by combinatorial barcoding of 16S rRNA gene amplification products of the high number of grown cultures (20).

Adaptations to Spatial and Temporal Heterogeneity: Adhesion and Chemotaxis

Bacteria experience a spatially heterogeneous habitat not only in soils and sediments but also in open waters where suspended particles provide discrete hot spots of growth substrates. Bacteria colonizing marine aggregates are either immotile or motile by gliding and can constitute up to 20% of the bacterial biomass in ocean water (130). They are adapted to high concentrations of biological polymers, use specialized uptake systems for high-molecular-weight substrates, excrete exoenzymes, and are capable of utilizing a broad spectrum of substrates at higher concentrations (102). In soils, the majority of bacteria (>84%) strongly adheres to particles (83). The attached lifestyle can be directly exploited for the cultivation of previously unknown types of bacteria. Thus, a selective enrichment of bacterial biofilms on chemically different surfaces yielded previously not-cultured *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, and *Bacteroidetes* from freshwater lakes, a marine microbial mat, and soil (46). Similarly, biofilms grown on cover slips incubated in peat water from the original habitat yielded enrichments of novel *Acidobacteria* and *Planctomycetes* species (29).

Bacterial habitats are also characterized by temporal variability of growth substrates. In open waters, a rapid enzymatic degradation of extracellular polymers and an incomplete uptake of the

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liberated compounds generate plumes of growth substrates behind sinking particles (2). Transient substrate gradients also occur in soils and sediments (37). A considerable number of bacteria have adapted to the temporal availability of resources through motility and chemotaxis; up to 70% of bacteria in plankton are motile, and chemotaxis also occurs among soil bacteria (37, 85). This feature can be exploited for the targeted enrichment of novel types of bacteria (66, 98).

Metabolic Interactions, Signal Compounds, and Cocultivation

In their natural environment, bacteria often occur in close proximity to each other. Cell-to-cell distances range between 5 and 29 μm in soils (111). Over these distances, diffusion is rapid and enables an efficient exchange of small molecules. Known metabolic interactions involve compounds participating in central metabolism, for example, amino acids, vitamins, certain fatty acids, reduced sulfur compounds, siderophores, and electron shuttles (30, 53, 100, 132). Quite often, reproducing such interactions can be straightforward and achieved by addition of these or chemically related compounds or by using complex supplements like yeast extract. Sometimes dependency on particular compounds is predicted by genome analysis (132).

A particular type of chemical interaction is the exchange of signal compounds. Intraspecific bacterial communication by quorum sensing involves density-dependent formation of acyl-homoserine lactone (AHL) autoinducer molecules or short peptides and controls light production, expression of virulence factors, swarming, biofilm formation, cell aggregation, and genetic competence. Autoinducer-2 (AI-2)-type molecules, γ-butyrolactones, and quinolones participate in bacterial cell-to-cell communication as well as communication between species (5). AHLs have been used only rarely in cultivation attempts (13, 53). Some actinobacteria secrete resuscitation-promoting factors that seem to enable growth of dormant cells through limited hydrolysis and turnover of the bacterial cell wall (90), but this concept has not been exploited for cultivating novel types of bacteria. AI-2 has not been observed to improve cultivation success (30).

Commonly, dilution-to-extinction cultivation yields mixed cultures at a frequency much higher than would be expected based on statistical considerations (75). Individual cocultures have been shown to involve specific partner bacteria (55, 73). Many novel and naturally abundant types of bacteria can only be maintained in the laboratory as cocultures with other bacteria but cannot be isolated with available techniques (29, 55, 73, 78). Different formats have been developed to maintain such unknown, but obviously crucial, biotic interactions during the cultivation of bacteria from natural environments.

Microbial cells have been incubated on membrane filters and in dialysis units, diffusion chambers, agar beads, and Gelrite plugs in their natural environments (8, 38, 73, 100, 147). Miniature diffusion chambers have also been produced (92). Membranes with sufficiently small pores (0.03 μm) (73) or inclusion of cells in macromolecular gel droplets (147) keeps the dividing cells separate from accompanying microorganisms while maintaining the exchange of compounds. Using these devices, phylogenetically novel bacterial lineages have been grown (38, 73, 92).

Novel sequencing technology now enables systematic analysis of cocultures obtained by high-throughput cultivation and revealed that cocultures frequently do not form by chance but involve particular types of bacteria (**Figure 3**). Phylotypes that partner with many other bacteria (*Pedobacter*, *Flavobacterium*, *Planococcus*, *Saccharopolyspora* spp. in **Figure 3**) may thus be employed as helpers in future cocultivation attempts (30).

A considerable number of bacteria are physically associated with other organisms (43, 59, 99, 100, 117). Bacteria with highly streamlined genomes depend on host functions, as indicated by the loss of genes encoding essential metabolic pathways (59, 84, 124). In these cases, cultivation in coculture with the interaction partner has been successful and, for instance, allowed laboratory

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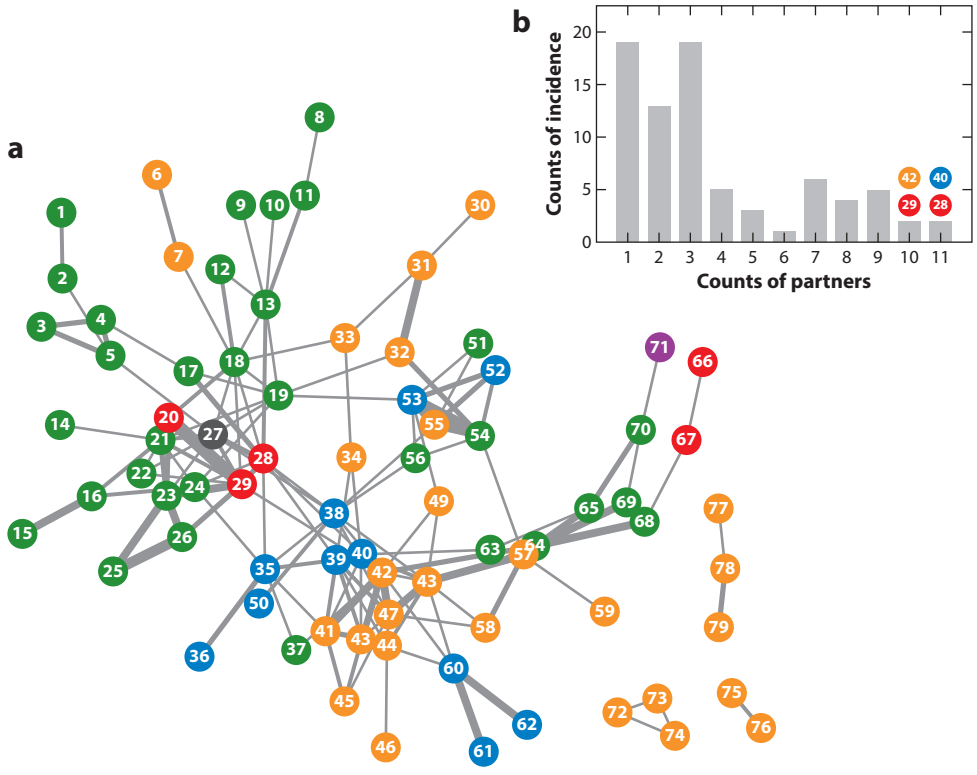

Figure 3

Specificity of cocultures established in a high-throughput cultivation series of bacteria from savannah soil as detected by Illumina sequencing of 16S rRNA genes. In total, 196 different genera were observed in 1,269 cultures. (*a*) Many—79—different genera of *Proteobacteria* (*green*), *Actinobacteria* (*orange*), *Firmicutes* (*blue*), *Bacteroidetes* (*red*), *Acidobacteria* (*purple*), and unclassified bacteria (*dark gray*) showed nonrandom (significance, $P = 0.05$) pairwise co-occurrence. The thickness of vertices connecting the 79 genera reflects the frequency with which each distinct pair was found to co-occur. (*b*) Counts of incidents of co-occurring partners. The majority of genera pair with 1–3 partners. The four taxa (highlighted in *orange*, *blue*, and *red* according to their affiliation) specifically grow in pairwise coculture with 10 or 11 different bacterial partners: 28, *Pedobacter*; 29, *Flavobacterium*; 40, *Planococcus*; 42, *Saccharopolyspora*.

cultivation of symbiotic phototrophic consortia 90 years after their discovery (43) and cultivation of *Babela massiliensis*, the first representative of the TM6 phylum, in its protozoan host *Acanthamoeba castellanii* (103).

Axenic cultures of bacteria that are associated with eukaryotic cells could only be achieved in a few cases. Auxotrophy for 16 amino acids was predicted by genome analysis for *Tropheryma whipplei* and provided the key for the subsequent successful axenic cultivation (112). Based on genome analysis and monitoring of cell activities in different media, axenic growth of *Coxiella burnetii* was achieved in a medium containing neopeptone, fetal bovine serum, and methyl-β-cyclodextrin (123). Axenic cultivation of *Chlamydia trachomatis* was successful at low oxygen partial pressures with energy-rich phosphate compounds (glucose-6-phosphate or ATP) (95). Groundwater bacteria of different phyla within the Candidate Phyla Radiation have very small genomes (usually <1 Mb) and limited metabolic capabilities. The genome streamlining resembles that of bacterial symbionts or parasites and features an incomplete TCA cycle, lack of electron transport chain

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complexes, and, frequently, incomplete nucleotide and amino acid biosynthesis pathways. It has therefore been concluded that these novel types of bacteria are obligate fermenters that live in association with other organisms (11). This hypothesis awaits confirmation.

FUTURE AVENUES

The Empirical Approach

Most previous cultivation approaches have been empirical, employing different media and incubation conditions, monitoring success of cultivation, and subculturing bacterial species of interest. This is particularly efficient if the cultivation conditions meet the requirements of a sufficient number of target bacteria. Prominent examples are the human and mouse intestinal microbiome, from which hundreds of novel bacterial species could be retrieved when applying different cultivation conditions in a high-throughput format (so-called microbial culturomics) (12, 80, 81).

Elucidating Suitable Cultivation Conditions

Inadvertently, cultivation work has remained highly biased toward a few well-characterized bacterial phyla (**Figure 2**). Future cultivation efforts need to target phylogenetic and functional novelty more efficiently. This can be achieved by combining ecological studies, meta-omics, and culture-independent physiological analysis with information on the physiology and biochemistry of the desired bacterial group as obtained from growing databases (**Figure 4**). Target phylotypes can be selected based on their high abundance or activity in particular environments. Correlating the relative abundance of target bacteria with a large number of abiotic and biotic environmental parameters in the corresponding environments provides initial information on unknown adaptations and cultivation requirements (41). Genome analysis has the potential to provide decisive information for successful cultivation but has rarely been exploited for this purpose (88, 112, 123, 139). Advanced metagenomic methods are independent of reference genomes and hence suitable for the analysis of distantly related taxa (93). The longer read lengths required for improved metagenomic analysis (131) can be gathered through novel long-read sequencing technologies (Pacific Biosciences single-molecule, real-time, or Oxford Nanopore sequencing).

As culture-independent approaches to the physiology of not-yet-cultured bacteria, changes in the metatranscriptome and metaproteome under varying environmental conditions can provide information on key physiological traits (6, 9). Growth substrates needed for subsequent cultivation can be identified by tracing incorporation of isotopically labeled compounds into specific marker macromolecules (76). On a single-cell level, physiology can be analyzed by tracking the incorporation of isotopically labeled substrates with high-resolution secondary ion mass spectrometry (NanoSIMS), or, alternatively, by monitoring the metabolic activity of cells under different incubation conditions by Raman microspectrometry or the incorporation of BrdU. A combination with fluorescence in situ hybridization allows concomitant phylogenetic identification of individual cells (33, 107, 135).

Finally, chemotaxis assays allow rapid and efficient screening of potential bacterial growth substrates (66, 98). Chemotaxis assays with thin, flat (0.1×1 mm inside opening), rectangular glass capillaries have proven particularly suitable. The capillaries are filled with test substrates and inserted in microscopic chambers, bottles containing natural bacterial communities, or directly in situ. Motile bacteria accumulating inside the capillaries can be monitored directly in the capillaries by microscopy and identified by 16S rRNA gene sequencing (98). Also, the enrichments can be used directly for subsequent enrichment and isolation trials of novel phylotypes of bacteria (43, 98).

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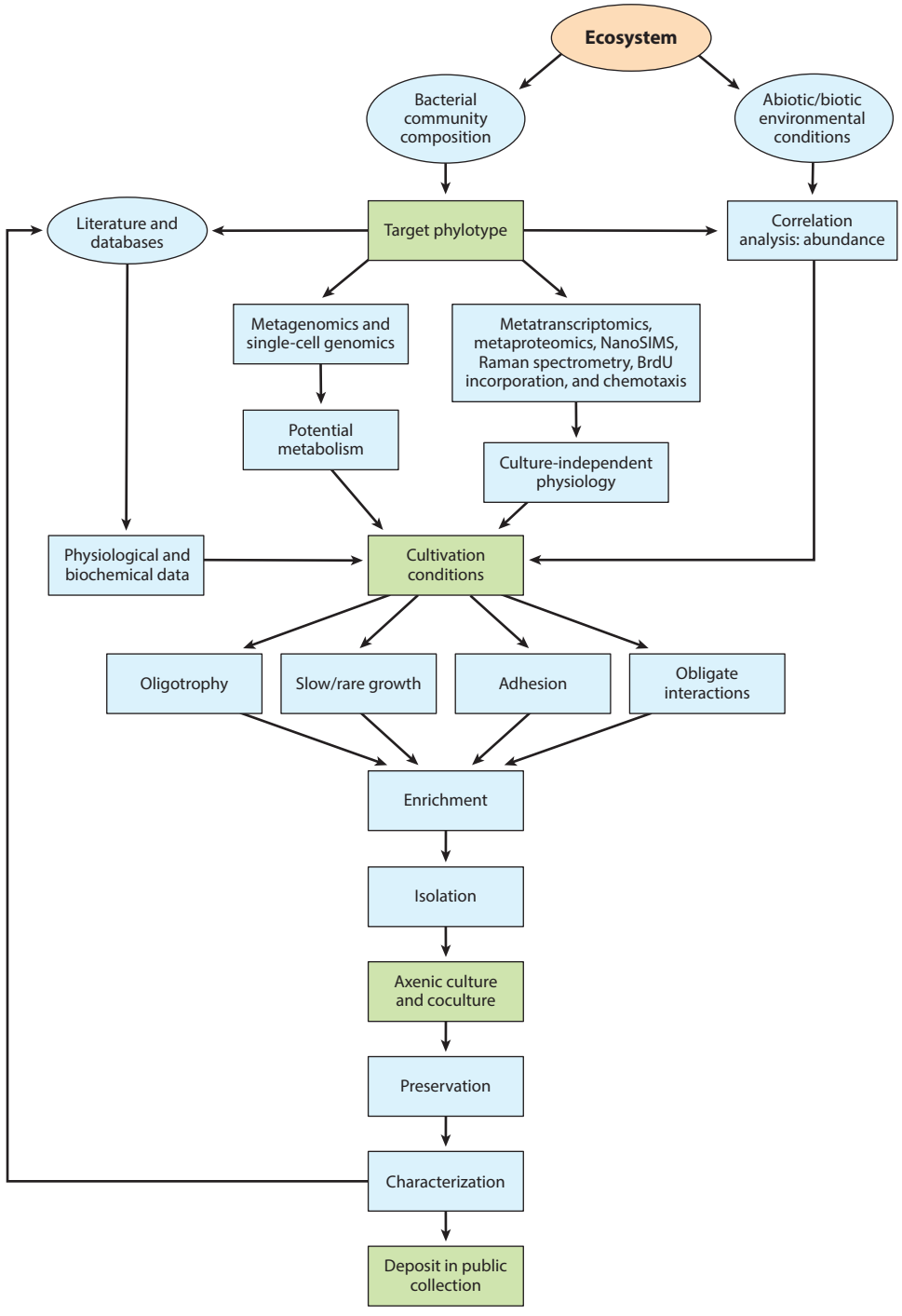

Figure 4

Flowchart of the different steps of the targeted cultivation of bacteria and archaea from environmental samples. Ovals mark the information that is required. Decisive steps are in green boxes. Abbreviation: BrdU, bromodeoxyuridine.

Target Bacteria of Future Interest

Based on their abundance and the availability of ecological and genomic information, target bacteria can be prioritized for future cultivation efforts. Most data have come from marine environments, where *Gammaproteobacteria* clade SAR86; *Deltaproteobacteria* cluster SAR324; *Actinobacteria*; *Chloroflexi*; and candidate phyla "Marinimicrobia," "Atribacteria," and "Poribacteria" provide highly interesting targets (45, 50, 94, 121).

Bacteria of the SAR86 clade seem to be aerobic chemoheterotrophs that degrade lipids and carbohydrates, utilize organosulfur compounds (DMSP, glutathione), and may be auxotrophic for some amino acids and B-type vitamins (31). Single-cell genomes of SAR324 cells from the deep Atlantic and Pacific oceans contain ribulose-1,5-bisphosphate carboxylase genes and genes for sulfur oxidation and C_1 metabolism and for a particle-associated lifestyle. Bicarbonate uptake and association with particles could subsequently be confirmed by microautoradiography and fluorescence in situ hybridization (128). Members of the class *Dehalococcoidia* (phylum *Chloroflexi*) in Baltic Sea sediments utilize fatty acids and aromatic compounds (136). Marinimicrobia are likely adapted to suboxic waters and the use of reduced sulfur compounds (138), whereas JS1 bacteria (candidate phylum Atribacteria) may grow anaerobically by fermentation employing syntrophic propionate catabolism (94). In addition, single-cell genomics of a symbiotic bacterium belonging to the Poribacteria and occurring in the marine sponge *Aplysina aerophoba* suggested the potential for degradation of *N*-acetylglucosamine-6-sulfate, *N*-acetylgalactosamine-6-sulfate, choline, mucin, and heparane by sulfatases; it also provided the first evidence of denitrification and urea utilization and the presence of CO_2-fixation capabilities, thereby allowing detailed predictions of the lifestyle of this bacterium (121).

Recent metagenomic and single-cell genomic analyses have expanded this list to as many as 21 bacterial and 6 archaeal candidate phyla, including several nonmarine groups (3, 35, 94, 113, 121).

The Significance of Safeguarding the Diversity of Cultured Bacteria

Notwithstanding increasingly frequent reports of the initial successful cultivation of previously not-cultured bacteria, valid descriptions of novel species or candidate species continue to be remarkably scarce. Successful initial cultivation does not guarantee subsequent successful isolation and characterization of novel types of bacteria. Indeed, a significant fraction of the initial enrichments cannot be subcultured (75). In fact, it may take years or even decades to isolate a single strain of a novel species from a coculture and to validly describe it (21, 56, 110). Even upon successful isolation, subcultivation of fastidious bacteria continues to be unpredictable (48). This clearly demonstrates that future cultivation work must also focus on improved subcultivation, purification, and preservation techniques (**Figure 4**). This is of prime importance since the effort necessary to enrich, isolate, and maintain novel, often rather fastidious, types of bacteria incurs substantial costs. A detailed compilation of costs, including person-hours, consumables, and depreciation of equipment, totaled €9,836 invested per bacterial isolate validly described (101). Thus, a considerable public funding is required to explore, recover, and utilize a larger fraction of microbial diversity.

FINAL REMARKS

Cultivation will remain an indispensable approach for the elucidation of biogeochemical cycles and for the study of the biochemistry, physiology, and cellular architecture of Bacteria and Archaea (50, 131). A more targeted cultivation of not-yet-cultured microorganisms will therefore drive the development of microbial ecology, biotechnology, and public health.

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DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

We thank Dr. Sixing Huang for supporting the analysis of genome statistics, and Alicia Geppert and Dr. Boyke Bunk for help with the high-throughput cultivation and subsequent phylogenetic analysis of cocultures. This work was supported by grants of the German Federal Ministry of Science and Education (No. 8000-105-3 through the German Center of Infection Research/DZIF, and grant No. 16GW0113K/ANoBiN) to Jörg Overmann.

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Volume 71, 2017

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