

Minireview

Nanobacteria, Ultramicrobacteria and Starvation Forms: A Search for the Smallest Metabolizing Bacterium

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Very small forms of bacteria have been reported from marine and freshwater systems as well as from soils, subsurface terrestrial environments' and more recently from samples of kidney stones. Also, such small cells could be obtained in the laboratory during starvation experiments, indicating that bacteria which survive periods of nutrient deprivation manifest a decrease of cell size. Even though the various reports brought about poorly defined designations for small bacteria, they have stimulated the discussion on how small a living bacterium could be. The information derived from the literature concerning starvation forms, ultramicrobacteria and nanobacteria is analysed in the light of own field and laboratory observations. It could be shown that despite conceptual shortcomings and problems with definitions of what is meant by "small", starvation forms and ultramicrobacteria are clearly distinguishable according to physiological characteristics, which could not be achieved for nanobacteria or nanobes. Furthermore, it is documented that ultramicrobacteria are not the smallest prokaryotes and that the size of bacterial starvation forms are much closer to the calculated minimal sizes required to ensure independent viable life. A bacterial cell which is growing and dividing needs to be large enough to accommodate DNA and RNA, enzymes for replication transcription and translation, solvent for substances as well as a minimum set of proteins and plasmitic space to run the operations. Many authors assume that this requires a cell with a diameter not smaller than 200 nm and with a volume between 0.014 and 0.06 μm^3 . Most cells with diameters equal or below 0.2 μm are rods while cocci are numerically of minor importance in natural aquatic systems. It seems that the rod morphotype has more potential to produce viable cells with minimal volumes than spherical morphotypes. This supports the assumption that not only size but also cell shape is important to achieve functional minimum cell volumes. Many cell parameters can be estimated with relatively high precision, but one should remember that absolute calibration is still not possible. Even if we assume that there are errors involved in most biometric measurements, there is still a trend which indicates that rod shaped cells can function with diameters below 0.2 μm and cell volumes well below 0.02 μm^3 . Such dimensions could be an indication that the lower size limit of a viable bacterium may be close to the size of the smallest hypothetical living cell, indicating that there is still more to know about the minimal required cell components allowing a bacterium the remain viable.

Key words: ultramicrobacteria, starvation forms, nanobacteria, nanobe, small

The existence of bacteria which are smaller in volume than those currently detected when determining bacterial numbers in aquatic samples or soils by AODC or via DAPI staining is well recognized and yet has not received the attention it deserves within the field of microbial ecology.

In spite of the large number of publications dealing with physiological responses of bacteria which reduce their size under various stress conditions²³⁾ or bacteria which do not increase their cell volumes despite exposure to multiple nutrient media⁵⁰⁾, most microbial ecologists and classical microbiologists seem to neglect the importance of small bacterial cells in research planning, in design for experimental set up, in bacterial production studies, and in quanti-

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tative microbial-loop models to be integrated in ecosystem models.

In the last 5 years, the topic of small bacteria has received much attention with the detection of small cell-like forms, resembling granules or inclusion bodies, in mars meteorites³⁴⁾, deep subsurface samples⁵⁴⁾ and human kidney stones²²⁾. The emergence of the so-called nanobacteria has re-opened the debate on the importance of small bacteria in various research fields and transported the topic from the field of specialists to the generalists in microbiology, molecular biologists, biogeochemists, physicists and to a certain extent, the scientific community at large. In this paper, an attempt is made to encourage and widen the debate on the global importance of small bacteria by showing that despite problems as to what exactly is meant by “small”, miniature or dwarf bacterial cells exist in abundance and a large amount of data is available concerning biometry, physiology and ecology for a number of taxa. Even though this short review will focus on investigations in aquatic environments, the results from studies of other environments are referred to when it is felt that it would help to improve the formulation of definitions or the understanding of the role small prokaryotes may play in the functioning of ecosystems.

Conceptual shortcomings and problems with definitions

It should be emphasized that there is no clear definition for the term “nanobacterium”, though such organisms are preliminarily grouped into the same phenotype class as bacterial starvation forms and ultramicrobacteria. Because starvation forms and ultramicrobacteria are well defined entities, however we are confronted with a conceptual shortcoming. As Hamilton¹⁸⁾ recommends adopting a working hypothesis, nanobacteria are defined as “extremely small cellular forms, widespread in nature and closely associated with the formation of inorganic precipitates and geological strata”. While this working definition is devoid of any quantification and does not facilitate decisions with respect to classification of small viable cells, it should be mentioned in this context that the term “nanobe” was formulated for similar structures⁵⁴⁾ which were also found on rock samples. The main criticism emerging after the description of the nanobes focused on the diversity of size and shape of these structures which may also suggest that these forms are nothing but fragmentation products of larger cellular life forms resembling actinomycetes or fungi. More regrettable, however, is not only the lack of reliable quantitative information concerning the size of these cells (the diameters range from 0.02 μm to 0.128 μm), termed nano-

bacteria or nanobes, but also the fact that we do not know whether the observed structures are (or were) living units. The controversies emerging from published observations on the size of potential nanobacteria or nanobes and the calculated required minimal size of a cell enabling independent viable life are ongoing^{16,33,39,47)}. It seems that the general consensus concerning the minimum size enabling independent life for a cell is a volume of between 0.014 and 0.06 μm^3 with a diameter ranging from 0.14 to 0.3 μm ^{26,33,47)}. This is supported by theoretical calculations of the minimum space required for DNA, RNA, ribosomes, enzymes and lipids and whatever else is needed to make up an organism, to be accommodated within a cell. Now, while we are confronted with an obvious definition problem with respect to nanobacteria and nanobes, we have a firm basis on which to define ultramicrobacteria and starvation forms even though there is still a great deal of confusion in the literature as to the use of these expressions. The main reason for dealing with these life forms in one and the same study is that their phenotypes often show the same cell features; they are both in a size range which is at the limit of resolution of an optical microscope, yet their physiology reveals clear distinctions (see following chapters). In this paper, a brief outline is presented, which shall not only allow one to clearly distinguish between these functional groups but also show that they have different roles within the microbial compartment. Also, it is recommended that terms such as “microcells”, “dwarf cells” and “Lilliputian cells” not be used unless they are employed as adjectives or quantitatively characterized by the author. The exception is the term “mini-cell” used to designate the DNA-less products of uneven cell division in an *Escherichia coli* mutant. For very detailed information concerning starvation of bacteria and starvation survival in oligotrophic environments, I would like to refer to the brilliant and valuable reviews by Kjelleberg²³⁾ and Morita³⁸⁾ and the papers cited therein.

Background Information

While the culturability of marine bacteria ranges between 0.1% and 0.01%^{2,11,14,60)} and is therefore restricted to rather large but culturable forms which are assumed to represent a minority within the bacterial community, the bulk of oligobacteria³⁾, seemingly able to sequester organic compounds from dilute ambient levels for growth but manifesting resistance to growth on agar plates, remain largely unknown. Yet, metabolic activity was recorded among the majority of these uncultured organisms^{10,11)}, and the incorporation of isotope by filter-fractionated populations showed that the activity is concentrated in the smallest

cells⁴). Many aquatic environments are characterized by a low bioavailability of organic and inorganic nutrients, and periods of starvation are suggested to be experienced by most free-living bacteria. However, marine bacteria are supposed to be well adapted to low nutrient environments^{4,24,42,44}, because of both the exceedingly low concentrations of organic matter in the open ocean ecosystems over most of the year and frequent fluctuations of nutrient levels in near shore waters³⁵ which may affect cell division and cell growth.

Much of the difficulties encountered in the isolation of these bacteria has been caused by use of media inappropriate to their growth, but a significant advance in the culturability of bacterial cells was made by Torella and Morita⁵³ as well as by MacDonell and Hood³⁰ who employed a less nutrient rich solid medium containing very low concentrations of peptone and yeast extracts. Taking a different approach, Button *et al.*⁵ proposed dilution cultures, demonstrating that they have advantages for studying oligobacteria, because culture selection then favors the most abundant, rather than the most nutrient-tolerant organisms.

Several research groups^{40,52,53} proposed that marine bacteria confronted with conditions of low solutes undergo certain changes. Among these are a decrease in cell volume and frequently a change in the cell morphology. It was suggested that these changes, initiated at the onset of starvation, were associated with the depletion of cellular reserves, the marked decrease in volume being a function of processes to scavenge non-essential cellular components and material. On starvation, the cells turn into small spheroids via fragmentation and continuous size reduction and survive for periods measured in months rather than days or weeks.

The presence of dwarf or miniature bacteria in aquatic systems was documented by both the improved technique of epifluorescent microscopy^{9,53,63} and electron microscopy^{61,62}, with cell diameters ranging from less than 0.2 μm to 0.3 μm . The fact that bacteria in 0.2 μm filtered seawater displayed uptake of ³H-amino acids and grew at high rates²⁹, and have been recovered from 0.2 μm filtrate in a viable state³⁰, raised interest in the physiology of "dwarfing" as a result of starvation²⁵ and its role within the bacterial community³⁷.

Ultramicrobacteria

The literature has supported the existence of miniature bacterial cells since 1974, but the term ultramicrobacterium (UMB) was first used by Torella and Morita⁵³ to describe "...very small coccoid forms (less than 0.3 μm in diameter) in the sea, and these may constitute a significant fraction of

the bacterioplankton of the sea". In addition, UMB were found to have a very low growth rate when inoculated onto nutrient rich agar surface and show no significant increase in cell size on this medium. This definition was adopted by MacDonell and Hood³⁰, but they made an important modification with respect to size¹⁹, defining UMB as bacteria that can pass through a 0.2 μm filter. This implies that according to the size classification scheme for components of pelagic ecosystems proposed by Sieburth *et al.*⁵¹, UMB belong to the femtobacterioplankton, assuming that the separation method with available filters is reliable. However, the formulation of a limit for cell diameter alone was not considered to be a satisfactory biometric criterium to define UMB. Because a nominal filter pore-size may not correspond, with few exceptions, to the nomination, and because cell walls of bacteria may be flexible and pass through such pore sizes despite having larger diameters, a volume limit, namely 0.1 μm^3 ⁵⁰ was introduced to complete the size definition of ultramicrobacteria. At this time, the diameter limit originally proposed by Torella and Morita⁵³ of 0.3 μm was reintroduced without further comment. However, the formulated size features do not allow one to distinguish UMB from the large number of small starving bacteria in oligotrophic environments as described below, having similar biometric features. Yet, there is one important trait which will clearly separate UMB from starvation forms when one is culturing small cells. UMB will retain their small cell size, namely a diameter below 0.3 μm ⁵³ and a cell volume below 0.1 μm^3 ⁵⁰, even when exposed to media with high nutrient concentrations. Such behavior was not observed for starvation forms from marine environments when confronted with full strength media.

To date, studies performed on UMB have been restricted to a *Sphingomonas* strain RB2256, isolated from Ressurrection Bay in Alaska⁴⁸, and as mentioned earlier, the strain was isolated by dilution of samples. It is argued that dilution factors resulting in inocula of approximately 1 to 2 cells per tube are ideal to obtain sufficient tubes that contain bacterial cultures while preventing outgrowth of atypically large cells. The advantage of this approach is that conditions for bacterial growth, using unamended filtered and autoclaved natural seawater, are probably as natural as possible, even when alterations during experimental handling of seawater occur. Surprisingly, storage of stationary phase cultures of UMB at 5°C for several months led to the development of facultative oligotrophic cells able to grow on high-nutrient media, a phenomenon which could not be achieved for the same small cells after detection in the dilution culture. Since the nature of this transformation is not known and this ob-

starvation conflicts with the definition of Ishida *et al.*²⁰, who claim that UMB should be considered obligately oligotrophic, much physiological work has been done to detect mechanisms allowing this transition. These investigations allowed us to summarize a number of characteristics which distinguish UMB from other small bacterial cells (i.e. starvation forms).

- 1) Isolated by dilution to extinction and usually present in relatively high numbers
- 2) Little variation in cell volume (0.05–0.09 μm^3) and cell diameter (equal or below 0.3 μm) even when exposed to high-nutrient media
- 3) Low DNA content (1.0 to 1.7 fg cell⁻¹)
- 4) Rich in protein (>800 mg ml⁻¹ cell volume: >60% vol/vol)
- 5) Low rRNA operon number
- 6) Obligately oligotrophic when first cultivated, but may turn into facultative oligotrophs
- 7) Contain a constitutive binding-protein-dependent “general” amino acid uptake system
- 8) Extremely high alanine uptake affinity with high maximum attainable rates
- 9) Well adapted to simultaneous substrate utilization
- 10) Able to grow in the ocean at realistic (i.e. measurable) rates
- 11) Internal storage of glucose during glucose-limited growth
- 12) Both growing and starving cells are extremely stress resistant
- 13) Absence of a typical starvation -survival response (see next chapter)

This list of characteristics shows that RB2256 is well suited to growth in low concentrations of nutrients and it was argued that this strain and UMB in general would not encounter nutrient levels that would cause starvation¹³. Consequently, a starvation-survival reaction of copiotrophic bacteria is not expected to take place and it was questioned whether the genetic potential to respond to marked nutrient deprivation was present in UMB. However, considering the total amount of bacterial carbon versus carbon available for microbial assimilation, the authors claim that cells of the strain RB2256 were likely to be starving at the time of sampling and therefore assume that bacteria in oligotrophic marine environments are potentially exposed to starvation. If this is the case, it implies that a physiological response to starvation could well be described for UMB.

Starvation forms of bacteria

Within the context of the above definitions, the decrease

in cell size is assumed to be a general phenomenon when bacteria are exposed to low nutrient or starvation conditions. Also, it is justified to assume that bacterial life contains intervals of non-growth with intermittent periods of unbalanced growth, indicated by exceptionally long generation times up to 210 days^{6,36}. Consequently, the process of cell miniaturization for non-differentiating bacteria is expected to take place frequently in aquatic environments, and the growing interest in non-growing bacterial morphotypes is the result of an increasing awareness that such morphotypes are common in natural ecosystems, which has led to physiological and molecular investigations of the stationary phase of the bacterial life cycle²⁷ and the starvation-recovery biology of bacteria^{15,21,28,40,41,43}. Although most of these authors restricted their investigations to marine bacterial strains of the genus *Pseudomonas* sp., *Pseudomonas* sp. S9 and *Vibrio* sp. DW 1 and CCUG 15956 (S 14), now synonymous with *Vibrio angustum* S14, it may be suggested that in general a nutrient deprived bacterium shows a sequential pattern of macromolecular synthesis that resembles the differentiation program of spore formation, implying that non-differentiating bacteria respond to starvation by a complex turn-on/turn-off pattern of protein synthesis*.*.*.*.*. Multiple nutrient starvation as well as carbon starvation were reported to induce the formation of miniature cells, also termed ultramicrocells which could be described in physiological terms^{43–45}.

Three phases could be distinguished on the basis of the pattern of macromolecular lysis/synthesis, in which physiological characteristics are event-specific, namely

- 1) the stringent control phase: a shut-down of macromolecular synthesis, the temporary accumulation of guanosine 5'-diphosphate 3'-diphosphate (ppGpp), and an increased rate of intracellular proteolysis, induction of high-affinity leucine uptake with broad substrate specificity to increase nutrient scavenging capacity, increase of exoprotease activity.
- 2) the reorganization phase: decrease of ppGpp content, partial recovery of macromolecular synthesis, marked transient shifts in the fatty acid composition of the membranes and degradation of reserve material³¹) but partial de novo synthesis of amino acids, chemotactic responses to different solutes³², the onset of development of resistance to a variety of stress conditions⁴⁵) and
- 3) a gradual decline in metabolic activities: notably the rate of respiration, and synthesis of RNA, protein and peptidoglycan, but nonetheless the continuous development of a persistent and recovery-orientated cell. Protein synthesis may still be detected after more than 1

week of starvation. In a number of experiments¹⁵⁾ it could be shown that carbon starvation induced the development of cells which remained highly responsive to nutrients during prolonged starvation, and that ribosomes existed well in excess of the apparent demand for protein synthesis in *Vibrio* sp. CCUG 15956. Furthermore, it was demonstrated that only a few genes were involved in the regulation of the C-starvation stimulon and that the function encoded by the *csrS* gene is essential for the successful development of starvation and stress resistance⁴⁶⁾.

Within the frame of these investigations, however, it was noticed that a) in most experiments dealing with a starvation-induced decrease in cell size, the cell volume of the bacteria at the initiation of the induction ranged between 1.0 and 2.0 μm^3 ^{24,31)}, thus being one to two orders of magnitude larger than the average marine bacterium in situ and b) the cell volume at the end of the experiment ranged from 0.4 to 0.5 μm^3 , which is still one order of magnitude above the size of marine bacteria.

Data on substrate uptake and utilization by a comparatively small marine bacterium, namely an ultramicrobacterium, as defined by Torella and Morita⁵³⁾, were provided by Schut *et al.*⁴⁹⁾ who obtained their strains by dilution culture⁴⁸⁾. But despite the small cell volumes of these rod-shaped bacteria, which ranged from 0.05 to 0.06 μm^3 , a cell diameter of 0.3 μm resulted in cell retention on a 0.2 μm pore size filter. Hence ultramicrobacteria with a width as defined by Hood and MacDonell¹⁹⁾ or bacteria with cell diameters of 0.2 μm were not produced during starvation experiments. Coincidentally, the 0.2 μm diameter represents a size which equates closely with the pore size of so-called sterilization filters and also with the functional boundary between particulate and dissolved organic matter, even though the concept of an organic matter continuum⁷⁾ has replaced the above mentioned dichotomy between the two phases of organic matter. This implies that none of the cells under investigation could be reduced to size observed in 0.2 μm filtrates of natural water samples from oligotrophic environments.

Consequently, important aspects of starvation physiology with respect to the synthesis of stress proteins or stability of transcripts¹⁾ may still be unknown. This speculation is supported by the findings of Eguchi *et al.*¹²⁾ who investigated responses to stress and nutrient availability by the marine ultramicrobacterium *Sphingomonas* sp. strain RB2256, obtaining evidence that its protein-synthesizing machinery is constitutively regulated and that the strain possesses novel physiological and molecular strategies that allow it to

predominate in natural seawater.

Starvation responses in ultramicrobacteria

Unlike copiotrophic bacteria and in comparison to *Vibrio angustum* S14⁴⁶⁾, the strain RB2256 did not manifest reductive cell division during starvation. The examination of protein profiles derived from two-dimensional polyacrylamide gel electrophoresis for logarithmic and stationary phase cells showed that protein spot intensity was regulated by up to 70-fold and a total of 72 and 177 spots showed increased or decreased intensity, respectively, by at least two-fold during starvation. Some 80 spots are present only in gels for logarithmic-phase cells¹³⁾. This may be indicative of growth phase-specific alterations in gene expression, supporting the presence of a genetically programmed starvation response. On comparing the obtained protein gels with those of *Vibrio angustum* S14, a relatively low number of protein spots which have a higher intensity was detected during starvation for RB2256. It is assumed that this is due to the repression of those genes involved in cell division, while they have to be expressed in *V. angustum* S14 to enable reductive cell division during the stationary phase.

Also, the regulation of protein and RNA synthesis differs from that in *V. angustum* S14 during starvation. When comparing the rate of protein synthesis at the onset of starvation with the rate after 7 days of starvation one registers a 2-fold decrease in the specific rates of synthesis for RB2256, while a 470-fold decrease was noted for *V. angustum* S14. A different behavior is observed for the rates of RNA synthesis; the rates show only small variations over 7 days and remain largely unchanged for RB2256, while they decrease abruptly during starvation in *V. angustum*. The authors argue that the results indicate clearly a difference in the physiological responses of the two classes of bacteria when exposed to starvation.

Further considerations concerning size and taxonomic status of small cells

A significant decrease of cell size, measured under in situ conditions, was first recorded in dialysis bag experiments for a predator-free bacterial population from the water column above a seagrass system (*Posidonia oceanica*) during the winter season⁵⁷⁾. In a 24 h experiment, designed to monitor bacterial production in the water column at a depth of between 5 and 15 m, the average volume of rods (0.083 μm^3), cocci (0.025 μm^3), vibrio (0.022 μm^3) and spirillae (0.026 μm^3) morphotypes decreased by 52%, 45%, 58.8% and 31.2% respectively, leading to a decrease in bacterial carbon production despite an increase in cell numbers. The

decrease in cell volume was not restricted to the winter period as it also took place in April, but such observations were not made in summer, and for most experiments from June to October we recorded increasing cell volumes and increasing cell numbers. However, these observations led to a pilot study on the biometry of bacterial morphotypes and the presence of cells with diameters equal and below 0.2 μm could be demonstrated by TEM for our samples.

The quantity of bacteria passing 0.2 μm pore size filters, determined for water column samples from the above-mentioned seagrass system varied from 4 to 11% of the total bacterial number⁵⁶). Moreover, we could show that bacterial secondary production in the 0.2 μm filterable fraction, measured by ³H-Tdr incorporation into the DNA, ranged from 0.4 to 18.5% of the total bacterial production.

To investigate the growth and degradation potential of the 0.2 μm filterable bacteria, subsamples from mesocosms with 0.2 μm filtered seawater and unfiltered control samples were supplied with isotopes and artificial fluorescent substrates in regular intervals over periods of between 96 and 120 h⁵⁸). In all experiments with filtered water we recorded enhanced ¹⁴C-Leu incorporation followed by a high ³H-Tdr incorporation into the DNA after 16 to 20 h, being 4 to 10 times higher than in control experiments. A similar trend was observed for enzymatic activities, especially for aminopeptidase, indicating intense substrate hydrolysis of fast growing cells.

First transfer experiments of our culture-grown and taxonomically characterized cells, which were rod shaped, had diameters up to 1 μm and reached cell volumes ranging from 1.7 to 1.9 μm^3 , gave the following results: The transfer into nutrient depleted seawater, i.e. multiple nutrient starvation allowed us to obtain an average size reduction of cell width ranging from 0.4 to 0.5 μm , leading to mean volumes between 0.16 and 0.35 μm^3 . Only occasionally a cell of 0.2 μm was found, and the majority of the cells, although originally derived from 0.2 μm filterable bacteria, remained well above the ultramicrobacterium size. It was assumed that sporadic cell lysis which was occasionally observed in the subsamples produced an uncontrolled DOC input. The cause of this lysis during the starvation experiment is presently under investigation.

Against expectations, a cell size reduction of 80% was observed in experiments done between 1999 and 2000 (Ulbricht *et al.*, in prep), during growth in multinutrient medium with subsequent depletion in an nitrogen/phosphorus-starvation medium, which led to an increase of the 0.2 μm filterable cells from 0.02 to 16% of the DAPI-stained cells. Yet the culturability of these 0.2 μm filterable cells,

(*Pseudoalteromonas* sp. strain RE10F/5) after 240 hours in these media was still as high as 70% as indicated by CFU on VNSS medium. On this occasion we could detect rod-shaped cells in the process of reductive division with diameters between 0.16 to 0.18 μm , and a length of 0.6 to 0.7 μm . Documentation of such cells was made by TEM and copies are available on request. DNA and protein synthesis rates could still be recorded after 240 h of starvation.

Although a great number of bacterial species were subjected to starvation experiments (for details see Kjelleberg²³), and Morita³⁸) which can not be reported here, it should be stated that even though a size reduction can be expected in species of non-differentiating bacteria, this does not imply that either a 0.2 μm filterability or a UMB size need to be reached. Also, despite the relatively large number of species which were investigated to describe starvation survival behavior under controlled conditions, we have little information on the relative number of species which are starving under in situ conditions. Adopting the working hypothesis that all 0.2 μm filterable bacteria would be either starvation forms or UMB, a preliminary investigation, dealing with the isolation and taxonomical characterization of 0.2 μm filterable bacteria from the Western Ligurian Sea, was initiated within the frame of a M. Sc. thesis in 1995 and published in 1999⁵⁹). According to the findings of Nyström *et al.*⁴⁵), we adopted the strategy of multiple nutrient starvation, using the minimal media of Torella and Morita⁵³) for growth of oligotrophic bacteria, and sampled over the summer season above and below the thermocline. We obtained a total of 33 isolates from 10 and 35 m depth and using SDS-PAGE, identified four different groups of strains which we subsequently characterized taxonomically. Among the strains which were identified as *Proteobacteria* of the γ -subclass, two were found to belong to the genus *Pseudoalteromonas* whereas one strain belonged to the family *Vibrionaceae*. One yellow pigmented strain belonged to the α -4 subclass of the *Proteobacteria*, being closely related to the genus *Erythrobacter*.

Unfortunately, our knowledge of the magnitude of microbial diversity is limited to culturable microorganisms and does not allow us to differentiate whether the strains of small bacteria obtained in culture are starvation forms or UMB.

Since there is a tremendous lack of information on the physiology and the diversity of the 0.2 μm filterable compartment and because it may consist of both starvation forms and ultramicrobacteria, we investigated pooled winter and summer samples from the oligotrophic region of the Western Mediterranean Sea (Corsica) by analyzing bacteria of size-fractionated water samples using denaturing gradi-

Table 1. Sequence homology of DGGE sequences (February 1997) derived from excised bands of the 0.2 μm filterable bacterial fraction of the Western Mediterranean Sea to their closest bacterial relatives available in the EMBL database. Sequences obtained after amplification of 16S rDNA fragments correspond to nucleotide position 341 to 534 (*Escherichia coli* numbering).

Sequence designation	Phylogenetic affiliation		Sequence homology ^a	Band detectable in fraction ^b		
				P _{total}	P _{>0.2 μm}	P _{0.1–0.2 μm}
CFW-1	<i>Cytophagales</i>	Unidentified eubacterium SCB37	98.7%	○	○	●
CFW-2	<i>Cytophagales</i>	<i>Antarcticum</i> sp.	100.0%	●	●	●
CFW-3	<i>Proteobacteria</i>	Unidentified proteobacterium BD1–5	94.1%	○	○	●
CFW-4	reamplification of the eluted DNA from excised bands failed			●	●	●
CFW-5	<i>γ-Proteobacteria</i>	<i>Alteromonas macleodii</i> IAM 12920 ^T	100.0%	●	●	●
CFW-6	reamplification of the eluted DNA from excised bands failed			●	●	●
CFW-7	<i>α-Proteobacteria</i>	“strain 303” (<i>α-proteobacterium</i>)	98.5%	○	○	●
		<i>Roseobacter algicola</i> ATCC 51440 ^T -FF3	98.5%			
CFW-8	reamplification of the eluted DNA from excised bands failed			●	●	●
CFW-9	reamplification of the eluted DNA from excised bands failed			●	●	●
CFW-10	<i>α-Proteobacteria</i>	Unidentified <i>α-proteobacterium</i> MBIC3865	100.0%	○	○	●
		<i>Stappia stellulata</i> IAM 12614	98.5%			

^a Highest obtained value of identity by comparison sequenced DGGE fragments with 16S rDNA sequences of EMBL database.

^b Band detectable (●), band not detectable (○) in DGGE profiles of the corresponding sample fraction.

ent gel electrophoresis (DGGE) on PCR-amplified 16S rDNA fragments¹⁷⁾ (Tables 1 and 2). The fragments were obtained after amplification of the variable V3-region of 16S rRNA genes from genomic DNA of uncharacterized mixtures of microorganisms. As DGGE separation of PCR products produces a band pattern in which each band represents a bacterial taxon, it allows the rapid assessment of the presence and relative abundance of different species and thus, the possibility to profile microbial populations. The advantage of this approach is that it allows identification of individual bacteria without the need for prior cultivation. Also, a comparative analysis of DGGE profiles from all bacterial size fractions, namely the fraction between 0.1 and 0.2 μm , the fraction of cells with a diameter of 0.2 μm and finally the total bacterial population, retained on a 0.1 μm pore filter, should help to answer which phenotype of bacteria, the starvation form or potential UMB, dominates in a specific marine habitat. It was reasoned that bandings being detected in the smallest size fraction, namely between 0.1 and 0.2 μm , and in the total fraction could represent potential UMBs, assuming according to Schut *et al.*⁴⁸⁾ that this phenotype is numerically important in the system. If UMBs were present but numerically of minor importance, one should detect bandings in the smallest size fraction only. However, species representing starvation forms should have bandings in all three size classes since individuals of one and the same species may be starving and of small size in the nutrient depleted part of the water column but be of large

er size and divide under favorable conditions in and around “hot spots” within the structured nutrient field in the environment. DGGE pattern indicated that most of the fragments representing 0.2 μm filterable bacteria were starvation forms rather than UMB. Even those few fragments which were only present in the 0.1–0.2 μm fraction, namely four cases out of 20, and which could have been UMB, were not definitely assigned to be potential UMBs because the absence of corresponding bands in the total bacterial population could indicate that a certain species may simply have a low relative abundance. This would therefore lead only to a small amplification signal which may be below the detection limit, and one cannot provide the proof that the signal is completely missing. The sequencing of excised and cloned DNA bands of the DGGE profiles showed that the 0.2 μm filterable bacteria we obtained clustered with known typical marine isolates of both the γ -subclass and the α -subclass of the *Proteobacteria* and the *Cytophaga-Flavobacterium-Bacteroides* branch.

While these findings on the relative abundance of starvation forms versus UMB in the Western Ligurian Sea are somewhat different from the findings that the only UMB RB2256 identified so far is a numerically dominant member of the indigenous community in Resurrection Bay (Alaska), an effort was made to characterize this bacterium and to determine its relatedness to six analogous isolates from seawater samples from the same habitat⁵⁵⁾. The bacterium was classified and described as a new species, namely

Table 2. Sequence homology of DGGE sequences (July 1997) derived from excised bands of the 0.2 μm filterable bacterial fraction of the Western Mediterranean Sea to their closest bacterial relatives available in the EMBL database. Sequences obtained after amplification of 16S rDNA fragments correspond to nucleotide position 341 to 534 (*Escherichia coli* numbering).

Sequence designation	Phylogenetic affiliation		Sequence homology ^a	Band detectable in fraction ^b		
				P _{total}	P _{>0.2 μm}	P _{0.1–0.2 μm}
CFS-1	γ -Proteobacteria	<i>Pseudomonas chlororaphis</i> LMG 5004 ^T	97.4%	●	●	●
CFS-2	reamplification of the eluted DNA from excised bands failed			●	●	●
CFS-3	γ -Proteobacteria	<i>Alteromonas macleodii</i> IAM 12920 ^T	100.0%	●	●	●
CFS-4	γ -Proteobacteria	<i>Ferrimonas balearica</i>	95.6%	●	●	●
CFS-5	γ -Proteobacteria	<i>Ferrimonas balearica</i>	96.3%	●	●	●
CFS-6	γ -Proteobacteria	<i>Aeromonas schubertii</i> ATCC 43700 ^T	97.5%	●	●	●
CFS-7	α -Proteobacteria	“strain 303” (α -proteobacterium)	98.5%	●	●	●
		<i>Roseobacter algicola</i> ATCC 51440 ^T -FF3	98.5%			
CFS-8	reamplification of the eluted DNA from excised bands failed			●	●	●
CFS-9	γ -Proteobacteria	Marine psychrophile IC067	94.4%	●	●	●
CFS-10	γ -Proteobacteria	Marine psychrophile IC067	93.0%	●	●	●

^a Highest obtained value of identity by comparison sequenced DGGE fragments with 16S rDNA sequences of EMBL database.

^b Band detectable (●), band not detectable (○) in DGGE profiles of the corresponding sample fraction.

Sphingomonas alaskensis, and the distance matrix tree, showing the phylogenetic relationships between *Sphingomonas alaskensis* and other species of the genus *Sphingomonas*⁵⁵) indicates that the new species is with *S. terrae* and *S. macrogoltabidus* in subgroup 4, *sensu lato*. As a consequence, a reclassification of this species into another genus can be expected in the near future. A further point deserving attention is that when *S. alaskensis* was grown on TSA, cells with diameters of up to 0.8 μm and lengths of 2–3 μm were observed⁵⁵), which conflicts with the claim for the size limit of UMB. It was argued that since this observation was not made in earlier studies, it may indicate that the size is either prone to variation on very rich media or that adaptations to maintenance in laboratory culture have occurred.

Conclusions

There are a number of diverging trends and conflicting results concerning cell size, abundance of specific cells, physiological responses and definitions when dealing with so called “small” bacteria.

- For nanobacteria or nanobes, as described for rock samples or meteorites, there is no proof that the described structures are really prokaryotes (or living units) or that one is not confronted with fragmentation products of a larger cellular life form.
- The investigations which were the most promising concerning small cells, namely the isolation of nanobacteria from human kidney stones²²), have to be considered with

caution since a study by Cisar *et al.*⁸) provides evidence that the biomineralization previously attributed to nanobacteria may be initiated by nonliving macromolecules and transferred on subculture by self-propagating microcrystalline apatite, while the previously described nanobacteria are probably identical to *Phyllobacterium mysinacearum* that has been previously detected as a contaminant in PCR.

- Ultramicrobacteria are not necessarily the smallest prokaryotes even though they may have the potential to occur in dimensions which are close to the calculated minimal sizes required to ensure independent viable life. At the moment, however, we have information indicating that UMB also have a tendency to “switch” between physiological states under laboratory conditions and to increase in size.
- *Sphingomonas alaskensis* was shown to a) change from an obligate oligotrophic bacterium into a facultative oligotroph under specific conditions as described above and b) increase in cell volume⁵⁵) which may be an indication that the previously stated “ultramicro” size is still prone to significant variation on very rich media or that adaptations to prolonged maintenance in laboratory culture have occurred.
- Also one can find contradictory indications concerning the relative abundance and biomass of UMB and starvation forms in natural environments. While Button *et al.*⁵) and Schut *et al.*⁴⁸) claim that UMB, if obtained by dilution to extinction, represent the most abundant bacterium in a

specific ecosystem, the above mentioned investigations using DGGE¹⁷⁾ have shown that the majority of the small cells occurring in an oligotrophic environment could be starvation forms.

- Most cells with diameters below 0.2 μm and volumes below 0.05 μm^3 are rods while cocci are numerically of minor importance, at least in natural aquatic systems, and it seems that the rod morphotype has more potential to produce viable cells with minimal volumes. This seems to confirm the assumption that not only size but also cell shape is important to achieve functional minimum cell volumes. A nonspherical shape increases the surface-to-volume ratio because a sphere has the least surface for the volume enclosed. A bacterium would be the least diffusion-limited of resources in the cell if it is as small as possible in cross section, even if this entails being longer.
- As far as the minimal size is concerned there seems to be a discrepancy between calculated minimal size, assumed to be necessary for allowing metabolism and growth of bacteria and the sizes of bacteria observed in seawater samples of oligotrophic environments or produced during starvation experiments. Even if we assume that there are errors involved in the biometric measurements, there is still a trend which indicates that rod-shaped cells can function with diameters below 0.2 μm and cell volumes below 0.02 μm^3 . Such dimensions could also be an indication that either the amount of “non-essential” cell components in bacterial cells has been underestimated or else, that there is still more to know about the cell components allowing a bacterium to remain viable.
- Consequently, the search for the smallest metabolizing bacterium continues. We expect little modification as far as the lower size limit of a viable bacterial cell is concerned and it may be somewhere between the size of the poorly defined nanobacteria and the calculated theoretical minimal size for a cell to remain functional.

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