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Proposed minimal standards for description of methanogenic archaea

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Abstract

Methanogenic archaea are a diverse, polyphyletic group of strictly anaerobic prokaryotes capable of producing methane as their primary metabolic product. It has been over three decades since minimal standards for their taxonomic description have been proposed. In light of advancements in technology and amendments in systematic microbiology, revision of the older criteria for taxonomic description is essential. Most of the previously recommended minimum standards regarding phenotypic characterization of pure cultures are maintained. Electron microscopy and chemotaxonomic methods like whole-cell protein and lipid analysis are desirable but not required. Because of advancements in DNA sequencing technologies, obtaining a complete or draft whole genome sequence for type strains and its deposition in a public database are now mandatory. Genomic data should be used for rigorous comparison to close relatives using overall genome related indices such as average nucleotide identity and digital DNA–DNA hybridization. Phylogenetic analysis of the 16S rRNA gene is also required and can be supplemented by phylogenies of the *mcrA* gene and phylogenomic analysis using multiple conserved, single-copy marker genes. Additionally, it is now established that culture purity is not essential for studying prokaryotes, and description of *Candidatus* methanogenic taxa using single-cell or metagenomics along with other appropriate criteria is a viable alternative. The revisions to the minimal criteria proposed here by the members of the Subcommittee on the Taxonomy of Methanogenic Archaea of the International Committee on Systematics of Prokaryotes should allow for rigorous yet practical taxonomic description of these important and diverse microbes.

INTRODUCTION

Anaerobic, methanogenic archaea are the only organisms that are able to produce methane as their main catabolic product. Methanogens play a critical role in the global carbon cycle, catalysing the final steps in degradation of biomass and other carbon compounds in anaerobic environments. Collectively, methanogens can produce methane from a variety of substrates, including H_2+CO_2, formate, acetate, short-chain alcohols, carbon monoxide, and methyl-containing compounds such as methylamines, methylsulfides and methoxylated aromatics [1, 2]. Along with recently discovered aerobic methanogenesis [3], biological methane production by methanogenic archaea is responsible for the vast majority of the annual methane flux into the atmosphere [4]. Because of this, methanogens are of prime importance in nature, contributing to global warming, climate change and production

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Abbreviations: ANI, average nucleotide identity; DDH, DNA–DNA hybridization; OGRI, overall genome relatedness index.

of biogas from waste [5, 6]. They are a diverse, polyphyletic group currently classified into five classes, eight orders, 16 families and 37 genera in the domain *Archaea* [6–8]. Recent culture-independent genomics data and taxonomic revisions suggest that the capacity for methanogenesis may not be limited to the archaeal phyla recently proposed for the Genome Taxonomy Database [9] that include cultured methanogens, *Methanobacteriota*, *Halobacteriota*, *Thermoproteota* and *Thermoplasmatota* but is also present in members of the proposed phyla *Korarchaeota*, *Bathyarchaeota* and *Verstraetearchaeota* [5, 6, 10–12].

It has been over three decades since Boone and Whitman [13] proposed the minimal standards for the description of new taxa of methanogens, at the time referred to as methanogenic bacteria. Although this subcommittee previously favoured the term methanoarchaea [14], here we will refer to these microbes as methanogens or methanogenic archaea because these terms are still much more widely used. Despite important advances in the field of microbiology, these standards have not been updated by the Subcommittee on the Taxonomy of Methanogenic Archaea of the International Committee on Systematics of Prokaryotes (ICSP). Chief among these advancements was the recognition that *Archaea* are evolutionarily distinct from *Bacteria* and the proposal of the three-domain system of *Bacteria*, *Archae*a and *Eukarya* [15, 16], although recent evidence suggests that eukaryotes may have originated from a lineage within the *Archaea* [17, 18]. Methanogens are now classified as *Archaea* rather than *Bacteria* and are known as methanogenic archaea. Additionally, significant advances have been made in methods of identification, classification and technologies, especially for DNA sequencing. Hence it is imperative to readdress the minimal standards for classification of methanogens based on the recent advances in taxonomical research. According to the International Code of Nomenclature of Prokaryotes (ICNP or simply the Prokaryotic Code [19]) and the Statutes of ICSP, minimal standards for description of any taxa should be proposed by experts in the field along with members of the corresponding subcommittee, revised as necessary based on advancements in the field and relevant technologies, and published in an appropriate journal. This paper reports the proposed updates and revisions to the minimal standards for taxonomic description of methanogenic archaea by the International Subcommittee on Systematics of Prokaryotes Subcommittee on the Taxonomy of Methanogenic Archaea.

OVERVIEW AND GENERAL COMMENTS

When devising the revised standards, major goals of the subcommittee were to provide important updates and deemphasize older techniques while retaining a rigorous approach to taxonomic, phylogenetic and microbiological principles. However, we felt that it was equally important to not erect barriers that might hinder or further complicate the description of new methanogenic taxa. Given the current commercial availability and affordability of whole genome sequencing, a standard microbiology lab that is equipped to cultivate methanogens should be able to perform a satisfactory taxonomic description based on these standards without access to specialized equipment. In this same spirit, side-by-side comparison of phenotypic traits using cultures of a novel isolate with type strain of the type species of the genus and all other closely related strains, although ideal, is not required; if pure cultures of the closely related strains are not easily accessible, data for comparison should be generated under similar sets of laboratory conditions as used in past publications for description of the closest relative(s), including appropriate negative and positive controls. Isolation and study of pure cultures has been and will always be a cornerstone of microbiology, and the ability to efficiently and effectively describe new taxa as they are discovered is key to this endeavour.

In comparison to the previous standards, those proposed here maintain many of the recommendations for phenotypic characterization, introduce new standards for genomic and phylogenetic characterizations, and somewhat deemphasize chemotaxonomic methods, although important updates are given for lipid and protein profiling. Many of the individual characteristics, broken down by different categories, are described below, with a focus on techniques or approaches that are novel or have undergone significant revision or improvement since the previous standards were published. We highly encourage researchers to also consult the previous standards [13] for additional details and perspective, especially on classical techniques.

The revised minimal standards proposed here are summarized in Table 1. Each individual characteristic or standard is listed as being mandatory, recommended or optional. Mandatory characteristics are considered essential for description of new taxa; if a mandatory standard cannot be met, e.g. due to unusual limitations or properties of a novel microbe, a strong justification for its exclusion should be given. Recommended standards should be met whenever practicable, and optional standards may be useful for certain taxa but are not of high priority. Researchers should take into account characteristics of close relatives, e.g. other species in a genus to which the new taxon belongs, when choosing which recommended and optional characteristics to describe.

ENRICHMENT, ISOLATION, AND CULTURE PURITY

Methods for environmental sampling and sample storage should be reported, as well as a description of the habitat sampled in terms of physico-chemical features like pH, salinity and temperature. The geological global positioning system location including latitude and longitude as well as altitude of the sampling site should be provided. In addition, for a better understanding of the geochemistry, climatic conditions as well as the structure and structure-related functions of microbes associated with sample and sampling sites, authors are encouraged to briefly review and cite previous work done by other researchers on the studied site. Enrichment medium and strategy should be described in detail as well as techniques for

Table 1. Proposed standards for pure cultures

AAI, average amino acid identity; ANI, average nucleotide identity; DDH, DNA–DNA hybridization; SDS, sodium dodecyl sulphate.

Category	Characteristic	Recommendation
Enrichment, isolation and culture purity	Description of habitat and sampling methods	Mandatory
	Enrichment, isolation strategy, verification of culture purity	Mandatory
Morphology and cellular observations	Cell morphology and arrangement by light microscopy	Mandatory
	Cell fluorescence at 420 nm	Mandatory
	Cell morphology and structure by electron microscopy	Recommended
	Characteristics of growth in liquid media	Optional
	Colony characteristics and ability to grow on solid media	Optional
	Motility	Optional
	Gram stain reaction	Optional
Methanogenesis, substrate utilization, and growth conditions	Proof of methane formation by gas chromatography	Mandatory
	Substrate range for methanogenic growth	Mandatory
	Temperature, pH and NaCl growth range and optimum	Mandatory
	Growth rate and yield (at least under optimal conditions)	Mandatory
	Growth in minimal medium	Recommended
	Growth stimulants (e.g. acetate, complex organics, trace metals)	Recommended
Stress and antibiotic susceptibility	Susceptibility to lysis (SDS, water)	Recommended
	Antibiotic susceptibility	Recommended
	Oxygen tolerance	Optional
Genomics	Complete or draft genome sequence	Mandatory
	Overall genome relatedness index (e.g. ANI, AAI, digital DDH)	Mandatory
	G+C content (*in silico*)	Mandatory
	Genomic evidence as a methanogen/physiological analysis	Recommended
Phylogeny	16S rRNA gene-based phylogeny	Mandatory
	McrA gene-based phylogeny	Optional
	Phylogeneomic analysis	Recommended
Chemotaxonomy	Lipid analysis	Optional
	Protein profiling	Optional
	Wet lab-based DDH	Optional
	Wet lab-based G+C content	Optional

isolation and verification of culture purity. Methods for purification can involve repeated streaking for isolation of single colonies on solid media on plates or in roll-tubes; a repeated dilution-to-extinction technique can be used for methanogens that do not grow well on solid media. Although it is difficult to verify with absolute certainty, culture purity should be checked carefully by microscopic observation and by observation of a single colony morphology if streak-plating or the roll-tube method is used. Checks for the presence of contaminating heterotrophic microbes can be done by testing for growth, without a methanogenic substrate, in the presence of different compounds that might serve as substrates for heterotrophic growth (e.g. sugars, complex carbohydrates, yeast extract, etc.). The purity of the isolated strain can be verified by demonstrating the absence of growth in a nonspecific medium that does not support the growth of methanogens. The addition of antibacterial antibiotics or antibiotics ineffective for methanogenic archaea is advisable during the enrichment; it assists in enrichment, improves the purity of methanogens and suppresses unwanted bacteria. However, antibiotics should be omitted during tests for purity. The purity of the isolate can further be checked by DNA extraction and 16S rRNA gene amplification with

bacterial primers. As is standard for the description of novel taxa, pure cultures must be deposited in at least two recognized culture collections in two different countries. Although isolation of multiple, independent strains can be informative in characterization of phenotypic diversity new species, this can often be problematic for strains isolated from difficult-to-obtain samples or for those that cannot be easily grown on solid media, and therefore it is acceptable for a single strain to be used for description of a new taxon.

MORPHOLOGY AND CELLULAR OBSERVATIONS

For cell morphology, electron microscopy is preferred because it provides more in-depth information about cell shape, flagellation pattern, adhering properties, cell-wall structure and cell–cell interaction [20, 21]; however, its universal availability is a matter of concern due to access and affordability. Therefore, scanning or transmission electron microscope images are not mandatory for description of new taxa, and a good quality image taken using a compound microscope is acceptable as long as it can sufficiently document cell morphology, size (diameter and length for rods/filaments, and diameter for spherical cells), and arrangement (e.g. single, pair, chain, aggregates/sarcina). Motility can also be assessed using light microscopy, however this is optional. It is important to note that optical microscopy, such as phase contrast with wet mount preparations, is a better option for cell size determination since cell size can change after technical manipulation and sample preparation for electron microscopic observation. Gram staining can also be performed; however, this is also optional because of its limited phylogenetic utility (in comparison to bacteria with true peptidoglycan) and the fact that some methanogens exhibit lysis during the Gram stain procedure [13]. Most methanogens have distinct autofluorescence due to the presence of coenzymes such as F_{420}, and detection of this property should be reported or at least attempted using epifluorescence microscopy [22]. Under excitation wavelength between 350 and 420 nm, most described methanogen species autofluorescence a blue-green colour due to an abundance of coenzyme F_{420}. However, the intensity of the fluorescence varies greatly [23], and some taxa exhibit weak fluorescence, such as *Methanothrix* species [24, 25], or no detectable fluorescence, such as *Methanomassiliioccales* species [26, 27]. Macroscopic appearance of growth can also be useful and simple to describe, but it is optional. If growth on solid media is achieved, then the colony characteristics (e.g. size, colour, morphology) can be reported along with a description of the growth conditions and incubation time. Any remarkable aspects of growth in liquid culture can also be reported, such as presence of cell aggregates or a pellicle, formation of precipitates, or changes of colour in the growth medium.

METHANOGENESIS, SUBSTRATE UTILIZATION, AND GROWTH CONDITIONS

As their name implies, methane production is a hallmark of methanogenic archaea, with rare exceptions under certain conditions [28]. Therefore, production of methane during growth should be measured. Detection and quantification of methane production, e.g. in the gas phase of the culture vessel, can be conveniently performed using gas chromatography coupled with flame ionization or thermal conductivity detectors [29]. The system must be calibrated using a standard gas. The temperature of the injector, column and detector must be indicated as well as the carrier gas used for the methane detection. For gas sampling from the headspace of the culture vessel, it is recommended to use a gas-tight syringe with a sliding valve (e.g. Hamilton Company) to avoid leakage during sampling. If the laboratory has gas chromatography equipment, the analysis can be performed directly after sampling. If there is no gas chromatography equipment in the laboratory, a defined volume of gas in the atmosphere of the culture bottle is sampled and injected into an appropriate storage tube (e.g. Venoject or Labco) for later analysis. The methane content in the headspace and medium is calculated according to the method of Mah *et al.* [30] using the appropriate Henry's law constant [31]. The methane produced is reported as mol methane produced per ml culture. The results must be corrected for the decrease in gas volume following repeated sampling. During the exponential growth phase of methanogenic archaea, the amount of methane produced also increases exponentially [32]. Direct cell counts can be used to demonstrate growth, and in cases where cell numbers are high enough, optical density/turbidity measurement using simple spectrophotometry can be included to support growth observation. The medium or media used for methane production and other characterization described below can depend on the specific context. In some cases, it may be appropriate to choose a medium that has been used for characterization of other species within the genus of the new isolate for purposes of comparison. It may also be useful to explore different media for optimal growth and use this for characterization. A minimal medium that might be used to test for growth on different substrates or stimulation of growth (see below) could instead be used for consistency throughout the characterization, if growth in this medium is sufficient. Ultimately, these choices should be at the discretion of the researcher(s), as long as the media and growth conditions for various tests are clearly reported.

Determination of substrates that support methanogenic growth is required for all new taxa. While methanogens are typically considered to be relatively restricted in their substrate range, recent research has expanded the potential compounds that can support methanogenic growth. For currently known methanogens, the most common substrates for growth and energy recovery are H_2+CO_2, formate and methylamines. Although the use of acetate as a sole source of carbon and energy is characteristic only of the genera *Methanosarcina* and *Methanothrix*, this compound is a necessary growth factor for some

other species, mainly growing on H_2+CO_2. The spectrum of substrates utilized by methanogens is slowly but continuously expanding and currently consists of H_2+CO_2, formate, acetate and methylamines (mono-, di-, or trimethylamine), dimethylsulfide, methanethiol, methanol, 1-propanol or 2-propanol + CO_2, ethanol + CO_2, 1-butanol or 2-butanol+CO_2, cyclopentanol+CO_2, H_2+CO, H_2+methanol or methylamines, betaine, choline, *N*,*N*-dimethylethanolamine, dimethylselenide, methylmercury, and methoxylated aromatic compounds [33–35]. There likely exist other methylated organic compounds that can be used as substrates and energy sources for methanogens that are yet to be tested. The list of substrates that are mandatory to check are: H_2+CO_2, formate, dimethylsulfide, methanol, 2-propanol + CO_2, H_2+methanol, H_2+methylamine, and mono-, di- and tri-methylamine. These substrates should be tested at levels that do not inhibit growth. The presence of growth must be verified by methane production or increase in cell abundance; successive transfers and no-substrate controls should be performed to ensure that observed growth is due to added substrates and not to nutrients carried over in the initial inoculum. Care should be taken if methane production is used as a proxy for growth, because some compounds such as methylmercury and dimethylselenide can support methane production but not growth [36].

The range and optimal values for growth at different temperatures, pH levels and NaCl concentrations should be determined. While it is ideal to determine the range and optimum based on specific growth rate during exponential phase (see below), it is acceptable to use total growth yield instead. When determining NaCl range and optimum, potential contributions to Na^+ or Cl^- from various medium components, such as buffers, reductants or growth substrates, should be taken into account. It is often convenient to prepare two media, one without NaCl and the other with a high concentration of NaCl. By mixing different volumes of the two media but keeping the final volume the same, a range of salinity may be obtained. For accurate measurement of pH range and optima, one approach is to add concentrated solutions of HCl or NaOH to the normal medium to achieve the desired pH. If this is done to the complete medium prior to inoculation, any significant changes in volume should be taken into account and adjusted for. After growth is complete, the pH of the culture should be determined again to ensure that it was not significantly changed due to growth of the methanogen. Depending on the type and concentration of buffering agent or agents present in the normal culture medium, additional buffers can be added to be effective at desired pH ranges. When choosing a buffer, the pK_a should be taken into account as well as any possible toxic effects that the buffer might have on growth; organic buffers should be considered, because some inorganic buffers may exert inhibitory effects [37–39]. Also, when characterizing pH range at excessively low or high temperatures, the effect of temperature on the pK_a of the buffer, which can vary considerably between buffers, should be considered. Additional recommendations in cases with bicarbonate-buffered media with CO_2 in the headspace can be found in Boone and Whitman [13]. The addition of NaOH and HCl can change the concentrations of mineral elements in the culture medium, and potential impacts of this, e.g. on salinity levels, should be considered.

The specific growth rate during exponential growth phase should be reported, at least under optimal growth conditions. The specific growth rate can be calculated based on methane production in the culture vessel headspace sampled at appropriate intervals or changes in cellular abundance as determined by direct cell counts, spectrophotometry (i.e., optical density) or other appropriate procedures. The mathematical expression $\mu=(\ln X_2-\ln X_1)/t$ can be applied to calculate the specific growth rate μ, where X_1 and X_2 represent the amount of methane or cellular abundance at the beginning and end of time interval t during exponential growth phase [40]. Growth yield can also be determined, either by total cell abundance as determined by spectrophotometry, direct cell counting (hemocytometer or other appropriate methods) or dilution plating, or by measuring dry cell mass. For the latter, cultures are passed through pre-weighed 0.2 µm polycarbonate filters, and deposited cells are then dried, first on the benchtop at room temperature and then in a desiccator overnight [41]; smaller pore size filters can be used in certain cases, e.g. where cells are thin filaments that might pass through 0.2 µm filter pores. Uninoculated medium should be filtered onto membranes to calculate the blank. All the determinations are performed in triplicate or higher replication, and either standard deviation or standard error of the mean is calculated. The quantification of dry cell mass can be correlated to other biomass quantification approaches such as spectrophotometry.

Characterization of growth in minimal medium and the effects of potential stimulants on growth is recommended. For minimal medium, any organic medium components other than the main catabolic substrate should be excluded, if possible; if the strain can grow using a completely inorganic substrate such as H_2+CO_2, then this should be used. As mentioned above, multiple transfers and no-substrate controls should be performed because of potential carry-over of nutrients. Enhancement of growth, as determined by increase in growth rate or total yield, by addition of various individual stimulants can be performed using this minimal medium, or another appropriate medium that does not contain the tested stimulant or complex organics. Typical stimulants tested include vitamin mixtures, acetate, and complex organic compounds like yeast extract, peptone and tryptone [13, 42]. Because complex medium supplements such as yeast extract and Casamino acids often contain small amounts of acetate [43], controls should be performed to distinguish requirements for acetate from those for amino acids, vitamins or other organic nutrients. Because yeast extract typically contains acetate, Casamino acids can be used to distinguish requirements for amino acids from acetate. Additional stimulants that can be considered include coenzyme M, metals like selenium and tungsten, and potential electron acceptors/sinks such as elemental sulphur or other oxidized sulphur compounds [13, 44, 45]. Researchers may also want to consider testing for use of different compounds as sole sulphur or nitrogen sources. For the latter, testing for growth

with N_2 as a sole nitrogen source is recommended if related taxa are capable of nitrogen fixation, an attribute which is broadly distributed in methanogens [46].

STRESS AND ANTIBIOTIC SUSCEPTIBILITY

Assessing susceptibility to various types of stresses can be useful characteristics for comparison to related taxa and can also be useful when considering the ecology of the isolate. For example, many methanogens have been described as being susceptible to lysis by sodium dodecyl sulphate (SDS) at various concentrations [13]. This susceptibility is likely due to the proteinaceous nature of their cell wall or analogous structure contributing to resistance to osmotic lysis (e.g. for those composed mainly of an S-layer). Typically this susceptibility can be tested by adding a concentrated solution of SDS to cultures in mid- or late-logarithmic phase to a desired final concentration, typically of 0.01% (0.1 g l^{-1}), and observing a decrease in turbidity or optical density; a decrease in cell number or appearance of spherical, empty cells can also be observed by phase contrast microscopy. Marine methanogens are also commonly susceptible to lysis after cultured cells are pelleted and resuspended in distilled water [13].

With the rise in prevalence of antimicrobial resistance (AMR), it is recommended that isolates be tested for their antibiotic susceptibility [47] and their AMR profile should be generated, especially for isolates derived from human or other animal hosts. These data are useful not only for comparative purposes but can also assist in selective cultivation of specific taxa. Similar to AMR assessment in bacteria, methods like disc diffusion, agar dilution, broth microdilution E-test, and broth macrodilution can be used for antimicrobial susceptibility testing of methanogens, depending on whether the test is to be done using solid or liquid media [48, 49]. It is recommended that tests be performed in accordance with internationally accepted procedure and guidelines, updated regularly by the Clinical and Laboratory Standards Institute (https://clsi.org/meetings/microbiology/clsi-and-ast/) or by the European Committee on Antimicrobial Susceptibility Testing (www.eucast.org). Any deviation or modification in cultivation medium, supportive nutrients, inoculum density, and incubation time and temperature and test condition should be accurately reported as mentioned in Schwarz *et al.* [50].

Methanogens can also differ in their susceptibility to oxidative stress. They are generally considered strict anaerobes based on the inability to use O_2 for respiration and O_2 suppression of methanogenesis that drives growth. Suppression is largely a consequence of the inactivation of O_2-sensitive metalloenzymes essential for methanogenesis [51]. However, many methanogen species remain viable from hours to days after O_2 exposure [52–56]. Further, the genomes of phylogenetically diverse methanogens are rich in genes encoding oxidative stress enzymes [57]. The literature describes a variety of methods for determining the oxidative stress tolerance of methanogens [52–56, 58]. However, the inability to initiate growth after exposure to O_2 or H_2O_2 is universally applicable and the recommended minimum for describing new species. In the interest of standardization, the following procedures are recommended as a guide. When applicable, it is preferable to investigate cells grown with each substrate which may be dependent on enzymes with different O_2 sensitivities. Washed cells should be suspended in medium without reducing agents and resazurin at the optimal temperature for growth and exposed to O_2 or H_2O_2 over time to determine the interval at which exposed cells are incapable of initiating growth when transferred to standard medium. Cell suspensions should be open to the atmosphere with mild agitation, rather than adding air to closed vessels, to ensure a maximum and consistent exchange of O_2 with time. For media with CO_2-bicarbonate buffers, an alternative pH buffer should be included, such as Tris-Cl or PIPES. As a guide, it is recommended to start with the previously described range of H_2O_2 concentrations [58, 59]. It is recommended to supplement the results by identifying genes encoding proteins for valuable phenotypic traits, including oxidative stress, substrate metabolism, methanogenesis, etc., in the draft or complete genome sequence (see below) [57].

GENOME SEQUENCING AND COMPARISONS

Although phenotypic analysis is important in characterization of novel microbes, genomic methods have now become essential to obtain a clear picture of their taxonomy and phylogeny. At present, most microbial systematics journals require deposition of the genome sequence in a public database for type strains of new species. Advancements in next generation sequencing platforms such as Illumina, Ion Torrent (Thermo Fisher Scientific) and Pacific Biosciences have made whole genome sequencing faster, cheaper and more accessible. Therefore, inclusion of whole genome sequences, as either a draft assembly or a complete, closed genome, is mandatory for description of novel species of methanogens. Recent development of long read sequencing technologies such as those from Pacific Biosciences and Oxford Nanopore can help to facilitate assembly of complete, closed genomes when used in combination with short-read technologies such as Illumina [60, 61] or on their own [62]. The quality of the sequenced genome should be verified using the criteria proposed by Chun *et al.* [63], and statistics such as the genome size, number of contigs and N50 (if a draft genome), and the sequencing depth of coverage should be reported. After annotation, genes such as the 16S rRNA gene and various single-copy conserved markers can be found for phylogenetic inference (see section below), and a variety of additional analyses can be performed, e.g. to determine the presence of genes for different types of methanogenesis pathways [64] and provide genomic evidence of methanogenic capacity. If the new taxon is represented by multiple isolates, it is only necessary to sequence the genome of the type strain

of a proposed novel taxon, although sequencing of additional isolates can be useful. Genome sequence data should be submitted to a public database.

One important use of genome sequence data is its application to overall genome relatedness indices (OGRI), which are of increasing importance in defining and delineating microbial taxa. Although experimental DNA–DNA hybridization (DDH) has long been considered a key criterion for definition of prokaryotic species [65], this can now be supplanted with pairwise whole genome comparisons. Delineation of prokaryotic species based on OGRI methods such as average nucleotide identity (ANI [66]) and digital DDH [67] have been adopted due to their rapidity, reproducibility and accuracy [63], and they can be calculated using online webtools such as http://enve-omics.ce.gatech.edu/ani/ for ANI [68] and http://ggdc.dsmz.de/ggdc.php for digital DDH [67]; see Chun *et al*. [63] for a list of additional tools. OGRI comparisons should ideally be carried out between the new taxon and the type strain of the type species of the genus and other members of the genus to which it belongs, or at least all closely related species based on phylogenetic analysis (see below) that have genome sequences available, where distinct species have ANI of <96% [69] and digital DDH of <70%. If ANI values are exceptionally low (<75%), it is possible to use average amino acid identity (AAI) in its place. While thresholds of <95, <65 and <45% AAI have been proposed as being characteristic of different species, genera and families, respectively [70], other characteristics, such as monophyly and important physiological or ecological properties, should also be considered when forming higher taxa [71, 72], as well as phylogenomic analyses (see below). Before genome sequencing was commonplace, biochemical determination of genomic G+C content was required [13]. Genomic G+C content should be reported, but it is now recommended to calculate G+C content from the sequenced genome.

PHYLOGENETIC ANALYSIS

Although the genomic comparisons described above are essential for delineation of species, phylogenetic methods are also necessary for classification at the genus and higher taxonomic ranks. It is thus mandatory that phylogenies of the 16S rRNA gene be inferred. 16S rRNA gene-based phylogenies have become a mainstay of description of new taxa, allowing for comparison to nearest relatives and potential assessment of the level of novelty as well as environmental rRNA databases for the distribution in nature [21, 47, 71]. In cases where the isolate contains multiple 16S rRNA genes with different sequences, each should be included in the phylogeny and discrepancies should be noted. Considering the declining interest of taxonomists in multilocus sequence analysis based upon only a few genes and accessibility of whole-genome sequencing, phylogenomic studies based on the core genome (translated to proteins) is recommended for phylogenetic analysis.

If genomes of the closest relatives are available, phylogenomics methods are recommended, using a concatenated alignment of multiple conserved, single-copy marker genes. Several methods and software packages for doing this are given in Chun *et al*. [63], some of which are available as online webtools (e.g. https://pitgroup.org/amphoranet/ for use of Amphora2 [73]). Recently, the Genome Taxonomy Database (GTDB) has also been developed, which can allow both for finding single-copy conserved markers and for assignment of phylogenomics-based taxonomy down to the species level using ANI [74]. The GTDB toolkit (GTDBtk [75]) can be run locally or on the KBase webtool (www.kbase.us [76]). If close relatives identified by 16S rRNA gene phylogenies do not have genome sequences available, it is recommended that researchers obtain cultures or isolated DNA from the strains in question for whole genome sequencing so that appropriate comparisons can be done. Any newly sequenced genomes of close relatives should also be included in the OGRI analysis and submitted to a public database as described above.

The *mcrA* gene is a unique functional marker of methanogens that encodes the alpha subunit of methyl-coenzyme M reductase, which catalyses the last step of methanogenesis [77]. Its sequence is highly conserved, and it is possible to obtain portions of the gene by PCR amplification of genomic DNA. As a protein-coding gene, it can contain both synonymous and nonsynonymous mutations, and therefore is typically more variable than the 16S rRNA gene. Hence, it is especially useful for comparing closely related species and strains or as a methanogen-specific probe in complex communities. However, when genomes became widely available, it became clear that many methanogens possessed multiple copies of *mcrA*, including species in the following genera: *Methanoculleus*, *Methanofollis*, *Methanoregula*, *Methanolacinia*, *Methanocorpusculum*, *Methanonatronarchaeum*, *Methanothermus*, *Methanobrevibacter*, *Methanobacterium*, *Methanothermobacter*, *Methanocaldococcus*, *Methanotorris* and *Methanothermococcus*. For at least some genera, the sequence of *mcrA* obtained from PCR amplification did not agree with the sequence from the genome, sometimes possessing less than 94.7% sequence similarity [78]. Presumably, the PCR amplicons were chimaeras with low similarity to the authentic genes. For this reason, the use of *mcrA* as a phylogenetic marker should be approached cautiously, especially in genera known to contain multiple copies. Considering these potential pitfalls, *mcrA* gene phylogenies can be included if desired, but they should supplement phylogenomic and 16S rRNA gene analyses rather than as a stand-alone approach.

CHEMOTAXONOMY

In comparison to the previous standards, the standards proposed here place less of an emphasis on chemotaxonomic methods, especially wet lab-based determination of genomic G+C content and DNA–DNA hybridization, which have largely been supplanted by *in silico* approaches using whole genome sequences. However, whole-cell protein and lipid profiling can be useful for characterization, especially of new higher order taxa (e.g. genera or families). In some cases, organisms show similarity when compared using phylogenetic methods, but when their whole-cell protein pattern and membrane lipid analysis [79] is carried out, significant differences are observed [20]. These methods can be sensitive to small differences in culture conditions or growth stage and need to be applied with care, and comparative analyses need to be performed in the same laboratory in parallel with other taxa. Additionally, lipid analysis in methanogens can be challenging because they are ether-linked isoprenoids and not amenable to the standard techniques used with Bacteria and, in some cases, because of biomass limitations for strains that do not grow to high cell densities. More details on protein and lipid profiling are given below.

Protein profiling of methanogens for chemotaxonomic purposes were mainly performed on whole-cell or ribosomal proteins primarily by gel electrophoresis [80] when the previous standards were published [13]. More recently, methods for whole-cell protein profiling using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) have been developed and applied to microbes, including archaea [81–83]. With the proper equipment, sample preparation, and expertise, MALDI-TOF can be used to cheaply and quickly identify microbes, and this technique has been increasing in popularity, especially in clinical identification of isolates [84]. To apply this technique, the isolated pure culture is cultivated in media until late log or early stationary phase. Conditions and medium for the growth, method of cell harvesting, protein extraction and profiling using MALDI-TOF/MS (Bruker Daltonics) can be adopted as from Shih *et al.* [83]. Proteins should be analysed in automated data acquisition mode using MALDI-TOF/TOF MS (Bruker Daltonics) and spectra should be collected in the linear positive mode with mass-to-charge ratio (m/z) from 2000 to 20000. Extracts from a well-analysed organism such as *Escherichia coli* DH5α can be used as a positive control, and only matrix, without any addition, can be treated as negative control. For comparative protein profiling, all compared taxa should be cultivated using the same conditions and medium.

Methanogenic archaea are characterized by specific membranes composed mainly of a polar head group linked to a glycerol dialkyl diether, such as archaeol phosphatidylglycerol or to glycerol dialkyl glycerol tetraether lipids [85]. The alkyl chains usually have 20 carbon atoms and show an isoprenoid structure. This distinguishes methanogens and other members of the *Archaea* from most *Bacteria* and from *Eukarya*, whose membrane is usually composed of ester-linked fatty acids with variable chain length and a non-isoprenoid structure. The cell membrane lipid inventory is therefore very useful in description of new taxa and, if the same growth conditions (e.g. incubation temperature, pH, salinity) are chosen, also for comparative analyses of different species. According to Mangelsdorf *et al.* [86] and references therein, intact membrane lipids from microbial cultures can be efficiently extracted by a modified Bligh and Dyer [87] method, applying a solvent mixture of methanol–dichloromethane (DCM)–ammonium acetate buffer (10 mM; 2:1:0.8 (v/v)). For phase separation, DCM and buffer are added to achieve a ratio of 1:1:0.9. Subsequently the organic extract is separated by a column chromatography to obtain a polar lipid fraction, which includes the membrane lipids. Intact lipids can be analysed in the extract by high-performance liquid chromatography–electrospray ionization-MS. So far, mainly *Methanobacterium* and *Methanosarcina* strains have been investigated for their membrane polar lipids [88–90]. The major polar lipids for instance in *Methanobacterium movilense* are archaeol phosphatidylethanolamine and diglycosyl archaeol and minor lipids are archaeol phosphatidylinositol and glycosyl archaeol. The membrane of *Methanosarcina soligelidi* as another example is dominated by archaeol phosphatidylglycerol, hydroxyarchaeol phosphatidylglycerol, archaeol phosphatidylethanolamine and hydroxyarchaeol phosphatidylethanolamine.

ENRICHMENT CULTURES AND *CANDIDATUS*

One of the essential criteria stated earlier was culture purity [13], but it is now recognized that the majority of prokaryotes have yet to be grown in pure culture. Methanogens can be challenging to isolate using available media, techniques and equipment, though they do remain active and produce methane in enrichments or co-cultures [91]. In these cases, enriched methanogens can be described using the concept of *Candidatus* [92, 93]. Here we focus on standards for description of *Candidatus* taxa in laboratory enrichment cultures, although this is not a strict requirement for *Candidatus* [19] and recent proposals have been made to stabilize *Candidatus* nomenclature and expand acceptable type material to DNA sequences [94].

The proposed methanogen-specific criteria for *Candidatus* designation are shown in Table 2, and in many ways mirror some of the standards for description of pure cultures (Table 1). The environment from which the enrichment was derived should be described, as well as the sampling methods and enrichment medium/media and conditions. To prove the presence of methanogens in enrichment or mixed cultures, methane production should be confirmed by gas chromatography as described above. Presence of methanogens in the enrichments should be demonstrated to be stable over multiple subcultures, and the abundance of methanogens and identity of other abundant taxa in the enrichments should be determined using 16S rRNA gene tag sequencing or shotgun metagenomics. Taxonomic composition of the enrichment culture as determined by 16S

Table 2. Proposed standards for *Candidatus* taxa in enrichment cultures

ANI, average nucleotide identity; DDH, DNA–DNA hybridization; FISH, fluorescence *in situ* hybridization.

Category	Characteristic	Recommendation
Characterization of enrichment	Description of habitat and sample	Mandatory
	Enrichment medium and conditions	Mandatory
	Documentation of stability of enrichment cultures (recommend different methods or criteria for this)	Mandatory
	Abundance of methanogen in cultures	Mandatory
	Identity of major members of enrichment cultures (metagenomics, 16S rRNA gene tag sequencing)	Mandatory
	Proof of methanogenesis	Mandatory
	Preservation and availability of enrichment culture	Recommended
Morphology	Fluorescence at 420 nm	Mandatory
	FISH with probe specific for putative methanogen	Recommended
Genomics	Genome sequence, checked for completeness/contamination	Mandatory
	Genomic evidence as a methanogen/physiological analysis	Recommended
	Overall genome relatedness index (e.g. ANI, digital DDH)	Mandatory
	G+C content (*in silico*)	Mandatory
Phylogeny	16S rRNA gene-based phylogeny	Mandatory
	McrA gene-based phylogeny	Optional
	Phylogeneomic analysis (multiple conserved markers)	Recommended

rRNA gene tag sequencing or metagenomics may offer clues for further enrichment and isolation strategies. For example, depending on the taxonomy and inferred physiology of other microbes in the enrichment, omitting electron acceptors such as sulphate, sulphur or nitrate from the enrichment medium may help to increase the relative abundance of methanogens. Addition of selected antibiotics that typically do not affect methanogens or excluding some organic nutrients (e.g. yeast extract, peptone, acetate) could also help to reduce the bacterial fraction in the enrichment. It could be useful to develop quantitative PCR primers targeting the methanogen to be isolated, tracking its abundance under different enrichment conditions and facilitating the selection of the most favourable enrichment conditions. Researchers may want to consider various types of isolation strategies such as using different types of solidification agents (agar, noble agar, gellan gum, phytagel), dilution-to-extinction if growth on solid media is not achieved, or novel isolation approaches [95, 96], although use of different methods should be weighed against the time and effort required. In cases where isolation is not achieved, it is nonetheless useful to describe any unsuccessful attempts that were made to further enrich for or obtain the methanogen in pure culture.

The stability of the culture, in the context of presence of methanogens, can be assessed using sequencing-based techniques, or by production of methane (i.e. methanogenesis). It is highly recommended to visualize the methanogen cells using techniques such as epifluorescence microscopy coupled with fluorescence *in situ* hybridization (FISH) using oligonucleotide probes [97] that are specific to the *Candidatus* methanogen being described in order to identify the cell morphology and confirm that the methanogen is actually cultivated. Alternatively, detection of cells with autofluorescence consistent with presence of F_{420} can be performed as described above. It is important to note that, at the time of writing, there is no data on the fluorescence properties of putative methanogen cells belonging to the newly described, uncultivated lineages *Bathyarchaeota* and *Verstraetearchaeota* [10, 11], and to keep in mind that some methanogens may exhibit little or no autofluorescence as described above. If the enrichment culture is available and can be preserved, then information on conditions for preservation of viable cultures and accessibility of preserved cultures should be provided; some microbial resource centres may allow deposition of defined, simple mixed cultures (e.g. co-cultures), but complex or undefined consortia are typically not accepted.

Reconstruction of a high-quality draft or complete genome sequence from an enrichment using metagenomics or single-cell genomics approaches should be mandatory and should follow appropriate standards on the Minimum Information about a Metagenome-Assembled Genome (MIMAG) or Minimum Information about a Single Amplified Genome (MISAG) as appropriate, including the percentage of genome completeness and contamination based on analysis of single-copy marker genes [98]. Phylogenetic analysis of the 16S rRNA gene should be performed; if a 16S rRNA gene is not present, then

phylogeny using single-copy conserved markers should be performed as described above. In particular, the GTDB and associated toolkit may be useful in helping to assign taxonomy at various levels from phylum to species [74]. Based on the results of phylogenetic inference methods, the genomic data should be used to assess taxonomic novelty in comparison to close relatives using OGRI techniques (e.g. ANI, AAI digital DDH) as described above for pure cultures. The genomic data should also be used to provide evidence that the microbe is indeed a methanogen and to predict physiological traits such as methanogenesis pathways [64] and substrates utilized for energy generation and conservation. Detailed information and criteria about designation of *Candidatus* taxa are described in Appendix 11 of the Prokaryotic Code [19].

CONCLUSION

In the past three decades, key concepts and methods have changed in the taxonomy of methanogenic archaea, including the methods for identification and description and technological advancements in DNA sequencing and other areas. The time is ripe to bring these advancements to bear on classification of methanogens. As such, the Subcommittee on Methanogenic Archaea has revised the proposed minimal standards for taxonomic classification during its virtual meetings (23 September 2020 and 8 October 2020) and further online discussion. The revised criteria described above and summarized in Table 1 for pure cultures and Table 2 for *Candidatus* taxa in enrichment cultures should enable practical identification and description of methanogens. Following these criteria will give a more complete profile of the isolate, enabling proper identification and assessment of phylogeny and novelty. Important changes and updates from the previous proposed minimal standards [13] are highlighted below.

(1) Cell morphology documented by electron microscopy is preferred but not mandatory; a good quality light-microscope image clearly depicting the cellular morphology and cell organization is acceptable.
(2) Simultaneous comparison of phenetic and physiological traits using cultures of closely related strains is preferable. However, if pure cultures of the closely related strains are not easily accessible, data for comparison generated under similar set of laboratory conditions as used in past publications is acceptable.
(3) A high-quality draft or complete whole genome sequence, available in a public database, is mandatory for all proposed type strains, and should be used for comparison to close relatives using OGRI, such as ANI and digital DDH.
(4) 16S rRNA gene phylogeny is mandatory; if whole genome data of closest relatives are available, then phylogenomic approaches using multiple single-copy, conserved markers is recommended.
(5) Whole cell protein analysis, membrane-lipid analysis and antimicrobial sensitivity testing are optional.
(6) If obtaining a pure culture of a given methanogen is not practically possible from an enrichment culture, the putative methanogen should be described as a *Candidatus* taxon. A high-quality genome should be generated and deposited in a public database, and additional information about habitat, detection method, phylogeny, and proposed catabolism should be provided.

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Author contributions
O.P. formulated the concept and edited the manuscript. I.S. wrote the first draft of the manuscript. Subsequent editing and writing was performed by J.A.D, X.D., J.G.F., S.L.H., H.I., Y.K., S.K.R., V.S., D.W. and W.B.W. according to their scientific expertise. All the members of the ICSP Subcommittee on Methanogenic Archaea read and approved this manuscript and contributed to the discussion.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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