Size Matters: Ultra-small and Filterable Microorganisms in the Environment

RYOSUKE NAKAI*†

1Applied Molecular Microbiology Research Group, Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), 2–17–2–1, Tsukisamu-Higashi, Sapporo, 062–8517, Japan

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Ultra-small microbial organisms are ubiquitous in Earth’s environments. Ultramicrobacteria, which are defined as having a cell volume of <0.1 μm³, are often numerically dominant in aqueous environments. Cultivated representatives among these bacteria, such as members of the marine SAR11 clade (e.g., “Candidatus Pelagibacter ubique”) and freshwater Actinobacteria and Betaproteobacteria, possess highly streamlined, small genomes and unique ecophysiological traits. Many ultramicrobacteria may pass through a 0.2-μm-pore-sized filter, which is commonly used for filter sterilization in various fields and processes. Cultivation efforts focusing on filterable small microorganisms revealed that filtered fractions contained not only ultramicrocells (i.e., miniaturized cells because of external factors) and ultramicrobacteria, but also slender filamentous bacteria sometimes with pleomorphic cells, including a special reference to members of Oligoflexia, the eighth class of the phylum Proteobacteria. Furthermore, the advent of culture-independent “omics” approaches to filterable microorganisms yielded the existence of candidate phyla radiation (CPR) bacteria (also referred to as “Ca. Patescibacteria”) and ultra-small members of DPANN (an acronym of the names of the first phyla included in this superphyla) archaea. Notably, certain groups in CPR and DPANN are predicted to have minimal or few biosynthetic capacities, as reflected by their extremely small genome sizes, or possess no known function. Therefore, filtered fractions contain a greater variety and complexity of microorganisms than previously expected. This review summarizes the broad diversity of overlooked filterable agents remaining in “sterile” (<0.2-μm filtered) environmental samples.

Key words: filterable microorganisms, ultramicrocells, ultramicrobacteria, candidate phyla radiation, minimal cell

How small may actual organisms be? This question has long fascinated scientists in various fields. Prokaryotic microorganisms (Archaea and Bacteria) constitute the smallest life forms. Bacterial cells range in volume from 0.2-μm filtrate (Levy and Jornitz, 2006). In fact, efforts to culture microorganisms remaining in the 0.2-μm filtrate (hereafter described as CPR/Patescibacteria) and freshwater Actinobacteria and Betaproteobacteria (Hahn, 2003; Hahn et al., 2003) as well as the candidate phylum termite group 1 (TG1) described as Elusimicrobia (Geissinger et al., 2009). The existence of UMB has expanded our knowledge of microbial life at the lower size limit.

In the last five years, filterable microorganisms have been attracting increasing interest with the discovery of other ultra-small members: the candidate phyla radiation (CPR) bacteria, also referred to as “Candidatus Patescibacteria” (hereafter described as CPR/Patescibacteria; Rinke et al., 2013; Brown et al., 2015), and some members of DPANN (an acronym of the names of the first phyla included in this superphyla, “Ca. Diapherotrites”, “Ca. Parvarchaeota”, “Ca. Aenigmarchaeota”, “Nanoarchaeota, and “Ca. Nanohaloarchaeota”; Rinke et al., 2013; Dombrowski et al., 2019). Several CPR members have an extremely small cell volume (approximately 0.01 μm³) that was unveiled by cryo-transmission electron microscopy imaging (Luf et al., 2015). Moreover, the emergence of these ultra-small prokaryotes has re-opened debate on the tree of life (Hug et al., 2016; Parks et al., 2018; Zhu et al., 2019). These members are ubiquitous in the environment and recent studies have provided insights into their contribution to the material cycle (e.g., carbon and nitrogen cycles; Danczak et al., 2017; Lannes et al., 2019). This review focuses on the phylogenetic diversity and complexity of filterable microorganisms in natural systems, with specific references to UMB and pleomorphic bacteria. Other reviews presented aspects of ultra-small microorganisms including CPR/Patescibacteria and DPANN members (e.g., terminology, biogeography, cosmopolitan freshwater Actinobacteria and Betaproteobacteria (Hahn, 2003; Hahn et al., 2003) as well as the candidate phylum termite group 1 (TG1) described as Elusimicrobia (Geissinger et al., 2009). The existence of UMB has expanded our knowledge of microbial life at the lower size limit.

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genomic diversity, and metabolic variety; Duda et al., 2012; Castelle et al., 2018; Ghuneim et al., 2018; Dombrowski et al., 2019). In this review, archaea with a cell volume of \(<0.1 \mu m^3\) are specifically referred to as ultramicroarchaea (UMA) to distinguish them from UMB.

**Filterable microorganisms**

To date, many studies have reported the presence of filterable microorganisms in various environments (mainly aqueous environments) including seawater (Haller et al., 2000; Elsaeid et al., 2001; Lannes et al., 2019; Obayashi and Suzuki, 2019), lake water (Hahn, 2003; Hahn et al., 2003; Watanabe et al., 2009; Fedotova et al., 2012; Maejima et al., 2018; Vigneron et al., 2019), terrestrial aquifers (Miyoshi et al., 2005; Luef et al., 2015), glacier ice and the ice cover of lakes (Miteva and Brenchley, 2005; Kuhn et al., 2014), deep-sea hydrothermal fluids (Naganuma et al., 2007; Nakai et al., 2011), and soil and sand (Nakai et al., 2013). However, the use of membrane filters with a small pore size (approximately 0.2 μm) was traditionally recommended for the retention of bacteria in the field of marine microbial ecology in the 1960s (e.g., Anderson and Heffernan, 1965) and is still widely practiced today in various fields. The existence of very small microorganisms has been well recognized since the 1980s. The term “ultramicrobacteria” was first used by Torrella and Morita (1981) to describe very small cocoid cell forms of \(<0.3 \mu m^3\) in diameter from seawater. MacDonell and Hood (1982) subsequently isolated and characterized viable filterable microorganisms potentially belonging to the genera Vibrio, Aeromonas, Pseudomonas, and Alcaligenes from estuarine waters. They concluded that these filterable microorganisms represented a state of dormancy for adaptation to low nutrient conditions and were not completely novel bacteria. Other studies also reported that external factors reduced cell sizes, such as Staphylococcus aureus and Pseudomonas syringae (~50% reduction in size as described in Table 1; Watson et al., 1998; Monier and Lindow, 2003). Therefore, the cells of miniaturized microorganisms need to be distinguished from true UMB and are described in this review as “ultramicrocells”, which has the synonyms dwarf cells and midget cells, according to Duda et al. (2012). Schut et al. (1997) and Duda et al. (2012) subsequently defined a cell volume index of \(<0.1 \mu m^3\) as being characteristic of true UMB.

Based on previous studies, filterable microorganisms have been classified into five groups (Fig. 1): (I) ultramicrocells that are miniaturized microorganisms because of external factors (e.g., environmental stress) as described above; (II) obligate UMB that maintain small cell volumes (<0.1 μm³) regardless of their growth conditions; (III) facultative UMB that contain a small proportion of larger cells with a cell volume >0.1 μm³ (note that the definitions of the terms “obligate” and “facultative” UMB follow those of Duda et al. [2012]); (IV) slender filamentous bacteria; and (V) ultra-small members among CPR/Patescibacteria bacteria and DPANN archaea. In contrast to UMB strains, the cell shapes and morphological characteristics of members in group V are largely unknown under different environmental or culture conditions because all of the members of CPR and DPANN are uncultivated, with a few exceptions of members belonging to the phyla “Ca. Saccharibacteria” (former TM7) and Nanoarchaeota (e.g., Huber et al., 2002; He et al., 2015). Incidentally, the groups presented in this review do not include filterable cell-wall-less mycoplasmas as well as “nanobacteria” or “nannobacteria” as microfossils, which are often referred to in geological literature (Folk, 1999), or as calcium carbonate nanoparticles in the human body, as reported in medical literature (Martel and Young, 2008). Representative cases of groups II to V are described below and Table 1 shows a summarized list.

**Obligate UMB**

Obligate UMB are often reported from aqueous environments. One of the most prominent representatives is “Candidatus Pelagibacter ubique” HTCC1062, which is a SAR11 clade bacterium that is ubiquitous in marine environments. Previous studies found that SAR11 members consistently dominated ribosomal RNA gene clone libraries derived from seawater DNA and estimated their global population size as 2.4×10²⁸ cells—approximately 25% of all prokaryotic cells—in oceans (Giovannoni et al., 1990; Morris et al., 2002). Despite their ubiquitous and abundant presence, it was not possible to isolate them. However, the first cultivated strain HTCC1062 was established in 2002 using a high-throughput dilution-to-extinction culturing (HTC) technique (Rappé et al., 2002). This HTC technique involves cultivation with serial dilutions of natural seawater samples into very low nutrient media (Connon and Giovannoni, 2002). The cell volume (approximately 0.01 μm³) of “Ca. P. ubique” was reported as one of the smallest free-living cells known. Subsequent studies characterized the SAR11 clade with the small, streamlined genomes (<1.5 Mbp) described below, an unusual mode of glycine auxotrophy, a light-dependent proton pump known as proteorhodopsin, and the ability to utilize various one-carbon compounds (reviewed in Tripp, 2013; Giovannoni, 2017). The SAR11 clade is highly divergent with multiple ecoretotypes and has freshwater members known as LD12 classified in SAR11 subclade IIB (Grote et al., 2012). An LD12 cultivated representative, “Ca. Fonsibacter ubiquis” strain LSUCC0530, was subsequently established (Henson et al., 2018), and its genomic characteristics promoted the hypothesis that gene losses for osmolyte uptake were related to the evolutionary transition, or metabolic tuning, of freshwater SAR11 (LD12) from a salt to freshwater habitat.

Another marine ultramicrobacterium, Sphingopyxis alaskensis (formerly known as Sphingomonas alaskensis) RB2256 was intensively investigated before the study of the SAR11 clade (e.g., Eguchi et al., 1996; Schut et al., 1997). This strain was also characterized as an obligate UMB (Duda et al., 2012). When the cultivation of this strain transitioned from low-carbon to highly-enriched media, the cell volume of S. alaskensis remained at \(<0.1 \mu m^3\) in most media; however, larger elongated cells, not UMB cells, were observed in trypticase soy agar medium (Vancanneyt et al., 2001). Furthermore, this strain possesses a larger genome of 3.3 Mb (DDBJ/ENA/GenBank accession no. CP000356) than other UMB (Table 1).
Table 1. An overview of ultra-small and filterable microorganisms in the environment

| Taxa                                      | Phylum (and class for Proteobacteria) | Isolation source | Cell shape | Cell size (length/width and/or volume) | Genome size (Mb) | Physiological and ecological trait(s) or its potential                                                                 | Reference                |
|-------------------------------------------|--------------------------------------|------------------|------------|----------------------------------------|------------------|---------------------------------------------------------------------------------------------------------------------------------|--------------------------|
| Ultramicrococci                           |                                      |                  |            |                                        |                  |                                                                                                                                  |                          |
| Staphylococcus aureus 8325-4              | Firmicutes                           | derivative of S. aureus NCTC8325 (patient's strain) | cocci      | cell size reduction from 0.60–0.08 to 0.410–0.08 μm | n.d.             | host cell invasion, starvation-associated cell size reduction                                                                 | Watson et al. (1998)     |
| Pseudomonas syringae pv. syringae B728a   | Proteobacteria (γ-proteobacteria)     | snap bean leaflet | rods       | cell length reduction from ~2.5 to ~1.2 μm | 6.09             | host cell invasion, leaf environment-induced cell size reduction                                                                 | Monier and Lindow (2003); Feil et al. (2005) |
|                                            |                                      |                  |            |                                        |                  |                                                                                                                                  |                          |
| Obligate ultramicrobacteria and related candidates |                                      |                  |            |                                        |                  |                                                                                                                                  |                          |
| “Candidate Paligibacter ubiquae”           | Proteobacteria (α-proteobacteria)     | coastal sea      | curved rods | 0.01 μm³ | 1.31 |                                                                                                                                  |                          |
| “Candidate Fusobacter ubiquus”             | Proteobacteria (α-proteobacteria)     | coastal lagoon   | curved rods | 1.0–0.1 μm | 1.16 |                                                                                                                                  |                          |
| Sphingopyxis alakentui RR2256             | Proteobacteria (α-proteobacteria)     | fjord estuary    | short rods | 0.05–0.09 μm² | 3.35 |                                                                                                                                  |                          |
| Aurantimonibium minutum KNC                 | Actinobacteria                       | freshwater river | curved rods | 0.7–0.8×0.3 μm; 0.04–0.05 μm³ | 1.62 |                                                                                                                                  |                          |
| Rhodolana lasica MWH-TaBas                 | Actinobacteria                       | freshwater lake  | curved rods | 0.85–0.30 μm; 0.053 μm³ | 1.43 |                                                                                                                                  |                          |
| Rhodolana limnophilie 27D-LEPI              | Actinobacteria                       | freshwater pond  | short rods | 0.49–0.28 μm | 1.40 |                                                                                                                                  |                          |
| “Candidate Planktophiila rubra”            | Actinobacteria                       | freshwater lake  | curved rods | 0.041 μm³ | 1.35 | catalase-dependent growth                                                                                                                  | Kim et al. (2019)        |
| “Candidate Planktophiila aquilis”          | Actinobacteria                       | freshwater lake  | curved rods | 0.061 μm² | 1.46 | catalase-dependent growth                                                                                                                  | Kim et al. (2019)        |
| Polynucleobacter necessarius subs.         | Actinobacteria                       | freshwater pond  | straight rods | 0.7–1.2×0.4–0.5 μm | 2.16 | utilization of low-molecular-weight substrates                                                                                   | Hahn et al. (2012); Meinecke et al. (2012) |
| Optitius sp. VeCBl                          | Verrucomicrobia                      | rice paddy soil  | ellipsoids | 0.49×0.33 μm; 0.030 μm³ | n.d. |                                                                                                                                  |                          |
| Faculative ultramicrobacteria              |                                      |                  |            |                                        |                  |                                                                                                                                  |                          |
| Endomicrobium proteum Rsa215               | Elasicmicrobium                      | gut homogenate of Reticulites santonensis     | cocci       | 0.3–0.5 μm (for cocci); 0.5–3.5×0.15–0.30 μm (for rods) | 1.59 |                                                                                                                                  | Zheng and Brune (2015); Zheng et al. (2016) |
| Chryseobacterium soloncola NF4             | Bacteroidetes                        | lake sediment    |              | 0.004–0.04 μm (for cocci); 0.1–0.3 μm³ (for rods) | ~1.7 | ectoparasite of Bacillus subtilis                                                                                                  | Suzina et al. (2011); Duda et al. (2012) |
| Slender filamentous bacteria               |                                      |                  |            |                                        |                  |                                                                                                                                  |                          |
| Hylemonella gracilis CB                    | Proteobacteria (β-proteobacteria)     | freshwater       | spirals    | 0.12 μm³ (smallest width=0.2 μm) | n.d. | n.d.                                                                                                                             | Wang et al. (2007, 2008) |
| Oligoflexus tantienensis Shi3              | Proteobacteria (Oligoflexaceae)*     | desert sand      | pleomorphic (rods, filaments, spirals, and spherical [or curled] cells) | various lengths×0.4–0.8 μm (for filaments) | 7.57 | multidrug resistance, incomplete dentrification**                                                                 | Nakai et al. (2014, 2016a) |
| Silanigrella aquatica MWH-Nonom- Wbed       | Proteobacteria (Oligoflexaceae)*     | freshwater lake  | pleomorphic (rods, filaments, and spirals) | 3.6×0.6 μm (for rods) | 3.51 | antimicrobial peptides, phospholipid-encoded type IV secretion systems**                                                                 | Hahn et al. (2017)       |
| Silanigrella paladina SP-Ram-0.45-NSV-11    | Proteobacteria (β-proteobacteria)     | freshwater pond  | pleomorphic (rods and filaments) | various lengths | 3.94 | utilization of limited substrates                                                                                               | Pitt et al. (2020)       |
| Flavispira multicolourata 33A1-SZDP5        | Proteobacteria (Oligoflexaceae)*     | freshwater creek | pleomorphic (rods and filaments) | various lengths | 3.39 | violacein-like production                                                                                                       | Pitt et al. (2020)       |
| CPR/Patesbacteria bacteria                 |                                      |                  |            |                                        |                  |                                                                                                                                  |                          |
| WVE3-OP11-OD1 bacteria                     | candidate division WVE3,             | deep aquifer     | cocci or oval-shaped | 0.009±0.002 μm³ | 0.69–1.05 | potential interaction with other bacterial cells via pilus-like structures                                                                 | Luef et al. (2015)       |
| “Candidate Somnebenhoria yantiaensis”       | “Candidate Parachlorobacteria” (OD1) | ciliated protist | Paramecium bursaria | straight rods | 1.6–1.9×0.5–0.6 μm | n.d. | endopolysymbiont of the ciliate Paramecium bursaria                                                                 | Gong et al. (2014)       |
| TM7s bacterium                             | “Candidate Cedarsbacteria” (TM7)     | human oral cavity | cocci      | 0.2–0.3 μm | 0.71 | ectosymbiont of Actinomyces odontolyticus                                                                                         | He et al. (2015)         |
| DPANS archaea                              |                                      |                  |            |                                        |                  |                                                                                                                                  |                          |
| Nanoarchaeum equitans                      | Nanoarchaeota                        | submersible hot vent | cocci     | 0.4 μm | ~0.5 | ectosymbiont of Igniococcus hospitalii                                                                                           | Huber et al. (2002)      |
| “Candidate Nanopuusillus acidilobi”         | Nanoarchaeota                        | hot spring       | cocci      | 0.1–0.3 μm | 0.61 | ectosymbiont of Acidilobus species                                                                                               | Wurth et al. (2016)     |
| “Candidate Nanoleptia minutus” Nc-1         | Nanoarchaeota                        | hot spring       | flagellated cocci | ~0.2 μm | 0.57 | ectosymbiont of Zesthaphera nihonrae                                                                                              | John et al. (2019)      |
| “Candidate Nanosalvaria” sp. J07AB43        | “Candidate Nanohalarchaeota”         | hypersaline lake | cocci-like | 0.6 μm | 1.23 | possible free-living lifestyle                                                                                                | Narasingarao et al. (2012) |
| “Candidate Nanosalvaria” sp. J07AB56        | “Candidate Nanohalarchaeota”         | hypersaline lake | cocci-like | 0.6 μm | 1.22 | possible free-living lifestyle                                                                                                | Narasingarao et al. (2012) |
| ARMAN-2, -4, and -5                        | “Candidate Micarchaeota”             | acid mine drainage | cocci     | ~0.5 μm | ~1.0 | potential interaction with Thermoplasmatidae cells via pilus-like structures                                                                 | Baker et al. (2010)     |
| “Candidate Manchuricaea acidiphilum” Mai14  | “Candidate Micarchaeota”             | acid mine drainage | n.d.     | n.d. | 0.95 | ectosymbiont of Cupulalia prima                                                                                                   | Golyshina et al. (2017) |

n.d.: no data.

* The proteobacterial class Oligoflexaceae is classified in the candidate phylum “Bdellovibrionota” in the Genome Taxonomy Database (GTDB).

** Putative physiological traits are inferred from their genomic and plasmid annotation.
Other prominent representatives of obligate UMB are freshwater actinobacterial strains. Typically, actinobacteria are among the numerically dominant groups in freshwater and their cells are found in smaller size fractions (Glöckner et al., 2000; Sekar et al., 2003). Hahn et al. (2003) first isolated nine filterable UMB of the class Actinobacteria from freshwater habitats and newly described a novel phylogenetic cluster (Luna cluster). This isolation was achieved by the “filtration-acclimatization” method of filter separation combined with an acclimatization procedure, which is a stepwise transition from low substrate conditions to artificial culture conditions. The important features of Luna cluster strains are their wide distribution in freshwater systems (Hahn and Pöckl, 2005) and their small cell sizes are stable and maintained in nutrient-rich media (Hahn et al., 2003). Our group also isolated an ultamicrosize actinobacterium related to Luna strains from river water in Japan and named it *Aurantimicrobium minutum* KNCT (Fig. 2; Nakai et al., 2015). This strain showed high 16S rRNA gene sequence similarity (>99%) to strains isolated from freshwater systems in other places in Japan as well as in Austria, Australia, China, Nicaragua, and Uganda (accession nos. AB278121, AB599783, AJ507461, AJ507467, AJ565412, AJ565413, and AJ630367), suggesting its cosmopolitan distribution in freshwater.

![Fig. 1. Diagram showing filterable microorganisms in the environment. (I) ultramicrocells; (II) obligate ultramicrobacteria; (III) facultative ultramicrobacteria; (IV) slender filamentous bacteria; (V) ultra-small members of CPR bacteria (also referred to as “Candidatus Patescibacteria”) and DPANN archaea indicated by the arrow in this Figure. See details in the text. This figure was created with BioRender (https://biorender.com/).](image1)

![Fig. 2. Scanning electron micrograph of c-shaped cells of *Aurantimicrobium minutum* KNCT. Cells were cultured in organic NSY (nutrient broth, soytone, and yeast extract; Hahn et al., 2004) medium for two weeks. Scale bar: 200 nm. This micrograph is an unpublished figure from the author; other micrographs of this species are shown in Nakai et al. (2013, 2015).](image2)
The other freshwater bacterium belonging to the Luna cluster, *Rhodoluna lacicola* MWH-Ta8, was also described as an obligate UMB (Hahn et al., 2014); an additional three *Rhodoluna* strains smaller than *R. lacicola* were subsequently reported (Pitt et al., 2019). From an ecophysiological point of view, the genomes of freshwater actinobacteria possess rhodopsin photosystems (Neuenischwander et al., 2018), while *R. lacicola* has an unconventional proton-pumping rhodopsin that requires external supplementation with the cofactor retinal (Keffler et al., 2015). The underlying cause is considered to be an inability to biosynthesize the cofactor (Neuenischwander et al., 2018), suggesting that *R. lacicola* obtains retinal from the surrounding environment. One potential source in freshwater appears to be retinoids produced and released by cyanobacteria (Ruch et al., 2005; Wu et al., 2013).

Freshwater actinobacteria, including UMB strains, were previously shown to be phylogenetically diverse and subsequent studies yielded nine lineages (acI, acTH1, acSTL, Luna1, acIII, Luna3, acTH2, acIV, and acV; Newton et al., 2011). Among these lineages, acI containing multiple tribes is considered to be the most successful and ubiquitous group (Kim et al., 2011). Among these lineages, acI containing multiple tribes is considered to be the most successful and ubiquitous group (Kim et al., 2011). The genomes of freshwater actinobacteria possess rhodopsin photosystems (Neuenischwander et al., 2018), while *R. lacicola* has an unconventional proton-pumping rhodopsin that requires external supplementation with the cofactor retinal (Keffler et al., 2015). The underlying cause is considered to be an inability to biosynthesize the cofactor (Neuenischwander et al., 2018), suggesting that *R. lacicola* obtains retinal from the surrounding environment. One potential source in freshwater appears to be retinoids produced and released by cyanobacteria (Ruch et al., 2005; Wu et al., 2013).

Freshwater actinobacteria, including UMB strains, were previously shown to be phylogenetically diverse and subsequent studies yielded nine lineages (acI, acTH1, acSTL, Luna1, acIII, Luna3, acTH2, acIV, and acV; Newton et al., 2011). Among these lineages, acI containing multiple tribes is considered to be the most successful and ubiquitous group (Kim et al., 2011). Among these lineages, acI containing multiple tribes is considered to be the most successful and ubiquitous group (Kim et al., 2011). The underlying cause is considered to be an inability to biosynthesize the cofactor (Neuenischwander et al., 2018), suggesting that *R. lacicola* obtains retinal from the surrounding environment. One potential source in freshwater appears to be retinoids produced and released by cyanobacteria (Ruch et al., 2005; Wu et al., 2013).

Freshwater habitats also harbor another obligate UMB belonging to the genus *Polynucleobacter* in the class Betaproteobacteria. Similar to some actinobacteria described earlier, UMB members of this genus also showed a cosmopolitan distribution in freshwater systems (Hahn, 2003). The relative abundance of the subspecies named PnecC was high, ranging between <1% and 67% (average 14.5%) of total bacterial numbers, in more than 130 lakes studied in Central Europe, as assessed by fluorescent in situ hybridization (Jezberová et al., 2010). Culture experiments and genomic characterization suggested that PnecC bacteria in nature can utilize low-molecular-weight products derived from photooxidation and/or the direct enzymatic cleavage of high-molecular-weight substrates, such as humic substances (Watanabe et al., 2009; Hahn et al., 2012). Certain PnecC strains sharing ≥99% similarity in 16S rRNA gene sequences differed in their ecophysiological and genomic features (e.g., the presence/absence of iron transporter genes), suggesting cryptic diversity among the abundant lineage not covered by 16S rRNA gene-based typing (Hahn et al., 2016).

The obligate UMB inhabiting sea and freshwaters described above were characterized by minute cell sizes, but also small genome sizes (<2 Mbp) with a low genomic guanine-cytosine (GC) content: this genome “streamlining” is considered to reflect an adaptation to nutrient-limited conditions (e.g., SAR11 members; 1.16–1.46 Mb; Giovannoni et al., 2003; Grote et al., 2012; Henson et al., 2018) (Table 1). This phenomenon of a reduced genome size with gene loss also indicates metabolic dependencies on co-existing microorganisms in nature, as described by the “Black Queen Hypothesis” (Morris et al., 2012). As another example, the reconstructed genomes of ultra-small and uncultivated marine actinobacteria (“Candidatus Actinomarinidae”) were very small (<1 Mbp) and had a very low GC content of 33% (Ghai et al., 2013). In addition, known obligate UMB of different lineages, such as “Ca. P. ubiquus” (Alphaproteobacteria), *Polynucleobacter* strains (Betaproteobacteria), and *A. minutum* and *R. lacicola* (Actinobacteria), showed similar “c-shaped” (curved-rod) cells (Table 1; *A. minutum* for Fig. 2; Hahn, 2003). This unique shape may be advantageous for the efficient acquisition of substances because of their increased surface-to-volume ratio of cells or grazing resistance against bacteriovorous protists for planktonic life in waters.

In contrast to aquatic environments, limited information is currently available on UMB, including the obligate type, from soil habitats. Janssen et al. (1997) previously reported anaerobic obligate UMB with very small ellipsoid to nearly spherical shapes (e.g., *Opitutus* sp. VeCb1 with a cell volume of 0.030 μm³) belonging to the *Verrucomicrobiales* lineage from rice paddy soil using dilution culture techniques. Nakai et al. (2013) isolated and cultivated filterable strains from soil and sand suspensions; however, obligate UMB were not found among these strains. High-throughput sequencing of the 16S rRNA gene revealed that the smaller size fractions in soils were more likely to harbor rare or poorly characterized bacterial and archaeal taxa, such as Acidobacteria, Gemmatimonadetes, Elusimicrobia, *Verrucomicrobia*, and *Crenarchaeota* (Portillo et al., 2013). However, further studies are needed to clarify whether the members detected in the small fractions contain UMB.

**Facultative UMB**

Facultative UMB that contain a small proportion of larger cells with a cell volume >0.1 μm³ have not yet been characterized in detail (Table 1) because morphological changes throughout the growth cycle have only been examined in a limited number of UMB. *Endomicrobiurn provitum* Rsa215 (now deposited as DSM29378=JCM32103T) belonging to the phylum *Elusimicrobia* appears to be a well-studied example of facultative UMB. The phylum *Elusimicrobia* (former termite group 1 candidate phylum) was initially established with the cultivated ultramicrobacterium of *Elusimicrobiurn minutum* strain Pei191T from the 0.2 μm-filtered filtrate—originally prepared as a growth promoting supplement for gut bacteria—of the gut homogenates of a scarab beetle larva (Geissinger et al., 2009; Herlemann et al., 2009). *E. provitum* Rsa215 was isolated from the filtrate of the gut homogenate and was identified as a free-
living bacterium of a novel class-level lineage in *Elusimicrobia* (Zheng et al., 2016). *E. proavitum* has an unusual cell cycle that involves different cell forms, i.e., cocci, rods, and budding-like cells, during the cell cycle. Under laboratory cultivation conditions, before growth commences, the cell population is comprised of a large population of UMB coccoid cells with a few rod-shaped cells (~3.5 μm in length); small cocci are formed from a bud-like swelling at one pole of the rod-shaped cells during growth. Although its morphological variation in the host gut currently remains unclear, cell characteristics as observed in the laboratory result in the classification of facultative UMB. Another important trait for *E. proavitum* is the ability to fix nitrogen gas with a group IV nitrogenase, which was considered to harbor functions other than nitrogen fixation (Dos Santos et al., 2012).

**Slender filamentous bacteria**

In addition to ultramicrocells and UMB, slender filamentous bacteria have frequently been found in 0.2 μm-filtered fractions of environmental samples. Slender spirillum-shaped *Hylemonella gracilis* was isolated from filtrates of freshwater samples (e.g., Hahn et al., 2004; Nakai et al., 2013) and passes through membrane filters with small pore sizes of not only 0.22–0.45 μm, but also 0.1 μm (Wang et al. 2007). The smallest widths of *H. gracilis* cells are approximately 0.2 μm and close to filter pore sizes, which may allow its slender cells to “squeeze” through these pores. Regarding the quality control and assessment of filter sterilization, Wang et al. (2008) proposed that filterable slender bacteria, such as *H. gracilis* with small cell widths, may be used for the microbiological validation of membrane filters instead of *Brevundimonas diminuta*, which is the current standard strain tested.

During a screening of UMB, our group isolated a slender filamentous bacterium from the filtrate of a suspension of desert sands collected in Tunisia, and described *Oligoflexus tunisiensis* Shr3, which represents the eighth novel class named *Oligoflexus* within the phylum *Proteobacteria* (Nakai et al., 2014; 2016a). The cell shape of this species is mainly slender, filamentous, and of variable lengths, but shows a pleomorphic with other shapes, such as a spiral, spherical (or curled), or curved rod morphology (Fig. 3; Nakai and Naganuma, 2015). This polymorphic flexibility of cells with small widths down to 0.4 μm appears to be related to their ability to pass through membrane filters; however, it has not yet been clarified whether each morphological shape is associated with a resting state or other states. Regarding filamentous formation, this shape may be related to resistance to protozoan grazing, as reported in previous studies (e.g., Jürgens et al., 1999; Suzuki et al., 2017a). The environmental sequences closely related (>97%) to the 16S rRNA gene sequence of *O. tunisiensis* were recovered from paddy soil, cyanobacterial bloom in lake water, bioreactors, and human skin using culture-independent approaches; however, their detection frequency was low, with at most ~0.6% (Nakai and Naganuma, 2015). Thus, *O. tunisiensis* and its relatives appear to be rare species, and their ecological roles are currently unclear; one possible role for *O. tunisiensis* may be incomplete denitrification to nitrous oxide, as inferred from its genome sequence (Nakai et al., 2016a).

Despite the potential rarity of its occurrence, the size filtration method led to the isolation of an additional slender filamentous strain, *Silvanigrella aquatica* MWH-Nonnen-W8red, with a pleomorphic morphology in the class (Hahn et al., 2017). Hahn et al. (2017) reclassified the order *Bdellovibrionales*, including *Bdellovibrio* spp. known as small “bacteria-eating” bacteria (reviewed in Sackett, 2009), from the class *Deltaproteobacteria* to the class *Oligoflexia* based on in-depth phylogenetic analyses. Incidentally, 0.45-μm filtrates of environmental samples are frequently used for the enrichment culture of *Bdellovibrio* predatory bacteria. In the Genome Taxonomy Database (GTDB) based on genome phylogeny (https://gtdb.ecogenomic.org/; Parks et al., 2018), the class *Oligoflexia* belongs to the candidate phylum “Bdellovibronota”, named after the genus *Bdellovibrio*, and not the phylum *Proteobacteria*; its taxonomic assignment will be discussed in future studies. *Oligoflexia* very recently gained two more species, *Fluviispira multicolarata* 33A1-SZDPOT and *Silvanigrella paludirubra* SP-Ram-0.45-NSY-1T, from freshwater habitats (Pitt et al., 2020). *Silvanigrella* spp. are phylogenetically closely aligned with “*Candidatus Spirobacillus cienkowskiii*” (Pitt et al., 2020), which is an uncultured pathogen of water fleas (*Daphnia* spp.) described morphologically almost 130 years ago (Metchnikoff, 1889). Since *Silvanigrella* spp. are isolated from the filtrates of micropore filtration, size fractionation may be an effective method for isolating the uncultivated pathogen as well as additionally overlooked agents in *Oligoflexia*. A detailed comparison within members of this class will also be important for pursuing the evolutionary acquisition and divergence of predatory and pathogenic behaviors.

**Diverse ultra-small members and their potentials**

Metagenomic investigations on microbial communities have generated genomes for an astounding diversity of bac-
eria and archaea; CPR/Patescibacteria inhabiting groundwater has attracted increasing attention in recent years. Traditionally, certain types of groundwater bacteria were known to pass through a micropore filter (e.g., Shirey and Bissonnette, 1991). Additionally, Miyoshi et al. (2005) phylogenetically characterized filterable microorganisms captured by 0.1-µm-pore-sized filters from deep aquifers of the Tono uranium mine, Japan and then discovered candidate phyla “Ca. Parcubacteria” and “Ca. Microgenomates”, respectively, enriched by approximately 44% in 16S rRNA gene clones from the filtered fraction. The specific occurrence of “Ca. Parcubacteria” (OD1) in the 0.2-µm filtrate was also detected in deep-sea hydrothermal fluid (Naganuma et al., 2007). It was previously unclear whether members of these candidate divisions OD1 and OP11 (now recognized as candidate phyla “Ca. Parcubacteria” and “Ca. Microgenomates”) were also present in the field of synthetic biology, the top-down approach has been employed to reduce and simplify the genomes of microbial cells by genetic engineering, and then to identify essential genes for living systems; the bottom-up approach, which is the opposite of the top-down approach, has been used to examine what is sufficient for living systems by assembling non-living components, such as nucleic acids, proteins, and lipids (e.g., Matsuura et al., 2011; Xu et al., 2016). In this context, DeWall and Cheng (2011) pointed out that the small genomes of microorganisms in nature may be models for the identification of a minimal genome. Since the ultra-small members described here as well as free-living obligate UMB already harbor small and sometimes streamlined genome structures (<2 Mb) through the loss of unnecessary components, the “middle-out” approach, referring to the metabolic pathway of these members (Fig. 4), which effectively combines traditional top-down and bottom-up approaches, will be useful for the rational design of artificial cells.

Conclusions

Numerous cultivation efforts have clearly shown that some previously uncultured members remain viable in
small-size fractions. Some obligate UMB are ubiquitous and dominant in water systems and may play important roles in natural microbiome functions. In parallel, the advent of high-throughput sequencing technology has greatly expanded our knowledge of ultra-small microbial diversity. Future studies are required to shed light on small microorganisms hidden in various environmental samples (e.g., soils and sediments) other than aqueous environments, and on the ecophysiological traits and biogeochemical roles of these members, including CPR/Patescibacteria and DPANN. Further studies on “extreme” microorganisms at the lower size limit will undoubtedly lead to new conundrums about life on Earth.

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