Prompt: Discuss the challenges involved in defining a microbial species and how HGT complicates matters, especially in the context of the evolution and phylogenetic distribution of microbial metabolic pathways. Can you comment on how HGT influences the maintenance of global biogeochemical cycles through time? Finally, do you think it is necessary to have a clear definition of a microbial species? Why or why not?"

If one was to sequence the genome of every cell derived from a millilitre of seawater or a gram of soil, they might be surprised by the number of different microbes they would find in the sample. However, classification of these microorganisms into separate species remains a controversial issue. Although there are various concepts that have been suggested for this process, microbiologists do not have a consensus on which species definition should be accepted (1, 2). This lack of a robust microbial species definition is in part due the advancements in DNA sequencing techniques and genetics analyses, which have unveiled the complexity and processes involved in prokaryotic variation (3-5). In particular, horizontal gene transfer (HGT) and homologous recombination make it difficult to properly demarcate species (5). In this paper, I will discuss the different ways microbial species are defined and their limitations. I will also consider the roles of horizontal gene transfer in shaping microbial genomes and distributing metabolic pathways, while highlighting the fact that this process further complicates classification of different microbial lineages.

Limitations of current approaches to microbial species definition

Today's gold standard for species delineation is inadequate as it uses arbitrary measurements. The current method for defining prokaryotic species is based on the phylo-phenetic species concept by Rossello´-Mora and Amann (6). It defines microbial species as, "monophyletic and genomically coherent cluster of individual organisms that show a high degree of overall similarity with respect to many independent characteristics, and is diagnosable by a discriminative phenotypic property" (6). Firstly, it uses a genetic marker such as the 16S rRNA gene to initially classify organisms into a taxa (i.e. family or genus level) (1, 7). Followed by DNA:DNA hybridization, organisms belonging to the same species are identified based on 70% genomic similarity set as the threshold (6). Then, phenetic measures are used to differentiate the microbial species in question from existing identified species (8). Although both genomic information and phenotype are examined, the use of empirical parameters (genetic markers for phylogeny, percentage of DNA:DNA hybridization, and phenotypic properties) at each step of

identifying phylo-phenetic microbial species is problematic as there are no theoretical evidences that prove these measures are correct (1). The 70% similarity in DNA:DNA hybridization and the 97% cut-off for 16S rRNA gene similarity are arbitrary numbers and were only adapted based on mounting previous work and experimental results (8). Hence, the phylo-phenetic species method may not be sufficient to guarantee microbial species identity.

Other current prokaryote species concepts listed in a review by Ereshefsky also highlight several points that make it even more challenging to choose a single method for species definition (1). For instance, the biological species concept states that "species are groups of interbreeding natural populations that are reproductively isolated from other such groups" (9). This traditional concept, however, does not appropriately apply to prokaryotes as they do not sexually reproduce. Instead, they use binary fission or vegetative means (1). As one may assume, a parent microbial species should then be genetically identical to its daughter cells, and yet this is not the case (10). Through horizontal gene transfer, prokaryotes can move genetic information between each other, creating chimeric genomes (11). Microbes can exchange homologous and non-homologous genes that recombine with its genome (4). HGT can occur via transformation (genetic material absorbed by a host), conjugation (cell to cell transfer), and/or transduction (viral transfer) (12). Thus, in the microbial world, evaluating what it means to be a species is more certainly not as absolute as the sexually reproducing (biological) species definition. It is instead much more fluid as it involves the movement and transfer of genetic material from one organism to another. Unfortunately, the current approach for defining species is relatively inadequate for detecting genetic rearrangement, gene amplification, mutation, and exchange of genetic material from different lineages (6, 8).

Another proposed prokaryote species concept by Cohan defines a species as follows: ''a species in the bacterial world may be understood as an evolutionary lineage bound by ecotype-periodic selection" (13). This suggests that, in defining microbial species, one must consider where a prokaryote lives or is found, as well as the adaptations it has to specific environments. According to Ereshefsky, this ecological approach for delineating species assumes that natural selection is the main process responsible for maintaining species as "a cohesive group" and eliminating those that are niche-specific (1). However, it is possible that selection and

recombination occur on the same group of microbes, and thereby resulting in cross-classification of those microorganisms (1). Evidently, a study by Nesbø *et al.* shows that in the genus *Thermotoga*, some groups of microorganisms form single species based on the recombination approach but several species are classified using the ecological approach (11). Thus, this further complicates defining microbial species because this empirical evidence suggests that one group of prokaryotes can belong to two different species, though species of different types (1).

HGT transforms microbial genomes

As previously established, the acquisition of genomic material and/or extrachromosomal elements are possible via horizontal gene transfer. Consequently, it can lead to misclassification or misidentification of prokaryotes because of its role in re-shaping microbial genomes (6, 14). With HGT, prokaryotic genomes have been reported to possibly be subjected to homologous recombination with related species that are up to 25% different in the sequences of homologous genes (6). Microbes can also receive and express new genes on plasmids from extremely divergent sources (6). Thus, HGT can link genomes from distant lineages or even introduce new genes, making it difficult to dissect and analyze parts of the organisms' genomes, especially in terms of determining their evolutionary histories. Furthermore, it is important to note that not all genes transferred via HGT occur with equal probability (15). For example, it has been observed that, compared to operational genes (needed for housekeeping), informational genes (involved in transcription, translation, and other related processes) are rarely transferred (15). As this finding may question the stability of microbial genome, an important consideration on the extent of gene transfer among prokaryotes is thereby necessary to improve our current approaches for species assignment.

HGT spreads metabolic functions

While HGT can influence and expand microbial genomes, this process also contributes to the distribution of microbial metabolic functions. Through HGT, it is possible that operons encoding parts of, or the entire metabolic pathway, may be spread to closely or distantly related organisms (14). Taking the nitrogen fixation pathway as an example, there is evidence suggesting that HGT played a role in spreading nitrogen fixation genes within the microbial world (14). A phylogenetic analysis of different nitrogen fixation proteins (NifHDKEN) has

shown that a group of bacteria, such as green sulphur bacteria, *δ-Proteobacteria* and *Chloroflexi*, cluster with *Methanosarcina* (*Euryarchaea*). Likewise, some *Firmicutes* (mainly *Clostridium* species) also cluster as a sister clade with the euryarchaeote *Methanoregula boonei* (14) This highlights the fact that different microbial lineages can acquire metabolic genes from one another (14).

Furthermore, even if a group of microbes containing genes of a certain metabolic pathway were eliminated by natural selection, supposing those genes were initially transferred to another microorganism, then the metabolic processes would be maintained through evolutionary time (14). Consequently, if microbial species are defined by metabolic repertoires encoded in their genome, the spread of metabolic functions via HGT could be a limitation to single out a microbe and call it a species. As an example, in a study by Welch *et al.*, three different *Escherichia coli* strains (CFT073, EDL933 and MG1655) which were categorized as one species, were found to only have 39% similarity with respect to its functional repertoire (16). In addition, sometimes metabolic pathways are not only within an organism; it could be within a population or even extend to a community level. Thus, the distribution of metabolic functions through evolutionary time and among different microbial groups make it more problematic to define species boundaries in prokaryotes.

Conclusion

While there are various species concepts available in microbiology, each of them has limitations. In addition, many microbiologists are challenged by the importance and influential role of horizontal gene transfer in microbial genome variability and divergence of metabolic functions, which all together complicates the issue of defining microbial species. For one thing, perhaps in order to fully capture genomic differences when defining species, whole genome analysis should be the basis for future microbial species classification. Nonetheless, having a clear definition of a microbial species is vital. It increases our understanding of microbial structures and communities, as well as surveying disease outbreaks (17). Most importantly, the convenience for communicating microbiology is undeniable and thus, defining microbes into species should continue.

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