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Functional characterization of prokaryotic dark matter: the road so far and what lies ahead

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ABSTRACT

Eight-hundred thousand to one trillion prokaryotic species may inhabit our planet. Yet, fewer than two-hundred thousand prokaryotic species have been described. This uncharted fraction of microbial diversity, and its undisclosed coding potential, is known as the “microbial dark matter” (MDM). Next-generation sequencing has allowed to collect a massive amount of genome sequence data, leading to unprecedented advances in the field of genomics. Still, harnessing new functional information from the genomes of uncultured prokaryotes is often limited by standard classification methods. These methods often rely on sequence similarity searches against reference genomes from cultured species. This hinders the discovery of unique genetic elements that are missing from the cultivated realm. It also contributes to the accumulation of prokaryotic gene products of unknown function among public sequence data repositories, highlighting the need for new approaches for sequencing data analysis and classification. Increasing evidence indicates that these proteins of unknown function might be a treasure trove of biotechnological potential. Here, we outline the challenges, opportunities, and the potential hidden within the functional dark matter (FDM) of prokaryotes. We also discuss the pitfalls surrounding molecular and computational approaches currently used to probe these uncharted waters, and discuss future opportunities for research and applications.

1. Introduction

Ever since the dawn of life ~3.5 billion years ago (Allwood et al., 2006; Blaser et al., 2016), the Earth’s environmental, geochemical, and biological systems of all levels of complexity have relied on microorganisms (McFall-Ngai et al., 2013). Microorganisms are the most abundant, ubiquitous, functionally and metabolically diverse forms of life (Locey and Lennon, 2016); they are responsible for a substantial part of our planet’s biomass (Solden et al., 2016), and an overwhelming fraction of its biodiversity (Locey and Lennon, 2016; Solden et al., 2016). Previous studies estimated that approximately four-hundred thousand bacterial and archaeal species exist (Yarza et al., 2014). More recent estimates range from eight-hundred thousand (Louca et al., 2019), to over one trillion (10^{12}) (Locey and Lennon, 2016). The actual number, however, is still a subject of debate (Louca et al., 2019; Pedrós-Alió and Manrubia, 2016; Willis, 2016; Zhang et al., 2020). Nonetheless, only a very small fraction of all prokaryotic species has been described to this day (approximately two-hundred thousand complete and draft genomes (Zhang et al., 2020)), thus exposing our ignorance regarding their diversity. Roughly 85-99% of prokaryotic taxa are unamenable to axenic culture (Lok, 2015), which precludes the *in vitro* characterization of such species. Yet, it has been known for decades that most microorganisms cannot be cultured (Jannasch and Jones, 1959; Jones, 1970; Rappé and Giovannoni, 2003). In 1985, Norman Pace’s group showed that the diversity of uncultured prokaryotes could be probed by molecular biology methods (Olsen et al., 1986), namely those relying on the 16S ribosomal RNA (16S rRNA) as a marker (Woese, 1987). Microbiology has benefited tremendously from further advances in this field, which became collectively known as “molecular microbial ecology” (Pace, 1995).

Next-generation sequencing (NGS) brought a breadth of new insights into microbial genomics. Through the lens of culture-independent methods like metagenomics, NGS has allowed to investigate the diversity and coding potential of microbial communities in the context of

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their environment (New and Brito, 2020). Concurrent advances in bioinformatics gave birth to metagenome-assembled genomes (MAGs) (Kayani et al., 2021), which are crucial for broadening our knowledge of the ecology, metabolism, and coding potential of uncultured prokaryotes (Sangwan et al., 2016). Third-generation sequencing (TGS) technologies (i.e., Pacific Biosciences and Oxford Nanopore Technologies) currently allow to gather massive amounts of data with unprecedented detail (van Dijk et al., 2018), and at low cost (Karlsson et al., 2015). The long reads produced by TGS, together with ever-improving *de novo* assembly algorithms (Dida and Yi, 2021), have streamlined (meta)genome sequencing and assembly endeavors (Athanasopoulou et al., 2021). These developments already allow to attain complete, closed, *de novo*-assembled prokaryotic genomes and MAGs (Loman et al., 2015; Moss et al., 2020; Somerville et al., 2019). Another approach, named single-cell genomics (SCG), relies on amplifying and sequencing the genome of individual cells isolated from environmental samples, instead of bulk sequencing the entire community (Solden et al., 2016), and it has provided numerous insights into the metabolism and evolutionary context of many uncultured groups of Archaea and Bacteria (Santoro et al., 2019). A different method that combines high-throughput culturing with MALDI-TOF mass spectrometry and 16S rRNA sequencing (i.e., culturomics), has allowed to isolate hundreds of new prokaryotic species (Lagier et al., 2018), and to decode their complete genome sequences thereafter. As a result of these advances, a modest part of the microbial "black box" was unveiled over the past decades. Together with continuous improvements in bioinformatics, it has been possible to discover new functions and metabolic features that have: (i) bolstered natural product discovery (Bull and Goodfellow, 2019; Chen et al., 2019; Goodfellow et al., 2018; Lackner et al., 2017; Ling et al., 2015b; Owen et al., 2015; Rust et al., 2020; Ziemert et al., 2016), (ii) challenged preconceived boundaries among the three domains of life (Hug et al., 2016; Parks et al., 2017; Rinke et al., 2013), and (iii) reshaped our understanding of microbial life forms (Brown et al., 2015; Nasir et al., 2015; Wiegand et al., 2020).

The unexploited fraction of microbial diversity, along with its functional and metabolic potential, is commonly referred to as the microbial dark matter (MDM). This term was coined by Marcy and colleagues in 2007 (Marcy et al., 2007), alluding to the large amount of unknown microbial taxa and respective genomes inferred by culture-independent approaches. Although most authors use the term interchangeably to refer to the taxonomic tapestry and the coding potential of MDM, we reckon that either concept should be employed separately. There are four categories of sequence novelty based on annotation (Bernard et al., 2018)Fig. 1(A): (i) sequences with known taxonomic provenance and molecular function (e.g., a hydrolase gene from *Escherichia coli*); (ii) sequences with known taxonomic provenance but unknown molecular function (e.g., an *E. coli* gene without ascribed function); (iii) sequences with unknown taxonomic provenance but known molecular function (e.g., a hydrolase gene from an unknown prokaryote); and (iv) sequences with unknown taxonomic provenance and unknown molecular function (e.g., a gene with no ascribed function from an unknown prokaryote, i.e., the true dark matter). Abiding by the rationale set forth by Bernard et al. (Bernard et al., 2018), we propose the classification of microbial genomic sequences with unknown taxonomic provenance as "taxonomic dark matter" (TDM), regardless of their functional annotation; and the classification of sequences with unknown molecular function as "functional dark matter" (FDM), regardless of their taxonomic context. Throughout this review we use the terms "microbial" and "microorganisms" to refer to prokaryotes only, comprising the Archaea and the Bacteria domains.

Researchers frequently use phylogenetic-driven techniques and canonical molecular markers (e.g., 16S rRNA (Yarza et al., 2014)) to study MDM through targeted community profiling. Still, the coding potential of these bacterial communities often remains inaccessible. Harnessing genomic information from these microorganisms is often limited by gene annotation methods that rely on sequence similarity to proteins characterized from microbial cultures (Michalska et al., 2015). This approach makes it difficult to study the FDM and identify unique functions particular to uncultured organisms (Michalska et al., 2015). Adding to these shortcomings, the genomes of these elusive microorganisms typically diverge from those of well-known species (Grötzinger et al., 2018; Miller et al., 2016; Sysoev et al., 2021). Notwithstanding, several remarkable studies have explored the phylogenetic novelty hidden in the TDM, offering insights into the putative functions it encodes (Gies et al., 2014; Lackner et al., 2017; Makarova et al., 2014; McLean et al., 2013; Mehrshad et al., 2017; Rinke et al., 2013; Wegner and Liesack, 2017). Some reports even predict the existence of community-wide metabolic profiles (Anantharaman et al., 2016; Hawley et al., 2017; Momper et al., 2017; Nobu et al., 2015; Thrash et al., 2017).

Equally impressive is the diversity of secondary metabolites and enzymes produced by microorganisms, well-known mediators of fundamental biogeochemical cycles (Blaser et al., 2016; Rust et al., 2020). Insights gained from microbiome research and innovation (Małyska et al., 2019) allow not only to discover new compounds and metabolic pathways with potential applications in agronomic, biotechnological, environmental, and pharmaceutical industries (Blaser et al., 2016; Bull and Goodfellow, 2019; Chen et al., 2019; Goodfellow et al., 2018; Rust et al., 2020); as they may also promote new biotic solutions to overcome pressing societal challenges (United Nations, Department of Economic and Social Affairs, 2015). Bringing this knowledge to light is critical, as the key to solve some of the current concerns may lie on novel metabolite-producing gene products and enzymes of biotechnological value, such as those hidden in the FDM (Danso et al., 2018; Ling et al., 2015b; Rashid and Stingl, 2015; Sysoev et al., 2021; Yoshida et al., 2016; Zrimec et al., 2021).

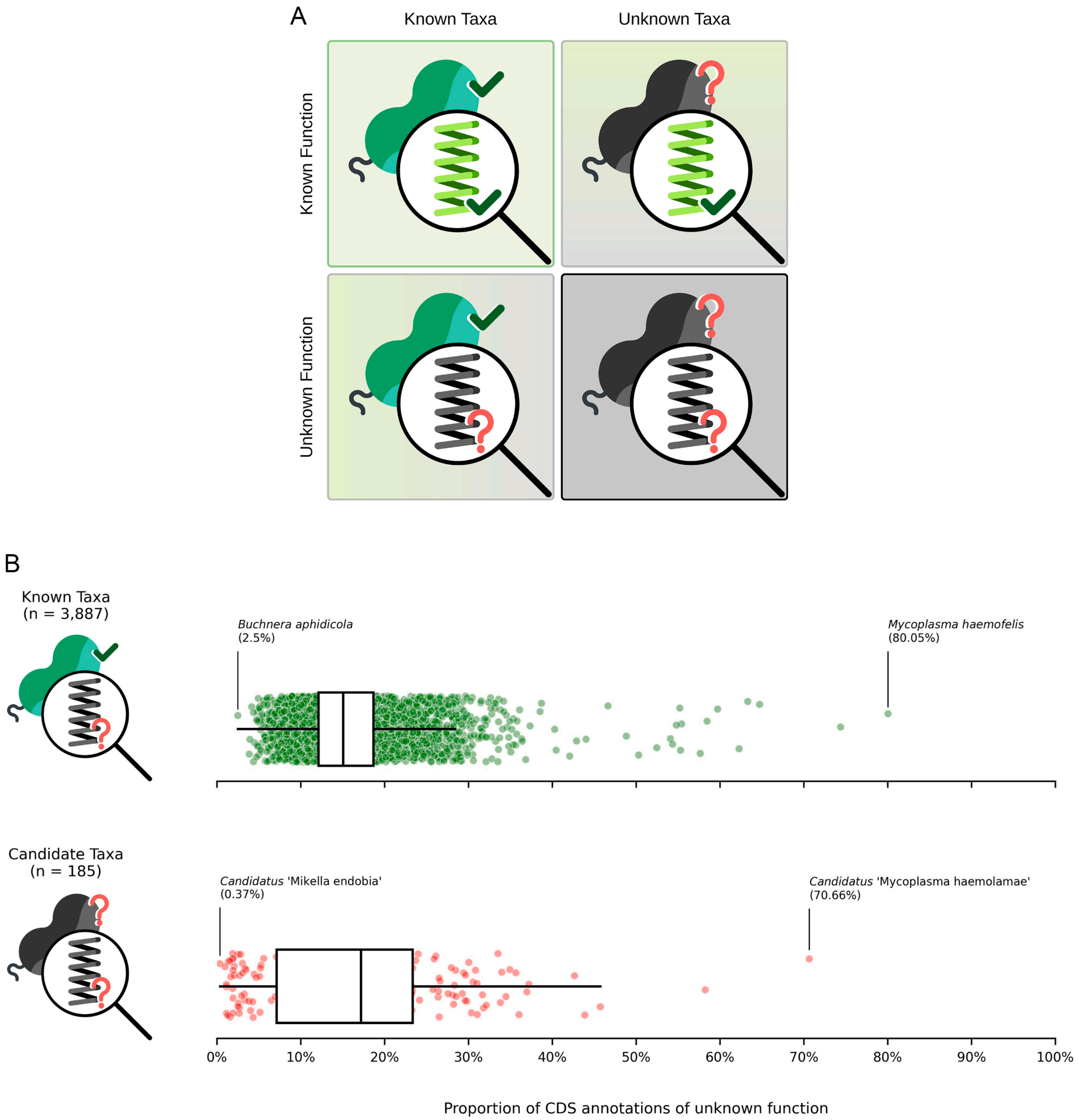
Here, we outline the challenges, opportunities, and unique potential of the hidden world of FDM. We also discuss some limitations of molecular and computational approaches that currently preclude the comprehensive characterization of the MDM. Finally, we offer a perspective on the potential opportunities and directions for future research.

2. Revealing the hidden potential of functional dark matter

Until the turn of the century, genome sequencing of microorganisms depended on their culturability. Researchers often had to isolate the microorganisms from an environmental sample and cultivate them in order to sequence their genome. The progress made in culture-independent DNA sequencing approaches, coupled to NGS technologies, and that of annotation pipelines, has improved our awareness of elusive microorganisms, and our understanding of the genomic content they encode. However, the functions this genomic content codes for are frequently unknown. In this section we will overview studies that aimed to quantify and characterize the FDM, and those that tried to exploit the functional richness it encloses, in order to unearth new knowledge with biotechnological implications.

2.1. Current estimates

The fraction of FDM genes ranges substantially among different prokaryotic genomes. Some authors estimate that the functions of ~35% of genes from a given genome remain a mystery (Piao et al., 2014). In other cases, this fraction is thought to amount to as much as 50%, like in newly sequenced genomes (Al-Shahib et al., 2007). Other authors report that, in genomes from uncultured candidate taxa, these proportions range from 46% to 60% (Becraft et al., 2015; Garza and Dutilh, 2015; Marcy et al., 2007; McLean et al., 2013). Makarova et al. disclosed that archaeal genomes encode from 30% to 80% of FDM (Makarova et al., 2019). In comparison to Bacteria, this represents a greater content of FDM harbored by archaeal genomes (Makarova et al., 2019). These occurrences were attributed to the difficulty in isolating and cultivating most Archaea, which in turn hinders the experimental characterization



(caption on next page)

Fig. 1. (**A**) Four possible combinations when addressing a protein regarding its taxonomic provenance and molecular function. Both rows and columns depict a binary range: known and unknown. The rows refer to the molecular function of the protein, and the columns refer to its taxonomic provenance. Each quadrant represents one combination that results from the intersection of the rows and columns. The bean-shape represents a prokaryotic cell and the coil-shape represents a protein. A green foreground with a green checkmark represents "known", and a grey foreground with a red question mark represents "unknown". Adapted from Bernard et al. (2018). (**B**) Proportion of CDS annotations of unknown function per genome, for "Known Taxa" (top boxplot, green data-points) and "Candidate Taxa" (bottom boxplot, red data-points). Each data-point corresponds to a genome. To calculate these percentages, we proceeded as follows. We gathered NCBI's assembly accessions for all complete genomes of Archaea and Bacteria that were either of reference or representatives, from `ftp.ncbi.nlm.nih.gov/genomes/refseq/archaea/assembly_summary.txt` and `ftp.ncbi.nlm.nih.gov/genomes/refseq/bacteria/assembly_summary.txt`, on the 8th of June 2022. This amounted to 239 Archaeal and 3,833 Bacterial genomes (n = 4,072). We subset these 4,072 genomes into the "Known Taxa" and "Candidate Taxa" categories according to their taxonomic description. A genome was placed in the "Candidate Taxa" category if its taxonomic description matched any of the following words: 'uncharacterized', 'unclassified', 'unidentified', 'endosymbiont', 'uncultured', 'metagenome', 'candidatus', 'candidate', or 'unnamed'. We downloaded the annotation file (i.e., `*_feature_table.txt.gz`) for each assembly accession, from the corresponding FTP server directory listed in the `assembly_summary.txt` files. We gathered the total number of CDS annotations for each genome from these annotation files. Each CDS annotation was matched against any of the following words: 'hypothetical', 'predicted', 'putative', 'uncharacterized', 'unknown function', or 'unnamed protein'. For each genome, the percentage of CDS' of unknown function corresponds to the number of CDS annotations that matched these words, divided by the total number of CDS annotations. For each of the "Candidate Taxa" and "Known Taxa" categories, we annotated the data-point whose corresponding genome had the highest percentage of FDM (and therefore, the lowest percentage of known function); and the data-point whose corresponding genome had the lowest percentage of FDM (and therefore, the highest percentage of known function). The icons used in this figure were retrieved from `flaticon.com`.

of their genes (Makarova et al., 2019). The same authors also accounted that in most of the Archaea, the amount of FDM scales linearly with the genome size (Makarova et al., 2019).

More recently, Lobb et al. reported an extreme variation in the proportion of genome annotation incompleteness across distinct bacterial species, according to different annotation tools (Lobb et al., 2020). These authors highlight that certain lineages issuing from the TDM (e.g., Patescibacteria), possessed greater content of FDM genes (Lobb et al., 2020). Moreover, they disclose that the proportion of FDM genes per genome can range from as little as 2.3%, to as high as 87.9% (Lobb et al., 2020). Akin to the results reported by Lobb et al. (2020), we also observe a wide range in the proportion of coding sequences (CDS') with unknown function for complete prokaryotic genomes from NCBI (Fig. 1B). Interestingly, *Mycoplasma haemofelis*, a known feline pathogen (Barker et al., 2011), lacks functional annotation for 80.05% of its CDS'; whereas this percentage is just 0.37% for *Candidatus* 'Mikella endobia' (Fig. 1B).

Nevertheless, in order to find FDM genes one does not need to venture into the uncultivated myriad of microorganisms, nor that of candidate taxa. Indeed, in 2016, a minimal synthetic bacterial genome based on that from *Mycoplasma mycoides*, a well-studied mammalian parasite, was generated (Hutchison et al., 2016). This synthetic genome contained only 473 genes, of which 149 had unknown functions. Yet, each of those genes was considered essential, as deletion of any of them was lethal (Hutchison et al., 2016). Therefore, even in a controlled laboratory environment, the function of nearly one-third of essential genes is unknown.

In more extreme cases, as indicated by metagenomic studies, these percentages can span from 85% (Lobb et al., 2015) to 99% of total gene content (Dutilh, 2014; Mokili et al., 2012). The reference gene catalog that stemmed from the Tara Oceans initiative, comprised more than 40 million non-redundant sequences from ocean metagenomes (Sunagawa et al., 2015). After mapping these sequences to clusters of orthologous groups, the authors of this study outlined that 40% of these groups were of unknown function (Sunagawa et al., 2015). Focusing on the human gut microbiome, Almeida et al. created the "Unified Human Gastrointestinal Genome" (UHGG), and the "Unified Human Gastrointestinal Protein" (UHGP) catalogs (Almeida et al., 2021). The UHGP contains more than 170 million protein sequences, however 40% of these could not be functionally annotated (Almeida et al., 2021). After exploring the core and accessory gene repertoires for each of the UHGG species, they report that the accessory cohort showed a greater proportion of genes of unknown function, and that ~21% of these genes failed to attain a match to any of the reference databases used in their study (Almeida et al., 2021). Other initiatives that aimed to broaden the phylogenetic coverage of prokaryotic genomes also report high percentages of FDM within their genomic catalogs (Mukherjee et al., 2017; Nayfach et al., 2021; Wu et al., 2009) (see subsection 3.4).

Nonetheless, researchers have striven to push the percentage of annotations for predicted proteins for a long time (Marcotte et al., 1999a; 1999b). Recently, Bileschi et al. developed a tool that, by learning known patterns of evolutionary substitutions among protein families, has allowed to extend the coverage of Pfam (Mistry et al., 2021) by more than 9.5% (Bileschi et al., 2022). Another study uncovered and characterized more than four-hundred thousand new protein families whose function was previously unknown from a global genomic dataset (Rodríguez del Río et al., 2022). The authors from this study report that these new protein families increase the total number of current prokaryotic orthologous groups by three-fold (Rodríguez del Río et al., 2022).

Fig. 2 shows the evolution over time of the number of protein sequences from NCBI's Protein database that belong to the Archaea and Bacteria FDM. Note that both the number of FDM sequences and the total number of sequences tend to increase over time, regardless of domain. As of 2020, the percentage of FDM sequences in NCBI's protein database is greater than 30% for Archaea, and nearly 20% for Bacteria. Since the commercialization of next-generation sequencing (NGS; i.e., 2005, see (van Dijk et al., 2014)), these percentages have been steady for Archaea, but decreased in the long run for Bacteria. We speculate that this decrease is a product of the representativeness of the total number of sequences from Bacteria in public databases; and consequently, the improvement and fine-tuning of annotation pipelines aimed particularly at this domain.

2.2. *Possible functions*

Prokaryotic genes of unknown function were initially considered to be mere "junk" elements, pseudogenes, or misannotations (Andersson and Andersson, 2001; Mira, 2002; Schmid and Aquadro, 2001) owing to the narrow understanding at the time of the functional sequence space (Lobb et al., 2015). But increasing evidence indicates that yet-unclassified elements do encode for specific functions (Hu et al., 2009). For instance, Hanson et al. showed that nearly 15% of *E. coli* enzymes of unknown function could play roles in metabolite repair (Hanson et al., 2016). Another role thought to be played by these elements is the addition and removal of posttranslational modifications (PTMs) (Ellens et al., 2017). In this respect, it should be emphasized that the enzymes responsible for recently discovered PTMs usually remain unidentified (Choudhary et al., 2014; Ellens et al., 2017). Likewise, one can speculate that some uncharacterized proteins may have been enzymes that lost their catalytic properties throughout evolution, and then acquired allosteric regulation functions thereafter (Ellens et al., 2017; Van Schaftingen et al., 2015).

Other proteins, commonly referred to as "moonlighting", play multiple biochemical and/or biophysical roles which are not associated with

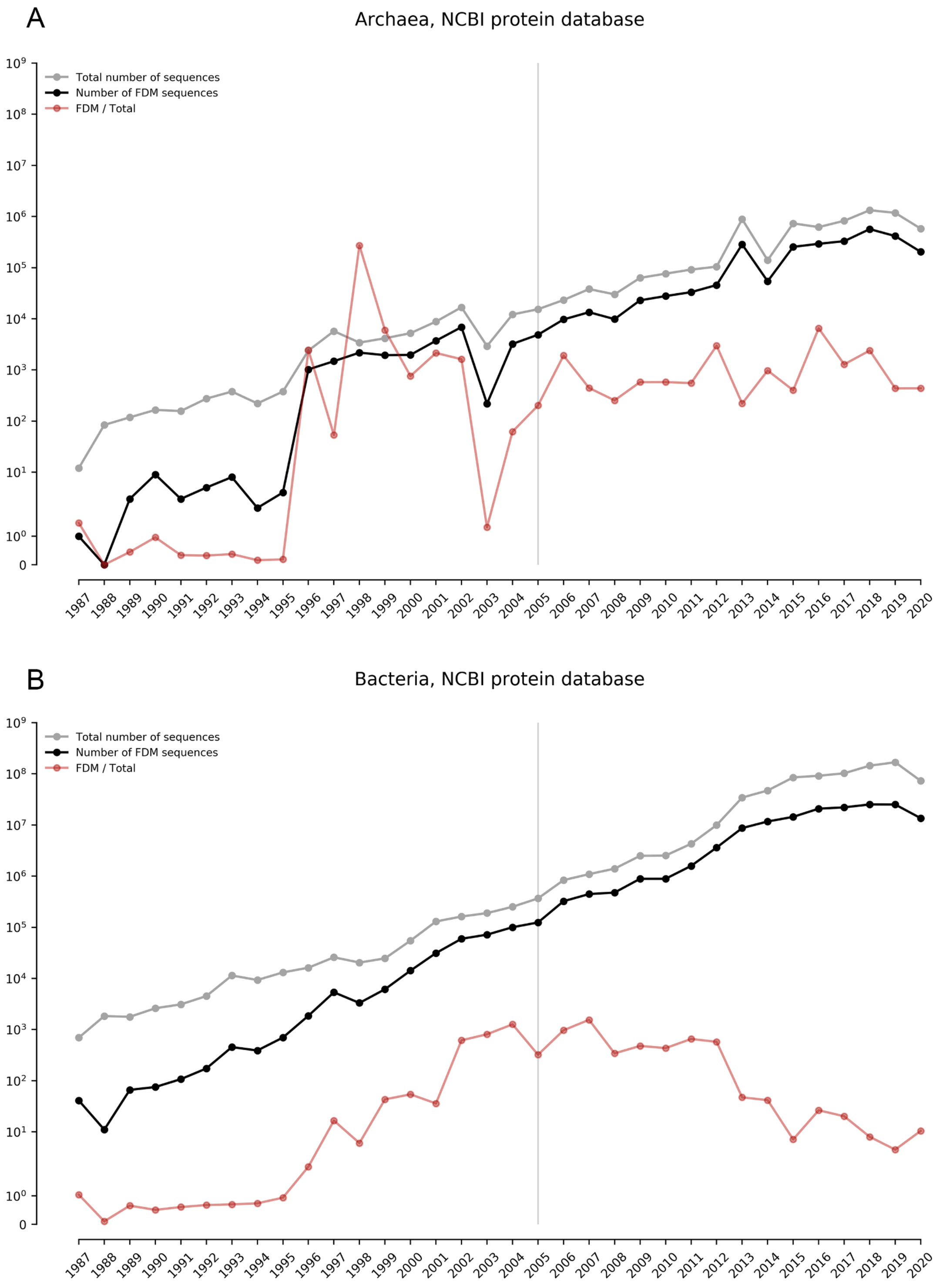


Fig. 2. Number of protein sequences of unknown function in comparison to the total number of sequences in NCBI's protein database. These counts refer to sequences in the Archaea (**A**) and Bacteria (**B**) domains from 1987 to 2020, inclusively. The x-axis represents the date of submission, the y-axis to the left represents the number of sequences in "symlog" scale (i.e., linear from 0 to 1, and logarithmic from 1 upwards), and the y-axis to the right represents the ratio between the number of FDM sequences and the total number of sequences. The light-grey vertical line placed in 2005 marks the commercial availability of the first NGS platform (the pyrosequencing method by 454 Life Sciences, now Roche). In order to obtain these counts we submitted queries to NCBI's Esearch utility at Entrez (`eutils.ncbi.nlm.nih.gov/entrez/eutils/esearch.fcgi?`). For each year and for each domain, we retrieved the total number of sequences with the following query: `db=protein&term=YEAR[pdat]+AND+DOMAIN[orgn]&rettype=count`; whereas as to gather the number of sequences of unknown function we used: `db=protein&term=YEAR[pdat]+AND+DOMAIN[orgn]+AND+(hypothetical[title]+OR+predicted[title]+OR+putative[title]+OR+uncharacterized[title]+OR+unknown+function[title])&rettype=count`.

gene fusion events nor proteolytic fragments (Jeffery, 2018). Moonlighting proteins are thought to include a few hundred members and play an extensive range of functions (Jeffery, 2018; Mani et al., 2015). We posit that the moonlighting phenomenon might explain why some proteins are left uncharacterized in the first place, given the added difficulty in classifying a protein with multiple molecular functions. Proteins of unknown function have also been presumed to encode for ecological or taxon-specific functions (Wilson et al., 2005), including morphological and developmental adaptations (Kaessmann, 2010; Lobb et al., 2015; Tautz and Domazet-Lošo, 2011), which could explain their lack of homology to annotated genes (Lobb et al., 2015). However, the latter can also be a consequence of the severe undersampling of Earth's microbiomes, despite the advances in metagenomics in the past decades.

Alternatively, some of these uncharacterized proteins are thought to be gene fragments, or pseudogenes arising naturally through gene degeneration (Makarova et al., 2019). Other studies hypothesized that sequences of unknown function can actually be of viral, integrative, or mobilomic origin (Cortez et al., 2009; Yin and Fischer, 2006). Dutilh et al. provided additional support for this possibility (Dutilh et al., 2014). This idea makes sense if one considers the fast mutation rates observed in viral DNA (and RNA), and the underrepresentation of sequences from viruses in most public databases (Lobb et al., 2015). Indeed, there is evidence that these unknown elements might be genes that evolve fast, like those associated with anti-parasite defense (Makarova et al., 2014), and those that encode small proteins (Makarova et al., 2019). As an illustration, Sberro et al. reported more than four thousand small (⩽ 50 amino acids in length) protein families, from human-associated metagenomes (Sberro et al., 2019). They describe an abundance of putative functions, namely: housekeeping, mammalian-specific, cell-cell crosstalk, adaptation, anti-parasite defense, secreted or transmembrane proteins, in addition to possible products of horizontal gene transfer (HGT) (Sberro et al., 2019). Despite this breakthrough, they outline that over 90% of these families lack a domain assignment, and nearly half of them are absent from reference genomes (Sberro et al., 2019).

2.3. Biotechnological significance

FDM sequences might encode any potential function waiting to be charted in the microbial sequence space. Some of these sequences may even code for new metabolite-producing proteins, and/or enzymes of biotechnological interest (Bernard et al., 2018; Chen et al., 2019; Rashid and Stingl, 2015; Sysoev et al., 2021). The FDM (and the MDM in a broader sense) could indeed be an outstanding asset for the discovery of novel biotechnological solutions in a world of ever-increasing societal demands. Pascoal et al. argued that bioremediation and bioprospecting are the two areas holding the most promise from within the MDM, where innovative approaches in biotechnology might arise (Pascoal et al., 2020), like new solutions for the decontamination of environments (Dvořák et al., 2017).

A prominent prospect of the latter is that of bioremediating ecosystems polluted with plastic (Danso et al., 2019). For instance, polyethylene terephthalate (PET) is reported to be the most abundant polyester plastic (Tournier et al., 2020), being mainly used in the textile and packaging industries (Danso et al., 2019; Tournier et al., 2020). Nearly 70 million tons of PET are manufactured worldwide per year (Tournier et al., 2020). The common recycling process of PET is through thermomechanical means, resulting in its loss of mechanical properties (Ragaert et al., 2017). As such, PET is preferentially synthesized *de novo*, and its waste continues to accumulate in ecosystems throughout the globe (Tournier et al., 2020; Yoshida et al., 2016). In 2016, a research team screened natural microbial communities at a PET bottle recycling site and managed to isolate a new bacterium named *Ideonella sakaiensis* (strain 201-F6) (Yoshida et al., 2016). Characterization of *I. sakaiensis* revealed that it could use PET as a primary energy and carbon source (Yoshida et al., 2016). After assembling the draft genome sequence of *I. sakaiensis*, the team identified an ORF that putatively encoded a hydrolase (Yoshida et al., 2016). Upon recombinant expression of this protein, the team observed that it exhibited PET-hydrolytic activity, thus naming it PET hydrolase (PETase) (Yoshida et al., 2016). PETase catalyzes the hydrolysis of PET into its monomeric component mono-2-hydroxyethyl terephthalate (MHET) (Yoshida et al., 2016). The genome of *I. sakaiensis* also coded for another enzyme that was capable of degrading MHET, which was designated MHET hydrolase (MHETase) (Danso et al., 2019; Yoshida et al., 2016). MHETase hydrolyzes MHET into its two monomers, terephthalic acid (TPA) and ethylene glycol (EG) (Yoshida et al., 2016), which are used by *I. sakaiensis* in its metabolism (Danso et al., 2019). Moreover, TPA and EG can potentially serve as novel substrates to be converted into value-added products (Franden et al., 2018). EG, for instance, can be used for numerous applications, such as a coolant in antifreeze (Franden et al., 2018). It is worth noting that even though multiple studies have described enzymes that can degrade PET, the connection of extracellular enzymatic PET degradation to catabolism in a single microbe was hitherto unheard of (Austin et al., 2018). Additionally, the reported crystal structure resolution of MHETase is likely to possess a scaffold that is unprecedented for plastic-degrading enzymes (Palm et al., 2019). This example shows that bioprospection of the MDM offers a promising source for the identification of pollutant-degrading enzymes that could be used for bioremediation (Danso et al., 2019).

Another relevant example of the biotechnological potential of the MDM is that of the isolation chip (Ichip) (Nichols et al., 2010). In 2010, the Ichip was developed for the *in situ* cultivation of microbes that had eluded previous standard culture efforts (Berdy et al., 2017; Nichols et al., 2010). The Ichip comprises several hundreds of miniaturized chambers, and each chamber harbors one or few cells from a given environmental sample. Each chip harbors cells from a single environment. Chamber incubation is carried out in the environment from where the cells were taken, allowing growth factors and other molecules to diffuse throughout the semipermeable membranes covering the chambers, thus facilitating growth and increasing the recovery of uncultured microbes (Rashid and Stingl, 2015). The discovery of the antibiotic Teixobactin (Ling et al., 2015b) not only demonstrates the efficacy of the Ichip, but also highlights the hidden potential of the MDM.

2.4. Catalytic prospectives

Enzymes are the backbone of numerous industries (Bruno et al., 2019; Cabrera and Blamey, 2018; Gurung et al., 2013; Li et al., 2012; Meghwanshi et al., 2020; Ramesh et al., 2020; Robinson, 2015; Singh et al., 2016; Verma et al., 2021). Reactions catalyzed by enzymes are thought to follow the rules of green chemistry-they are safer, faster, and generate less waste than traditional methods (Sysoev et al., 2021). The unmatched eco-friendly potential of enzymes is of vital use in the industry to mitigate the rampant overconsumption of our planet's resources (Sysoev et al., 2021). Presently, there are more than two-hundred types of enzymes of microbial origin that are commercially available (Meghwanshi et al., 2020), of which about 20 types are produced on industrial scales (Li et al., 2012; Verma et al., 2021). The discovery of novel enzymes of biotechnological interest is critical for the growth of the industrial enzymes market. This market amounted to 9.9 billion USD in 2019, and it is projected to reach 14.9 billion USD by 2027 (Sysoev et al., 2021).

Most industrial enzymes currently originate from fungi or mesophilic bacteria (Grötzinger et al., 2018; Sysoev et al., 2021), and the majority of enzymes of industrial relevance are hydrolytic in nature (i.e., hydrolases) (Verma et al., 2021). Examples of industrially-relevant enzymes that were unearthed from the MDM include cellulases (Piao et al., 2014), lipases (Verma et al., 2021), alcohol dehydrogenases (Akal et al., 2019; Grötzinger et al., 2018), carbohydrate-active enzymes (Stewart et al., 2018), enzymes that catalyze organophosphorus compounds (Singh, 2009), along with other enzymes displaying enhanced stability

Table 1
Enzymes of biotechnological and/or industrial interest and their applications sorted by alphabetical order. Adapted and manually curated from (Bruno et al., 2019; Cabrera and Blamey, 2018; Gurung et al., 2013; Li et al., 2012; Robinson, 2015; Singh et al., 2016).

EC number	Enzyme name	Application
EC:4.1.1.5	Acetolactate decarboxylase	Converting α-acetolactate to acetoin directly. Decreasing fermentation time by avoiding formation of diacetyl.
EC:2.7.4.3	Adenylate kinase	Biological indicator for validation of procedures to inactivate transmissible spongiform encephalopathy agents.
EC:1.4.1.1	Alanine dehydrogenase	Candidate for enantioselective production of optically active amino acids.
EC:1.1.1.1	Alcohol dehydrogenase	Candidate for asymmetric synthesis. Reduction of C-O and C-C bonds.
EC:3.1.3.1	Alkaline phosphatase	Candidate for molecular biology application: dephosphorylation of DNA.
EC:3.2.1.212	Alpha-L-fucosidase	Establishing glycosidic bonds.
EC:3.2.1.1	Alpha-amylase	Additive in food, textile, detergent and bioremediation industries. Waste-water treatment, drainage. Molecular biology applications. Treatment for digestive disorders.
EC:3.2.1.22	Alpha-galactosidase	Additive in soybean foodstuff.
EC:3.5.1.4	Amidase	Acylation, deacylation, enantioseparation. Degradation of nitrile-containing wastes.
EC:3.2.1.3	Amyloglucosidase	Glucose production. Increasing glucose content in beverages. Additive in toothpastes, mouthwashes, and bioremediation.
EC:4.3.1.1	Aspartase	l-aspartic acid production.
EC:3.2.1.2	Beta-amylase	Producing low-molecular weight carbohydrate. Starch hydrolysis. Cleaving α-1,4-linkages from non-reducing ends of amylose, amylopectin and glycogen molecules.
EC:3.2.1.21	Beta-glucosidase	Production of ginseng compounds for medical applications.
EC:3.5.2.6	Beta-lactamase	Molecular biology applications by conferring antibiotic resistance to Beta-lactam antibiotics.
EC:3.4.22.32	Bromelain	Additive in the cosmetic industry.
EC:4.2.1.1	Carbonic anhydrase	Candidate for biomedical applications.
EC:1.11.1.6	Catalase	Candidate for textile and cosmetic industries. Antioxidants. Bleach termination. Cheese processing.
EC:3.2.1.4	Cellulase	Additive in food, detergent and textile industries. Deinking. Drainage improvement. Degradation of cellulose in the textile industry.
EC:3.4.23.4	Chymosin	Cheese manufacturing.
EC:3.1.1.74	Cutinase	Triglyceride removal. Degradation of plastics, polycaprolactone. Additive in the textile industry.
EC:6.5.1.1	DNA ligase (ATP)	Candidate for molecular biology applications.
EC:2.7.7.7	DNA-directed DNA polymerase	DNA amplification used in the polymerase chain reaction and recombinant DNA technologies.
EC:3.3.2.10	Epoxide hydratase	Candidate for the production of enantiopure epoxides in the pharmaceutical industry.
EC:3.1.11.1	Exodeoxyribonuclease I	Candidate for molecular biology application: 3'-5' exonuclease specific for single-stranded DNA.
EC:3.1.13.1	Exoribonuclease II	Antiviral agent. Candidate for molecular biology applications.
EC:1.17.1.9	Formate dehydrogenase	Oxidation of alcohols and oxygenation of C-H and C-C bonds.
EC:4.1.2.13	Fructose-bisphosphate aldolase	Establishes C-C coupling.
EC:1.1.3.4	Glucose oxidase	Dough strengthening. Used in toothpastes and mouthwashes. Oxygen removal from beer. Polymerization of anilines. Detection of glucose in blood. Bleaching agent.
EC:3.5.1.2	Glutaminase	Cancer chemotherapy, particularly for leukemia.
EC:1.8.3.3	Glutathione oxidase	Used in hair waving.
EC:1.11.1.9	Glutathione peroxidase	Antioxidant properties.
EC:1.8.1.7	Glutathione reductase	Candidate as an antioxidant enzyme in heterologous systems.
EC:3.2.1.68	Isoamylase	Hydrolyzing α-1,6-linkages in glycogen and amylopectin.
EC:3.5.1.1	L-asparaginase	Cancer chemotherapy, particularly for leukemia.
EC:1.10.3.2	Laccase	Non-chlorine bleaching, delignification. Additive in food, textile, cosmetic, and pesticide industries. Degradation of waste containing olefin unit, polyurethane and phenolic compounds.
EC:3.2.1.108	Lactase	Lactose hydrolysis in dairy products or whey to avoid lactose intolerance. Antitumor agent.
EC:1.1.2.4	Lactic acid dehydrogenase	Reduction of C-O and C-C bonds.
EC:1.4.1.9	Leucine dehydrogenase	Candidate for medical and pharmaceutical industry applications.
EC:1.11.1.14	Lignin peroxidase	Degradation of phenolic compounds.
EC:3.1.1.3	Lipase	Additive in the food, detergent, cosmetic, textile, pharmaceutical, polymer, biodiesel, biosurfactant, pulping, and fossil-fuel industries.
EC:1.13.12.8	Luciferase	Molecular biology applications such as bioluminescent assays involving ATP.
EC:3.2.1.17	Lysozyme	Antibiotic. Disruption of mucopeptide in bacterial cell walls. Cheese manufacturing.
EC:1.1.1.37	Malate dehydrogenase	Candidate for detection and production of malate.
EC:3.2.1.20	Maltase	Additive in detergent and food industries. Production of glucose from maltose.
EC:3.2.1.133	Maltogenic alpha-amylase	Enhances shelf life of bread.
EC:1.11.1.13	Manganese peroxidase	Degradation of phenolic compounds.
EC:3.2.1.25	Mannanase	Additive in food, detergent and textile industries.
EC:3.4.24.3	Microbial collagenase	Treatment for skin ulcers. Wool finishing.
EC:3.1.1.102	Mono(ethylene terephthalate) hydrolase	Conversion of PET monomers into terephthalic acid and ethylene glycol.
EC:3.5.1.14	N-acyl-aliphatic-L-amino acid amidohydrolase	Production of L-amino acids.
EC:3.2.1.40	Naringinase (alpha-L-rhamnosidase)	Acting on compounds that cause bitterness in citrus juices. Debittering.
EC:3.2.1.135	Neopullulanase	Acting on both α-1,6- and α-1,4-linkages.
EC:4.2.1.84	Nitrile hydratase	Degradation of nitrile-containing wastes. Used in acylation, deacylation, enantioseparation. Synthesis of acrylamide, butyramide, and nicotinamide.
EC:3.4.22.2	Papain	Additive in the cosmetic industry.
EC:4.2.2.2	Pectate lyase	Bioscouring. Candidate for the detergent industry.
EC:3.2.1.15	Pectinase	Destabilizing the outer cell layer to improve fiber extraction via depectinization. Additive in food industries, such as clarification of juice and increasing its overall production, in the process of vinification, and the mashing of fruits.
EC:3.5.1.11	Penicillin acylase	Semi-synthetic penicillin production/broad-spectrum antibiotic production.
EC:1.11.1.7	Peroxidase	Hair dyeing. Quantification of hormones and antibodies.
EC:1.11.1.24	Peroxiredoxin	Candidate for food and pharmaceutical industries.
EC:3.1.3.26	Phytase	Candidate for feed applications, especially in aquaculture. Hydrolysis of phytic acid to release phosphorus, calcium, and magnesium cations.
EC:3.1.1.101	Poly(ethylene terephthalate) hydrolase	Biodegradation of PET polyester plastic into monomers.

(continued on next page)

Table 1 (*continued*)

EC number	Enzyme name	Application
EC:1.10.3.1	Polyphenol oxidase	Hair dyeing.
EC:5.3.4.1	Protein disulfide-isomerase	Hair waving.
EC:3.2.1.41	Pullulanase	Additive in food and biofuel industries. Attacking α-1,6-linkages, liberating straight-chain oligosaccharides of glucose residues linked by α-1,4-bonds.
EC:2.8.1.1	Rhodanese	Cyanide poisoning treatment.
EC:1.10.3.6	Rifamycin-B oxidase	Antibiotic synthesis.
EC:3.1.2.12	S-formylglutathione hydrolase	Candidates for chemical synthesis and industrial pharmaceutics.
EC:5.3.1.28	Sedoheptulose-7-phosphate isomerase	Candidate for biocatalysis under low water conditions.
EC:2.1.2.1	Serine hydroxymethyltransferase	Candidate as a pharmaceutical, agrochemical and food additive.
EC:3.4.24.40	Serralysin	Antiviral and anti-inflammatory properties.
EC:3.2.1.18	Sialidase	Hydrolysis of glycosidic linkages of terminal sialic acid residues in oligosaccharides, glycoproteins, glycolipids, colominic acid and synthetic substrates.
EC:3.4.21.62	Subtilisin	Additive in food, textile, leather, detergent, and cosmetic industries. Degrading protein into its constituent peptides and amino acids to overcome antinutritional factors.
EC:1.15.1.1	Superoxide dismutase	Anti-inflammatory and antioxidant properties. Free radical scavenging. Candidate for applications in agriculture, cosmetics, food, healthcare products and medicines.
EC:3.4.24.27	Thermolysin	Aspartame production.
EC:2.3.2.13	Transglutaminase	Hair waving. Protein cross linking. Laminated dough strengthening.
EC:5.3.1.1	Triosephosphate isomerase	Candidate for biocatalysis under low water conditions.
EC:3.4.21.4	Trypsin	Anti-inflammatory and anti-coagulant properties. Molecular biology applications. Food processing.
EC:1.14.18.1	Tyrosinase	Tumor-associated antigen. Polymerization of lignin and chitosan.
EC:3.2.2.27	Uracil-DNA glycosylase	Candidate for molecular biology application: release of free uracil from uracil-containing single-stranded or double-stranded DNA.
EC:3.5.1.5	Urease	Urea quantification in body fluids.
EC:1.7.3.3	Uricase	Treatment of hyperuricemia.
EC:3.4.21.73	Urokinase	Removal of fibrin clots from bloodstream. Anti-coagulant properties.
EC:3.4.24.25	Vibriolysin	Additive in food, textile, leather, and detergent industries.
EC:3.2.1.32	Xylanase	Additive in food, textile, detergent, pulp and bioremediation industries. Hydrolyzing pentosans of malt, barley and wheat. Enhancing pulp-bleaching efficiency.
EC:5.3.1.5	Xylose isomerase	Production of high-fructose corn syrup. Catalyzing isomerization of glucose to fructose.

under industrial conditions (Sysoev et al., 2021). Table 1 presents a list of enzymes of known biotechnological and/or industrial interest, gathered and manually curated from the literature.

Science has profited tremendously from numerous breakthroughs that relied on enzymes of microbial origin, as substantiated by the work of numerous Nobel Prize laureates (Bernard et al., 2018). Many of these early studies relied on restriction enzymes (Smith and Wilcox, 1970), then on DNA polymerases (Brock and Freeze, 1969) coupled to the advent of the polymerase chain reaction (Saiki et al., 1988), and more recently on the CRISPR-cas9 system (Jinek et al., 2012). Hence, microbial gene discovery can greatly push progress and development of new mechanisms and compounds of pharmaceutical, biotechnological, and biomedical relevance.

In a seminal report, Grotzinger et al. proposed a workflow targeting protein production based on single amplified genomes (SAGs) from species that we cannot culture yet (Grötzinger et al., 2018). As a proof-of-concept, they used the method described in their paper to unearth an alcohol dehydrogenase (ADH) from an uncharacterized polyextremophilic archaeon sampled from a brine pool at the bottom of the Red Sea (Grötzinger et al., 2018). ADHs are of industrial interest, given their ability to produce chiral compounds for pharmaceuticals and fine chemicals (Akal et al., 2019). ADHs can also be used in biosensor-based diagnostics and fuel-cell technologies (Grötzinger et al., 2018). Characterization of this ADH not only demonstrated its thermostability, halotolerance, and the ability to withstand the presence of different solvents, but also the prospect of it being stored and used as a powder; all of which are features of utmost biotechnological significance (Grötzinger et al., 2018). A more recent paper identified and characterized another ADH of similar polyextremophilic nature and with solvent tolerance, presumed to be a member of a rare enzyme family-that of microbial cinnamyl alcohol dehydrogenases (Akal et al., 2019).

Another notorious example is that of Stewart et al., who assembled 913 near-complete and draft-quality prokaryotic genomes from a rumen metagenome sequencing study (Stewart et al., 2018). These assembled genomes encoded over 69,000 novel enzymes presumed to be carbohydrate-active, and over 90% of which lacked a significant match in public databases (Stewart et al., 2018). These authors further highlight that their rumen metagenomic dataset not only offers a valuable resource for the discovery of biomass-degrading enzymes, but also that these novel enzymes might be potential candidates for application in the biofuels and biotechnology industries (Stewart et al., 2018).

More recently, there were studies that created hidden Markov models (HMMs) based on the protein sequences of enzymes whose plastic-degrading abilities had been experimentally validated (Danso et al., 2018; Zrimec et al., 2021). By mining ocean and soil metagenomes, Zrimec et al. were able to compile a catalogue with more than 30 thousand non-redundant sequences that potentially coded for enzymes with the ability to degrade 10 different types of plastic (Zrimec et al., 2021). They report a significant correlation between the abundance of these enzymes in the two sampled biomes, and both marine and country-specific plastic pollution measurements (Zrimec et al., 2021). They suggest that these results might indicate signs of adaptation to current global plastic pollution trends by the Earth's microbiome (Zrimec et al., 2021), thus emphasizing the potential within global microbiomes in providing solutions to contemporary concerns.

2.5. Biosynthetic Gene Clusters

Prokaryotes and other microorganisms (e.g., Fungi) are known to produce many secondary metabolites (SM) (Chen et al., 2019). SMs are natural products that encompass diverse chemical structures (Chen et al., 2019). This chemical diversity allows SMs to perform a plethora of functions (Chen et al., 2019). SMs may have antibiotic, anti-cancer, anti-viral, antifungal, antioxidant, anti-trypanosome, cholesterol-lowering, immunosuppressant, insecticide, and herbicide properties, among many others (Bull and Goodfellow, 2019; Chavali and Rhee, 2018; Chen et al., 2019; Newman and Cragg, 2016). Biosynthetic gene clusters (BGCs) are the physical grouping of the genes in a given genome that encode all enzymes required to produce a SM (Medema et al., 2015). Previous evidence has suggested that microorganisms may harbor up to one million BGCs (Hadjithomas et al., 2015; Ziemert et al., 2016), few of which have been thoroughly described (Ziemert et al., 2016).

Two major biosynthetic systems are those of polyketide synthases (PKS), and nonribosomal peptide synthases (NRPS) (Chen et al., 2019;

Weber and Kim, 2016). PKS and NRPS synthesize the two major classes of SMs: polyketides (PK), and nonribosomal peptides (NRP) (Chen et al., 2019). PKS and NRPS are popular targets for bioprospection, given their reputation as producers of a broad range of SMs with important applications in healthcare and research (Chen et al., 2019). PK and NRP, together with terpenoids and alkaloids, were regarded as the four major groups of SMs throughout the 20th century (Arnison et al., 2013).

At the turn of the 21st century, the NGS revolution unveiled another major class of SMs, that of ribosomally synthesized and post-translationally modified peptides (RiPPs), which have since attracted increasing interest (Arnison et al., 2013; Hetrick and van der Donk, 2017). This interest stems from academic and industrial sectors alike, due to the structural variability and functional diversity shown by RiPPs (Zhong et al., 2020). The chemical space of RiPPs is determined by their nucleotide sequence, therefore linking the diversity of these small molecules with that of genes (Zhong et al., 2020). The genetically-encoded nature of RiPPs enables researchers to freely manipulate the scaffolds of the peptides by site-directed mutagenesis, and efficiently screen the targets for those possessing characteristics of interest (Zhong et al., 2020).

Several computational tools are able to accurately identify BGCs (see Chavali and Rhee (2018)), albeit not without limitations. These tools might rely on external databases, and/or rules extracted from previous knowledge, implying that only known biosynthetic pathways whose rules are implemented in the software are detected (Chen et al., 2019). Thus, biosynthetic pathways that make use of enzymes from the FDM will be missed (Blin et al., 2019; Chen et al., 2019). Bioprospecting BGC data from metagenomes is also challenging, as the computational tools that do so commonly require high-quality genomes, or those resolved from metagenomes as input (Blin et al., 2019; Nayfach et al., 2021; Skinnider et al., 2016; Youngblut et al., 2020). Another major challenge in natural product discovery is that a substantial portion of BGCs are transcriptionally silent, or expressed at very low levels when in a standard laboratory setting (Chen et al., 2019; Ren et al., 2017). Strategies designed to activate these silent BGCs are crucial for discovering new chemical scaffolds (Goodfellow et al., 2018).

Nonetheless, there are successes arising from the systematic interrogation of BGCs from within the FDM (Bull and Goodfellow, 2019; Goodfellow et al., 2018; Nayfach et al., 2021; Skinnider et al., 2016; Youngblut et al., 2020). As an example, a research team developed a novel algorithm that catalogs RiPP biosynthetic gene clusters (Skinnider et al., 2016). Upon analyzing 65,000 prokaryotic genomes, they unearthed RiPP BGCs that coded for more than two-thousand novel natural products (Skinnider et al., 2016). Bull and Goodfellow have studied BGCs while focusing their bioprospecting efforts on the phylum Actinobacteria (Bull and Goodfellow, 2019). Their rationale for focusing on this taxon is fivefold: (i) the recurrent and foundational role of Actinobacteria in soil ecosystems; (ii) the size and diversity of the taxon; (iii) the ceaseless discovery of new taxonomic radiations; (iv) the BGC-rich genomes of Actinobacteria; (v) and their unparalleled track record as producers of bioactive compounds of notable ecological and economic value (Bull and Goodfellow, 2019). SMs discovered from Actinobacteria–especially *Streptomyces* strains–account for two thirds of known antibiotics (Bérdy, 2012), including those in clinical use today (Goodfellow et al., 2018). Actinobacteria are also known to produce roughly tenfold as many specialized metabolites as those known from laboratory experiments (Goodfellow et al., 2018). Consequently, this ability has renewed interest in these prokaryotes as producers of new chemical entities (Goodfellow et al., 2018).

Bull and Goodfellow emphasize that SMs have a significantly greater diversity and quantity of chemical scaffolds than those produced by combinatorial synthetic compounds (Bull and Goodfellow, 2019). Thus providing a compelling reason for prioritizing them in the search of novel drugs (Bull and Goodfellow, 2019). Their research into Actinobacteria recovered from two extreme environments has uncovered a remarkable assortment of new chemical class members, and each of those products is either a new-in-a-class or first-in-a-class chemical entity (Bull and Goodfellow, 2019). The most widely distributed bioactivity of these compounds is that of antibacterial and anticancer activities (Bull and Goodfellow, 2019). They report that these compounds are also putative drug hits that could provide potential therapeutic targets for inflammatory diseases, Alzheimer's disease, and type II diabetes (Bull and Goodfellow, 2019). The discoveries disclosed above further elevate the foregoing rationale: the exploitation of biological know-how from within the FDM can offer an unprecedented range of biotechnological solutions. These solutions might not only be at the core of new markets and business models (Cornelissen et al., 2021), but also at the bleeding edge of innovation in therapeutics, industrial applications, and bioremediation strategies.

2.6. *Additional insights into the functional dark matter*

Deep investigation of the FDM has prompted an exciting scientific revolution. Besides presenting evidence for biotechnological exploitability, this revolution has provided invaluable insights into the MDM in a broader sense. For instance, in a study of uncharacterized genomic "islands" from archaeal genomes, Makarova et al. found that besides being abundant and comprising a heterogeneous gene pool of diverse putative functions; these islands also code for defense systems, along with new variants of the CRISPR-Cas genome editing system (Makarova et al., 2014). Other articles described synthrophic networks in anaerobic methanogenic consortia of uncultured microorganisms (Gies et al., 2014; Nobu et al., 2015), as well as in benzene-degrading settings (Luo et al., 2016). In this way, they highlight the potential applications for anaerobic bioreactors aimed at bioremediation and energy generation (Gies et al., 2014). Another study predicted metabolic roles for multi-faceted chemoorganoheterotrophic bacterioplankton, that would be involved with degradation of complex carbon compounds and the nitrogen cycle (Thrash et al., 2017). A different study revealed that uncultivated ultra small marine prokaryotes encoded for a wealth of gene homologs associated with diverse metabolic pathways, such as: carbon, methane, nitrogen, and sulfur (Lannes et al., 2021). These authors highlight that these little known prokaryotes presumably contribute to elemental cycling (Lannes et al., 2021).

Wong et al. reconstructed 115 genomes assembled from hypersaline microbial mat metagenomes (Wong et al., 2020). They uncovered novel eukaryotic signature proteins in the Asgard archaeal superphylum, many forms of RuBisCo (ribulose-1,5-bisphosphate carboxylase-oxygenase), high hydrogen production capacity, putative schizorhodopsins, and diversity-generating retroelements, among many other findings (Wong et al., 2020). Around the same time, Wiegand et al. characterized and sequenced the genome of 79 bacterial strains from the enigmatic bacterial phylum Planctomycetes (Wiegand et al., 2020). These authors identified previously unknown modes of bacterial cell division, such as lateral budding and binary fission; as well as new cell signaling and small-molecule production processes (Wiegand et al., 2020). Their study also advanced that the vast majority of putative BGCs encoded by planctomycetes differ from known BGCs, hinting at an untapped potential for small-molecule production (Wiegand et al., 2020). Two articles reported extensive drug discovery potential amidst the microbiome of distinct marine sponges, evidencing a wealth and breadth of untapped resources for novel chemistry (Lackner et al., 2017; Rust et al., 2020).

Also worthy of mention are several other studies that: identified hundreds of metalloproteases with signature catalytic motifs within ORFs of previous unknown function (Lobb et al., 2015); probed uncharacterized groups of Acidobacteria displaying extensive carbon catabolic abilities, including polysaccharide breakdown and metabolism of lignin derivatives (Wegner and Liesack, 2017); characterized new members of the *Oceanospirillales* order whose genomes code for enzymes capable of metabolizing crude oil (Mason et al., 2012); and described exceptionally high diversity of actinobacteria in the arid Atacama desert, outlining its remarkable significance for future biodiscovery campaigns (Bull et al., 2018; Idris et al., 2017).

3. Progress and pitfalls

There are numerous reasons why the function of many proteins has not been characterized yet. This problem is manifold. It starts with DNA extraction protocols, and sequencing technologies, which have limitations. Yet, also significant are most mainstream public databases, and associated computational tools, that in many cases are prone to unsupervised dissemination of (mis)information. In the following section we will briefly overview the progress made by current approaches and technologies, and discuss "how" and "why" they might be fostering an ever-increasing inflation in the number of protein sequences of unknown function.

3.1. Shotgun metagenomics

Shotgun metagenomics is the untargeted DNA sequencing performed from an environmental sample (Quince et al., 2017). This approach consists on extracting the DNA from a sample, which can be made into libraries, and then sequencing that DNA using either a short-read (i.e., NGS) or long-read platform (i.e., TGS) (New and Brito, 2020). A metagenome by itself, represents a bulk of fragmented genomic data, from a multitude of microorganisms, at different abundances. Two of the major assets of shotgun metagenomics are its versatility, as it can be used with different types of samples, and applied when other approaches such as SCG, have failed (Hedlund et al., 2014); and its simplicity in terms of sample preparation and data acquisition, provided that a suitable amount of DNA has been extracted.

Many discoveries were only made possible by the advances in metagenomics. Examples of this are the identification of bacteria that can perform complete oxidation of ammonia to nitrate (Daims et al., 2015; van Kessel et al., 2015), and BGCs that code for antibiotics in the human gut microbiome (Donia et al., 2014), among many others. Metagenomic studies have disclosed a momentous portion of MDM. One study created a ocean microbiome gene catalog comprising more than 40 million nonredundant and mostly new sequences from prokaryotes, viruses, and picoeukaryotes (Sunagawa et al., 2015). More recently, a different study has created a non-redundant gene catalogue of 303 million genes from 13,174 metagenomes spanning 14 major biomes (Coelho et al., 2022). Another project allowed to gather genomic information on hundreds of bacterial species with no sequenced representatives, enabling their subsequent use in reference-based metagenomic studies (Human Microbiome Project Consortium, 2012). Outstandingly, metagenomics allowed to uncover a novel branch of bacteria: the Patescibacteria, previously known as Candidate Phyla Radiation (CPR) (Brown et al., 2015; Parks et al., 2017); and two new archaeal superphyla: the DPANN (i.e., Diapherotrites, Parvarchaeota, Aenigmarchaeota, Nanoarchaeota, and Nanohaloarchaeota) (Castelle and Banfield, 2018; Dombrowski et al., 2019; 2020; Parks et al., 2017; Rinke et al., 2013), and the Asgard superphylum (Eme et al., 2018; Imachi et al., 2020; López-García and Moreira, 2020a; 2020b; Spang et al., 2015; Zaremba-Niedzwiedzka et al., 2017).

Even though metagenomics has expanded our knowledge on the function and diversity of microbial communities, it has its limitations. Metagenomic data suffers from several confounders that can render it incomplete, biased, and difficult to interpret (New and Brito, 2020). These confounding factors include, but are not limited to, sequencing noise, phenotypic noise, spatial microenvironments, and contaminant DNA (Ackermann, 2013; Doud and Woyke, 2017; Quince et al., 2017). In addition, a metagenome only renders a snapshot of a microbial community at a given time, and it may not depict the active microbial population (i.e., the DNA might issue from dead or dying cells) (Bellali et al., 2021). These factors may consequently: (i) mask population (i.e., operational taxonomic units–OTUs) and (ii) genetic diversity (e.g., SNPs); (iii) hinder taxonomic assignment, (iv) functional profiling, and (v) the recovery of individual genomes; (vi) overlook mobile genetic elements; (vii) neglect rare taxa; (viii) misconceive spatially divergent populations; (ix) ignore ecological relationships,(x) phenotypic traits, and (xi) metabolic output; and (xii) restrain genomic context analysis (D Ainsworth et al., 2015; Doud and Woyke, 2017; Engel et al., 2014; New and Brito, 2020; Quince et al., 2017). This makes it so that some information relating to microbial communities, and its constituents, is prone to be lost among these data.

Arguably, the greatest limitation of metagenomes is the difficulty in annotating the function of most of the genomic data (Dutilh, 2014; Lobb et al., 2015; Mokili et al., 2012). There are several variables that influence metagenome function annotation besides those enumerated above. These include the complexity of the metagenome (i.e., richness and homogeneity of species), read length and format (i.e., single or paired end), sequencing depth, coverage, the size and quality of the data, taxonomic novelty, environmental context, and the sensitivity and accuracy of the computational methods used for downstream annotation (Lobb et al., 2015). The foremost reason however stems from metagenomic data itself, which consists on short-reads, in case of NGS-sequenced metagenomes. Usually, these short-reads are assembled into longer fragments named contigs, then genes are predicted and mapped to reference sequence databases (Treiber et al., 2020). However, this process oftentimes generates many short contigs, chimeric contigs (i.e., assemblies between different species), and reads that could not be assembled at all. Given that contig function prediction usually relies on homology-estimation algorithms (Doud and Woyke, 2017), the efforts to annotate these short contigs, chimeric contigs, or unassembled reads may prove unsuccessful. The reasons for this include the fact that short query lengths could lead to inaccurate assignments (Wommack et al., 2008), be deprived of significant matches against reference sequence databases (Prakash and Taylor, 2012), or fail to differentiate between functions, because they might align to promiscuous domains shared among distinct proteins (Tamames et al., 2019).

3.2. Metagenome-assembled genomes

Gene neighborhoods allow to deduce the function of FDM genes (Cotroneo et al., 2021). This may be achieved by analyzing the genes of known function with whom the FDM genes are consistently linked (i.e., across multiple genomes) (Koonin et al., 2021). Contiguous genomic data also enhances both functional profiling and taxonomic classification (Kayani et al., 2021; Somerville et al., 2019; Tamames et al., 2019). Nonetheless, metagenomic reads lack genomic context, because they have yet to be associated with their neighboring genes, and ultimately their genome of origin. To reconstruct the genomic context of metagenomic reads, two main computational procedures are usually required: metagenomic assembly, and "binning". Metagenomic assembly allows not only to gather contigs from metagenomic data, but also operons, gene arrays, syntenic blocks, or even putative metagenome-assembled genomes (MAGs) (Almeida et al., 2021; Olson et al., 2019). Contigs are usually grouped (i.e., "binned") together after assembly, so taxon-specific gene inventories can be created (Teeling and Glöckner, 2012). Binning is achieved by grouping contigs according to their intrinsic nucleotide composition and/or co-abundance profiles (i.e., unsupervised binning) (Hedlund et al., 2014; Nielsen et al., 2014; Plaza Oñate et al., 2019); or by assigning a taxon to a contig based on its homology to sequences of known taxonomy (i.e., supervised binning) (Teeling and Glöckner, 2012). When combined, metagenomic assembly and binning have allowed to obtain closed MAGs from candidate phyla (Brown et al., 2015; Kantor et al., 2013). However, supervised binning often leads to numerous genomic fragments that cannot be mapped to reference genomes (Quince et al., 2017). This is mainly due to the magnitude of prokaryotes from the TDM that lack representative genomes (New and Brito, 2020). Thus, the genomic fragments from these prokaryotes cannot be classified by supervised approaches.

Furthermore, the assembly and binning processes often fail to distinguish between related community members, resulting in MAGs that erroneously include contigs from distinct strains and/or species (Parks et al., 2017). This has repercussions on downstream function imputation and taxonomic assignments (Almeida et al., 2021).

An alternative consists in reconstructing MAGs directly from read data (i.e., *de novo*) (Olson et al., 2019). Prominent discoveries have been made based on *de novo* reconstruction of MAGs (Almeida et al., 2019; Anantharaman et al., 2016; Delmont et al., 2018; Nayfach et al., 2021; Parks et al., 2017; Pasolli et al., 2019). Despite these developments, many NGS-sequenced MAGs–and prokaryotic genomes in a general sense–consist on draft assemblies spanning hundreds to thousands of contigs (Koren et al., 2013), from which even core genes might be absent (Schmid et al., 2018). The primary reason for these incomplete assemblies is that NGS-generated reads are usually shorter than intragenomic repeats (Kingsford et al., 2010). Assembly complexity has also been directly associated with the ratio between the length of reads and that of repeats (Nagarajan and Pop, 2009). Repeat elements pose a challenge to *de novo* assembly algorithms (Koren et al., 2013), that typically fail to resolve them (Derakhshani et al., 2020), leading to fragmented assemblies, or misassemblies altogether. This challenge becomes even harder to overcome in MAG *de novo* assembly. In this case, the assemblers must not only resolve intragenomic repeats, but also intergenomic repeats (i.e., genomic fragments shared across strains) (Olson et al., 2019; Somerville et al., 2019). Failure to resolve intergenomic repeats may lead to the creation of composite MAGs, which complicate the ensuing interpretation of results (e.g., inaccurate functional profiles and taxonomic diversity, and distorted population abundance and prevalence) (New and Brito, 2020). Other factors that contribute to MAG incompleteness are sequencing depth and coverage. The coverage of a MAG depends on the abundance of the prokaryote of origin within the sampled community, and low-abundance prokaryotes may give rise to fragmented MAGs if the sequencing depth does not allow for genomic contiguity (Olson et al., 2019; Quince et al., 2017). Draft assemblies are also error-prone (Derakhshani et al., 2020; Nayfach et al., 2021). This limitation is irrespective of assembly algorithm and/or sequencing method–it is a result of the inherent complexity of the metagenomic assembly process (Olson et al., 2019).

MAG quality is usually assessed by measures, namely completeness and contamination (Bowers et al., 2017). Completeness and contamination have the standard English meanings, and these measures can be estimated in different ways (see Bowers et al. (2017); Nayfach et al. (2019); Orakov et al. (2021); Vollmers et al. (2022)). As an example, completeness might be calculated as the ratio between the number of observed and total single-copy marker genes from a marker gene set; and contamination as the ratio between the number of observed copies and total single-copy marker genes from a marker gene set (Bowers et al., 2017). Nonetheless, these estimates might be more appropriate to assess the quality of MAGs from known prokaryotes, as they might fail to attain the same resolution with MAGs issuing from the TDM (New and Brito, 2020), leading to MAGs whose quality cannot be accurately ascertained. The compilation of MAG-associated problems described hitherto might compromise downstream prediction of coding sequences, and the imputation of their function (Mavromatis et al., 2012).

To circumvent MAG fragmentation and incompleteness, some studies increase the minimum contig length threshold for MAG *de novo* assembly (Almeida et al., 2019; Nayfach et al., 2021; Pasolli et al., 2019). Other studies use sequencing data gathered from TGS technologies in the first place (Moss et al., 2020; Somerville et al., 2019). TGS is characterized by single-molecule, real-time sequencing of nucleotides, without the need for template amplification (Athanasopoulou et al., 2021). These traits allow to overcome amplification, sequencing, and compositional biases inherent to other molecular approaches (Sims et al., 2014). The most attractive feature of TGS is the production of long reads, with average read lengths surpassing 10kb (van Dijk et al., 2018), and reported maxima of ~1Mb (Jain et al., 2018). Increasing read length and contiguity improves the quality and accuracy of *de novo* assemblies (Koren et al., 2013), because fewer contigs are required to close a genome (Moss et al., 2020). The ability of long reads to span past repeat elements and anchor their copies to unique parts of the genome has allowed to overcome the major drawback of NGS-generated assemblies (Loman et al., 2015). The long reads produced by TGS, together with ever-improving *de novo* assembly algorithms (Dida and Yi, 2021), have already allowed to attain complete, closed, *de novo*-assembled prokaryotic genomes (Loman et al., 2015), and MAGs (Moss et al., 2020; Somerville et al., 2019). Fully resolved genomes are particularly valuable (Somerville et al., 2019; Varadarajan et al., 2020). Namely because these offer a complete landscape where the ensuing characterization of functional elements can take place. However, one should mind that even though MAGs may act as genomic placeholders for the TDM, these should eventually, and ideally, be replaced by genomes sequenced from clonal isolates (Nayfach et al., 2021).

3.3. Nucleic acid extraction

In studies of molecular microbial ecology, it is reasonable to favor the enrichment of prokaryotic DNA with a specific functional activity, prior to sequencing (Healy et al., 1995). However, in many cases (e.g., metagenomics) the interest may not be in favoring the DNA extraction of a particular type of prokaryote, but rather to recover the bulk DNA of the whole community. In these cases, the extraction method must allow to recover DNA from prokaryotes with distinct characteristics (e.g., cell wall morphology). Otherwise, sequencing data might be biased towards the prokaryotes that were the most susceptible to lysis (Quince et al., 2017). Thus, the DNA extraction method must suit the goal of the study, without compromising downstream sequence data interpretation.

When extracting DNA from an environmental sample, two main approaches are usually considered. Either the DNA is directly extracted in bulk from the sample (i.e., "direct extraction"); or cells are recovered from the sample, and only then are they lysed and the DNA extracted (i.e., "indirect extraction") (Kakirde et al., 2010). The "direct extraction" method has significant advantages: it is less time-consuming, and yields greater amounts of DNA (Kakirde et al., 2010). However, this method also isolates a high yield of non-prokaryotic contaminant DNA, and it produces shorter DNA fragments as a result of harsh lysis and purification steps (Kakirde et al., 2010). The "indirect extraction" method can overcome these limitations to a certain extent, since it tends to preserve the genomic DNA in larger fragments that may be further used to build metagenomic libraries and large prokaryotic DNA templates (Kakirde et al., 2010). It has also been shown to yield less non-prokaryotic DNA than the "direct extraction" method, while still collecting DNA from diverse prokaryotic taxa (Gabor et al., 2003). Nonetheless, the "indirect extraction" method is more laborious and yields less DNA, as it is potentially biased towards microorganisms that are not assorted in an environmental matrix, biofilm, or aggregated together with sample material (Kakirde et al., 2010).

Consequently, metagenomics has typically relied on short fragments from "direct extraction" protocols, followed by high-throughput NGS. Despite being a robust approach, it falls short when factors such as sequencing depth are considered (Sims et al., 2014), as its potential to unveil the taxonomic and functional profiles of a given community is restrained by read length (Brown et al., 2017). Although DNA extraction protocols can be optimized for a particular prokaryote of interest, this is more difficult to achieve when dealing with metagenomic mixtures (Olson et al., 2019). Additionally, in order to increase DNA yield, extraction protocols that achieve cell lysis through physical disruption (e.g., bead beating and sonication) have been preferred over those that use chemical lysis (Yuan et al., 2012). Yet, not only do these approaches vary in efficiency (Kennedy et al., 2014), they also shear the DNA, thus contributing to its loss during library preparation methods that select for fragment size (Quince et al., 2017). The efficacy of long-read TGS technologies depends on pure high-molecular-weight (HMW) DNA being provided to the sequencing instrument in the first place (Jones et al., 2021). Therefore, DNA extraction methods that generate small fragments for short-read sequencing are inappropriate for TGS (Mayjonade et al., 2016). To circumvent this issue, protocols to create bacterial artificial chromosome (BAC) libraries might be adapted; but these are laborious, and the commercial kits that are available are expensive (Mayjonade et al., 2016). Extraction methods that make use of agarose gel matrices (i.e., agarose plugs) can yield megabase-sized DNA molecules (Zhang et al., 2012); yet they span several days, and it is difficult to retrieve the DNA from the agarose plug (Mayjonade et al., 2016).

With these constraints in mind, Mayjonade et al. developed a protocol for fast and inexpensive extraction of HMW DNA that is suitable for TGS (Mayjonade et al., 2016). First, cell lysis is achieved through a combination of mechanical and chemical disruption (Mayjonade et al., 2016). The biological material is homogenized with SDS-based buffer, which disrupts cell membranes and inactivates DNA-degrading proteins (e.g., nuclease); and RNase A is added into the mixture, thereby digesting RNA (Mayjonade et al., 2016). Then, potassium acetate is added to the solution, leading to the formation of potassium dodecyl sulfate precipitate (Mayjonade et al., 2016). This compound binds contaminants like proteins and polysaccharides, that are removed via centrifugation (Mayjonade et al., 2016). DNA purification ensues by binding it to carboxylated magnetic beads with polyethylene glycol (Mayjonade et al., 2016). The authors emphasize that this combination is preferred, because carboxylated magnetic beads are inexpensive and cause minimal shearing; and polyethylene glycol precipitates less contaminants than other reactants like isopropanol and ethanol (Mayjonade et al., 2016). After ethanol washing, and resuspension in a buffer solution, highly pure HMW DNA is retrieved (Mayjonade et al., 2016). Many other DNA extraction protocols targeting TGS have been recently developed, adapted, and outlined (Jones et al., 2021; Maghini et al., 2021; Moss et al., 2020; Trigodet et al., 2022). With TGS technologies at the forefront of current advancements (Jones et al., 2021), one can envision that DNA extraction protocols directed towards long-read sequencing will keep being improved and refined, allowing for increased levels of DNA purity and yield, and thus enhancing downstream sequencing resolution and read contiguity.

3.4. Rare taxa

Culture-independent methods are bounded by protocol heterogeneity (e.g., DNA extraction protocols, primers used for amplification), and are especially susceptible to depth bias (Lagier et al., 2018; Sims et al., 2014). Thus, they make it difficult to detect prokaryotes that exist in low numbers, also known as the microbial “rare biosphere” (Sogin et al., 2006). Although scarcely represented, this minority might perform important functions, like that of a “seed bank” (i.e., dormant or metabolically inactive cells that behave as a genomic reservoir), or as “keystone species” (i.e., disproportional ecological roles in relation to their numbers) (Pascoal et al., 2020). A significant proportion of this observed, yet unculturable, diversity might result from stochastic phenomena, like random dispersal, or transient existence (Pascoal et al., 2020). These prokaryotes might also require long incubation times to form visible colonies, or require specific nutrients and physical conditions for growth (Bellali et al., 2021). Alternatively, they might be composed of dead or dying cells (Pedrós-Alió, 2012).

As an example, Bellali et al. set out to test the hypothesis that some species from the human gut microbiota remain uncultured because their cells are dead before reaching the end of the gastrointestinal tract, and not exclusively because of culture limitations (Bellali et al., 2021). By combining fluorescence-activated cell sorting (FACS) and culturomics, these authors were able to discriminate between live, injured, and dead bacterial groups (Bellali et al., 2021). They outlined that minority species constituted a substantial portion of the live TDM in human fecal samples, and that 28% of bacterial OTUs in the total fecal samples were either dead or injured (Bellali et al., 2021). Moreover, roughly two-thirds of the latter group were members of the TDM, consisting in part of anaerobic bacteria (Bellali et al., 2021). This result might explain why some taxa were missing in culture, given that culturing obligate anaerobes often requires specific laboratory equipment (e.g., anaerobic chamber) so as to provide an anoxic environment for these organisms (Lagier et al., 2018). Techniques like metatranscriptomics, or compounds that bind free DNA (e.g., propidium monazide), coupled to “indirect” DNA extraction protocols, might be useful to prevent potential biases arising from injured or dead cells (Quince et al., 2017).

The unculturability of most prokaryotes is clearly a deterring factor towards a comprehensive characterization of the microbial realm. Yet, one might argue that the underrepresentation of rare, uncultured, or unclassified prokaryotic taxa among reference genome catalogs is a more pressing concern (Zhang et al., 2020). This predicament impairs our ability to classify genomic sequences that were gathered from culture-independent approaches to begin with (Carr and Borenstein, 2014; Quince et al., 2017; Rinke et al., 2013). Most reference genome catalogs are biased towards pathogens, model organisms, and those amenable to cultivation (Quince et al., 2017). Taken together, these circumstances provide a distorted view of prokaryotic functional and taxonomic diversity (Mukherjee et al., 2017). Accurate functional annotation itself depends on the phylogenetic breadth of reference genomes (Carr and Borenstein, 2014). In addition, the discovery of functional novelty has been correlated with phylogenetic distance (Kunin et al., 2003; Mukherjee et al., 2017; Wu et al., 2009). By broadening our genome repertoire one might not only improve functional annotation and taxonomic assignment of genomic sequences, but also extend the genomic landscape where genes and functions of interest can be mined (Nayfach et al., 2021), or even unravel biologic novelty altogether (Rinke et al., 2013). While doing so, one also provides phylogenetic anchors for the posterior identification of genomic fragments by forthcoming (meta)genomic studies (Mukherjee et al., 2017). One way of rectifying the underrepresentation of rare prokaryotic taxa is to specifically target these organisms for genome sequencing (i.e., if cultivable); or alternatively single-cell sequencing, and metagenomic-assembly (i.e., if unculturable).

Numerous initiatives have been carried out to meet the need for a broader phylogenetic coverage of prokaryotic genomes (Almeida et al., 2021; Anantharaman et al., 2016; Brown et al., 2015; Mukherjee et al., 2017; Nayfach et al., 2021; Parks et al., 2017; Pasolli et al., 2019; Rinke et al., 2013; Tully et al., 2018; Wu et al., 2009). Wu et al. sequenced the genomes of 56 prokaryotic species, thereby creating the “Genomic Encyclopedia of Bacteria and Archaea” (GEBA) (Wu et al., 2009). By analyzing this genome set, these authors discovered new protein families, and improved function prediction for genes issuing from other organisms (Wu et al., 2009). More than 10% of the protein families identified in this study showed no significant sequence similarity to any known proteins at the time, thus hinting at functional novelty (Wu et al., 2009). Rinke et al. created GEBA-MDM, an extension of GEBA, that targeted candidate phyla (Rinke et al., 2013). They recovered draft SAGs of 201 uncultivated prokaryotic cells, issuing from 29 major branches of the phylogenetic tree (Rinke et al., 2013). The GEBA-MDM dataset enabled a twofold increase in phylogenetic diversity in relation to GEBA, the disclosure of 20 thousand new hypothetical protein families, among many other notable findings that significantly expanded the boundaries of biological knowledge (Rinke et al., 2013). Mukherjee et al. presented a significantly augmented version of the GEBA catalog (GEBA-I) (Mukherjee et al., 2017). GEBA-I includes reference genomes for 974 bacterial and 29 archaeal type strains, which increased the number of type strains at that time by twofold, and the overall phylogenetic diversity of prokaryotes by 25% (Mukherjee et al., 2017). By comparing the GEBA-I genomes with previously available ones, they reported an increase of 10.5% in new protein families as a consequence of increased phylogenetic diversity (Mukherjee et al., 2017). They disclose that the GEBA-I genomes improved the phylogenetic and functional interpretation of 25 million previously unassigned proteins from 4,650 metagenomes (Mukherjee et al., 2017). These authors also predicted more than 23 thousand BGCs from these genomes, most of which were of unknown biosynthetic function, highlighting the biotechnological potential encased in these organisms (Mukherjee et al., 2017). Nayfach et al. created the "Genomes from Earth's Microbiomes" (GEM) catalog (Nayfach et al., 2021). GEM comprises 52,515 MAGs, representing 12, 556 new candidate species, and spanning 135 phyla, as gathered from more than 10 thousand metagenomes, covering a myriad habitats worldwide (Nayfach et al., 2021). They report that the GEM catalog increases the known phylogenetic diversity of prokaryotes by 44% (Nayfach et al., 2021). To ascertain the potential for new functions, these authors gathered more than 5 million protein clusters from the GEM catalog (Nayfach et al., 2021). Upon annotating these clusters, they found that nearly 70% could not have their functions predicted by either TIGRFAM (Haft et al., 2013), KEGG Orthology (Kanehisa et al., 2016), nor the Pfam database (Mistry et al., 2021); and that 47% had no significant similarity to UniRef (Nayfach et al., 2021).

The results reported by these studies reinforce the hypothesis that functional novelty is far from saturated among sequence space (Mukherjee et al., 2017; Nayfach et al., 2021; Rinke et al., 2013; Wu et al., 2009). They also strongly suggest that future endeavors aiming to increase the genomic representation of elusive taxa will continue to provide improvements towards the functional and taxonomic assignment of unclassified genomic sequences throughout public databases.

3.5. Culturomics

Metagenomics and SCG do not allow for the effortless differentiation among strains of the same species, nor do they provide biological material for subsequent research (Lagier et al., 2018). Axenic culture remains indispensable in the complete characterization of a given prokaryote and its physiology (Wiegand et al., 2020). An approach that aims to reignite the pure culture of microorganisms, particularly those that have eluded previous cultivation efforts, is that of culturomics (Lagier et al., 2012). Culturomics is a high-throughput culturing method that makes use of multiple culture conditions, together with MALDI-TOF mass spectrometry and 16S rRNA gene amplicon sequencing (Sood et al., 2021). So far it has allowed to identify hundreds of new species (Lagier et al., 2012; 2018; 2016). Besides expanding our knowledge of the repertoire of the prokaryotic biosphere, culturomics provides pure, viable cultures that can be further used for *in vitro* and *in vivo* experiments (Bellali et al., 2021; Lagier et al., 2018).

Culturomics itself is a workflow composed of several steps. The first step consists on dividing and diversifying the environmental sample of interest into multiple culture conditions (e.g., complex media, broad range of temperatures and incubation times, addition of growth factors, supplements or metals, aerobic/anaerobic setting, etc) (Lagier et al., 2018). These conditions are designed to suppress the growth of the most represented populations, and to promote the growth of fastidious or rare prokaryotes that exist at lower concentrations (Lagier et al., 2018). Targeted culture conditions can also be employed to promote the growth of specific taxa (Lagier et al., 2018). Secondly, taxa identification is achieved by MALDI-TOF mass spectrometry, i.e., comparing the protein mass spectra of the target isolate with that of a preexistent database (Lagier et al., 2018). If MALDI-TOF fails to identify the isolate, the latter undergoes 16S rRNA gene amplicon sequencing (Lagier et al., 2018). Finally, the discovery of novelty is confirmed by sequencing the isolate's genome (Lagier et al., 2018).

However, no technique is without drawbacks. In the case of culturomics these include: (i) the sheer workload posed by the multiplex culturing conditions; (ii) the inability to test a multitude of samples like in metagenomics; and (iii) its incapability to provide data on gene expression and/or the functional potential enclosed within prokaryotic species by itself, as this always requires genome sequencing of the newly isolated organisms (Lagier et al., 2018).

3.6. Heterologous expression

Another problem associated with FDM proteins is that these might be spurious predictions–as discussed later on subsection 3.7. The (supposed) CDS' predicted for these sequences might instead encode genomic information that is not transcribed. One way of overcoming this problem is through large scale experimental functional genomics initiatives, like the Keio collection of *E. coli* single-gene knockout mutants (Yamamoto et al., 2009), and the transposon mutant library of *Pseudomonas aeruginosa* (Jacobs et al., 2003). These endeavors allow for an accurate characterization of proteins whose function is yet unknown. Nonetheless, these initiatives are costly and resource-demanding.

Likewise, heterologous expression of gene products is a promising approach for the study of particular gene products from uncultivated taxa (Lloyd et al., 2013). Together with increasing developments in synthetic biology, heterologous expression offers great promise as a strategy for re-coding entire operons for expression in model organisms (Hedlund et al., 2014; Temme et al., 2012). *In silico* analyses have predicted that *E. coli* can transcribe roughly 40% of genes from well-studied cultivated taxa if cloned in expression libraries (Doud and Woyke, 2017; Gabor et al., 2004).

However, there are many obstacles inherent to this approach. Examples of these obstacles are: the presence of accessory proteins, ambiguous decoding or recoding, apoprotein activation, codon bias, codon reassignment, different promoter structures, inaccurate protein folding, low expression rates, lack of essential post-translational modifications, lack of essential protein secretion, rare codon utilization, shared metabolic pools, or even unforeseen genetic codes (Ling et al., 2015a; Sysoev et al., 2021). These limitations indicate that only a minute percentage of the functional sequence space can be easily identified with this approach (Doud and Woyke, 2017). These problems become even more acute when taking into account uncultured taxa, which may harbor extremely divergent and mostly uncharted DNA sequences and physiologies. This is particularly true for extremophiles, because the experimental characterization of their gene products is further restrained by the lack of expression systems that allow for the production of recombinant proteins under the same extreme conditions required by these organisms to begin with (Grötzinger et al., 2018). These issues reduce the success rate of current methods in accessing the functional novelty that may lie within the FDM, leaving most of unknown functions from uncultivated microorganisms concealed within their native expression hosts (Doud and Woyke, 2017).

3.7. Annotation, public databases, and canonical computational approaches

Over the past decade, the growth in (meta)genomic data acquisition has been staggering. Previous estimates indicated that the total amount of sequencing data doubles approximately every seven months, spanning from small groups producing a couple of terabases per year, to large, dedicated institutes generating several petabases a year (Stephens et al., 2015). Yet, this enormous amount of sequence data is useless, unless it is accompanied by information regarding where the CDS' are, what ORFs do they belong to, and what products might they code for (Koonin et al., 2021; Salzberg, 2019). Besides allowing to understand the biology of genomes (Salzberg, 2019), functional annotation is critical for the growth of biological knowledge itself (Danchin et al., 2018). Unfortunately, it has not kept up with the progress witnessed for sequencing technologies (Salzberg, 2019), arguably due to two interdependent factors. First, the rampant accumulation of (meta)genomic data as described above. Second, a shift from automatic annotation, complemented by manual curation; to complete, unsupervised, automation of the entire annotation process (Koonin et al., 2021)–as currently witnessed for sequence submission portals and public databases.

Sequence submission portals often redirect sequence data to integrated annotation pipelines (Clum et al., 2021; Li et al., 2021; Overbeek et al., 2014). Sometimes, this data is manually curated thereafter (Famiglietti et al., 2014). After annotation, the information is added to, or updated, in different databases harboring distinct biological sequence data (e.g., NCBI's Nucleotide database (NCBI Resource Coordinators, 2018), and the Translated EMBL Nucleotide Sequence Data Library (Boeckmann, 2003)). A previous report on the Reference sequence database (RefSeq) at NCBI (O'Leary et al., 2016), established that prokaryotic genomes and proteins form most of this dataset, even though only a fraction of their reference bacterial genomes were manually curated. Even though small-scale community-based manual curation was reported to drastically improve gene annotations (Rödelsperger et al., 2019), it is still not a viable option to deal with large amounts of data, for two main reasons: it does not scale, nor does it always offer a solution (Salzberg, 2019). To characterize and validate the function of each individual protein in an experimental setting (e.g., gene-specific screening assays), is not a suitable alternative either (Ellens et al., 2017; Quince et al., 2017). These constraints leave no choice but to rely on computer-automated and unreviewed annotation methods as the sole alternative to predict the function of these sequences. However, the crux of the problem lies when annotation tools cannot assign a molecular function to a given sequence. In these cases, the imputed gene products are often classified as "uncharacterized", "hypothetical", or simply "unknown" (i.e., FDM) (Erdin et al., 2011; Makarova et al., 2019). There are a multitude of factors that influence our current inability to infer the function of these sequences. These factors include, but are not restricted to, the annotation methods and tools that are used, the taxonomic classification of the organism of origin, the size of the genome, genome completeness, research bias towards model organisms and pathogens, HGT events, mobile elements (e.g., transposons), location in core- versus accessory-genome, contamination of the sequencing data, sequencing errors, data and metadata collection, pre-processing, quality, and structure, among countless others (Arkhipova, 2020; Danchin et al., 2018; Dimonaco et al., 2021; Lobb et al., 2020). Below we will discuss the most blatant ones, with a focus on standard annotation workflows.

Given a genomic fragment, an annotation workflow will often start by identifying CDS genes, and then translating them (Lobb et al., 2020). Several problems may arise just in these two steps alone, such as: incorrect gene calling (e.g., prediction of spurious ORFs), prediction of genes in the wrong DNA strand, incorrect prediction of ORFs in non-coding sequences (e.g., CRISPR arrays), erroneous start codon assignment, under-prediction of short ORFs (sORFs) and alternative ORFs, and the failure to identify non-standard codon usage, as well as overlapping genes (Dimonaco et al., 2021; Makarova et al., 2019; Omasits et al., 2017; Orr et al., 2020). Besides severely compromising downstream functional annotation, these issues might also lead to inconsistent numbers of reported CDS genes among distinct annotation centers, over-prediction of non-coding genes, and the absence or under-representation of many gene types from public databases (Dimonaco et al., 2021; Omasits et al., 2017). As an illustration, increasing evidence shows that sORFs code for a broad range of important functions (see Duval and Cossart (2017); Khitun et al. (2019); Plaza et al. (2017); Saghatelian and Couso (2015) and references therein). Moreover, there are studies providing evidence for the existence of these sORFs using proteomics data (Omasits et al., 2017; Sberro et al., 2019). Yet, true protein-coding sORFs are poorly covered in public databases to begin with (Dimonaco et al., 2021). In addition to–and perhaps because of–their poor coverage, it is often difficult to discriminate between true sORFs, random in-frame genomic fragments, spurious sORFs, and pseudogenes (Lobb et al., 2020; Omasits et al., 2017; Orr et al., 2020; Sberro et al., 2019). Therefore, gene calling and annotation tools frequently impose a minimum sequence length cutoff to prevent spurious ORF annotation (Orr et al., 2020). In turn, this keeps perpetuating the under-representation of true sORFs in sequence databases (Sberro et al., 2019).

Most often, after the gene-calling and translation steps, a function is predicted for a given putative protein based on the similarity of its sequence to those of other proteins whose function is already known (Zallot et al., 2016). This is usually achieved by homology-based methods, also known as sequence similarity-based methods (e.g., BLAST (Altschul et al., 1990)). Annotation pipelines frequently rely on homology-based methods because these are straightforward to use and well-established. These methods also allow for huge numbers of query sequences to inherit the function annotations of homologous proteins from a given database (i.e., inheritance through homology) (Lee et al., 2007), provided certain conditions are met (e.g., percent identity and e-value thresholds). Yet, in many cases, the function of a given gene-product cannot be inferred by homology-based methods, due to a lack of recognizable homologs in sequence databases (Lobb et al., 2015). This may happen in the cases of highly divergent genes, or truly novel ones (Lobb et al., 2020). As such, annotation tools that rely solely on inheritance through homology are not applicable if homologous regions among targets of interest are undetectable (Coutinho et al., 2015). An alternative to homology-based methods is to compare the query sequence against position-specific scoring matrices (PSSMs), or HMMs. PSSMs and HMMs are gathered from curated collections of protein families (e.g., Clusters of Orthologous Genes (Galperin et al., 2021)), and protein domains (e.g., Conserved Domain Database (Lu et al., 2020)). These methods are more sensitive, because they are able to detect remote matches to protein or domain families that were conserved throughout long evolutionary distances (Koonin et al., 2021; Lobb et al., 2020). Nevertheless, numerous protein and domain families lack functional annotation themselves–i.e., the so-called domains of unknown function (DUFs), and uncharacterized protein families (UPFs) (Mistry et al., 2021). This entails that, even if a FDM protein manages to attain a match to a distantly related protein family or domain, there is a chance that the function for that protein family or domain is also unknown–e.g., as of 2021, 23% of all Pfam families were either DUF or UPF families (Mistry et al., 2021).

In many cases, when function is inferred solely via inheritance through homology, erroneous predictions arise leading to incorrect annotations (Promponas et al., 2015). The same can happen with more sensitive methods that rely on protein domains instead–in these cases, annotation inheritance might even occur in the absence of a full protein match (Lobb et al., 2020). Misannotations are specially pernicious for FDM sequences, as they might contribute to false-positive predictions, and therefore inaccurate annotation coverage reports. The quality of an annotation depends not only on the tools that generated it, but also on the fast and comprehensive inclusion of new data on the preexistent database annotations that are used by these tools in the first place (Makarova et al., 2019). This implies that when a misannotation is identified, not only should this error be corrected, but also that every other (mis)annotation that relied upon the latter as a source should be corrected as well (Salzberg, 2019). One can thus make the case that homology-based methods are only as good as the databases they rely upon (Dimonaco et al., 2021). Unfortunately, misannotation has not only become commonplace in public databases, as it is also an ongoing problem (Arkhipova, 2020; Girardi et al., 2020; Impey et al., 2020; Meyer et al., 2020; Nobre et al., 2016; Rembeza and Engqvist, 2021; Schnoes et al., 2009). To make matters worse, misannotations are seldomly confined to the database where the error first occurred, they often percolate throughout several databases as well (Promponas et al., 2015). Previous reports showed that the percentage of incorrect functional assignments in public databases soared from less than 5% in 1998 to as high as 40% in 2005 (Schnoes et al., 2009). More recently, it was reported that up to 50% of protein sequences from public databases contain at least one error (Meyer et al., 2020). As a case in point, some authors express particular concern towards error propagation issuing from draft genome assemblies (e.g., MAGs) (Arkhipova, 2020; Koonin et al., 2021; Salzberg, 2019). However, this issue is not exclusive to predicted genes issuing from draft genomes nor newly-discovered proteins. Well-known enzyme classes of industrial relevance (Rembeza and Engqvist, 2021), and genes encoding antibiotic targets from clinically important pathogens (Impey et al., 2020), also suffer from annotation error. Therefore, the degree to which one may rely on annotations from public databases is, for the most part, currently unknown (Rembeza and Engqvist, 2021). One may conclude that, in addition to the predicaments discussed throughout this section, the accumulation of misannotations in public databases may also restrain our ability to uncover biological novelty. Hence, truly innovative frameworks, spanning the entire process from functional (re)annotation to public data delivery, are desperately needed.

3.8. Logistical challenges

Besides the foregoing reasons, there is a more logistical factor for this large quantity of (meta)genomic data to be left unannotated: only a few research projects target the FDM in public datasets (Dutilh, 2014). This low number of projects is understandable. Searching for patterns amidst the data, or aiming to discover new phenomena, is essentially an exploratory science venture, following a “bottom-up” approach that goes from the data to the hypotheses, and that not always produces publishable results (Bernard et al., 2018; Burian, 2013). Since exploratory science is not hypothesis-driven, it can either reveal new knowledge or simply fail in revealing any knowledge at all.

In addition to this, researchers commonly find themselves under pressure to publish their results and move to the next project, leaving numerous uncharacterized sequences drowned amidst the contigs that were able to be annotated via inheritance through homology. This happens since using previously annotated sequences leads to straightforward conclusions (Dutilh, 2014). Thus, scientists contemplating the investment in purely exploratory studies take great risk and face a precarious situation (Bernard et al., 2018), that in turn perpetuates the FDM problem, as explained in this review.

4. Outlook

The rate at which proteins from the FDM keep accumulating in public sequence data repositories is nothing short of alarming. To experimentally validate the function of each of these proteins would take so long, that it would be impossible to keep abreast with the pace at which they are being discovered. There are numerous ways to tackle the FDM without resorting to *in vitro/in vivo* experimental characterization (see Danchin et al. (2018)). Moreover, these measures can be undertaken either during the upstream stage of a given (meta)genomic workflow (e.g., by improving the quality and interpretability of the data being generated); or downstream from that (e.g., by refining function prediction methods).

An example of a technique that can be applied upstream, and although not properly addressed in this review, is SCG. SCG is a powerful tool that holds great promise, not only towards MDM disclosure (Marcy et al., 2007; Martijn et al., 2015; Rinke et al., 2013; Woyke et al., 2009), but also in bioprospecting, natural product discovery, and in the search for heterologous expression systems, among many other applications (Kaster and Sobol, 2020; Mauger et al., 2022). When SCG and metagenomics are combined in the same study, they provide a highly complementary and synergistic approach, allowing for a fine-scale analysis and high throughput (Alneberg et al., 2018; Mauger et al., 2022; Probst et al., 2018). SAGs can improve metagenomic bins (Pachiadaki et al., 2019), as the former are able to capture HGT events, and allow to cast light on rare prokaryotes that might have been otherwise overlooked during the binning process, in addition to cell-cell associations missed in the course of MAG assembly (Dam et al., 2020; Wiegand et al., 2021; Woyke et al., 2017). In the same way, metagenomic data enables the recovery of genomic fragments that may be missing from SAGs, thus improving subsequent assemblies (Becraft et al., 2015; Dodsworth et al., 2013; Kaster and Sobol, 2020; Nurk et al., 2013; Xu and Zhao, 2018). Functional-driven SCG allows to isolate and characterize individual cells that exhibit a functional trait of interest, before and/or during genome sequencing (Couradeau et al., 2019; Doud and Woyke, 2017; Hatzenpichler et al., 2020; Kaster and Sobol, 2020; Lee et al., 2015; Woyke and Jarett, 2015). In this way, it has enabled to uncover gene functions that were previously encased in the FDM (Woyke et al., 2019). The development of new SCG-based technologies like the “mini-metagenomic” approach (Alteio et al., 2020; Geesink et al., 2020; Grieb et al., 2020); and the use of a single-cell printers (Gross et al., 2013), to investigate elusive taxa from complex environmental samples (Wiegand et al., 2021), emphasize on the exciting opportunities stemming from SCG research.

A multitude of *in silico* approaches have been designed to predict the function of the gene products inferred downstream from a given workflow, without the need to rely on inheritance through homology. Some examples include, but are not restricted to, predicting protein function by (i) inferring known patterns of evolutionary substitutions (Bileschi et al., 2022); (ii) integrative mapping of metabolic pathways (Calhoun et al., 2018); (iii) using domain-function associations (Rojano et al., 2022); (iv) leveraging template-based protein structure prediction (Zheng et al., 2022); (v) integration of multiple data sources (Zohra Smaili et al., 2021); and (vi) genomic context analysis (Cotroneo et al., 2021), among many others. Most current protein function prediction tools are based upon machine-learning (ML) (Bernardes and Pedreira, 2013; Bonetta and Valentino, 2020; Libbrecht and Noble, 2015), to some extent. ML approaches are built upon substantial knowledge of the subject at hand, and possess the ability to identify unforeseen patterns within the data itself (LeCun et al., 2015). These approaches often use features gathered from metadata associated with the primary protein sequences in order to train their models, and to predict the features of the protein sequence used as input, including its domains, protein-protein interactions, subcellular location, physico-chemical properties, secondary structure, and so forth (Cao et al., 2017). In Fig. 3 we show an overview of some of the metadata features that can be gathered for protein sequences.

The state-of-the-art of protein function prediction makes use of a broad family of ML methods that rely on artificial neural networks, called Deep Learning (DL) (LeCun et al., 2015). DL has already revolutionized many scientific fields, with particular emphasis on the biological sciences (Ching et al., 2018), especially computational biology (Sapoval et al., 2022). Recently, it has allowed for a groundbreaking advance towards the solution of the protein folding prediction problem (Jumper et al., 2021). Furthermore, DL model-based methods are substantially faster than non-DL methods, and can be leveraged for large-scale (meta)genomic data mining towards accurate and efficient MDM disclosure (Zha et al., 2022).

It is critical to understand however, that DL suffers from restraints just as other computational approaches. For instance, the classification outputs are inherently intertwined with the methodological design, and the duality of correlation and causality are still present (Ching et al., 2018). In the same way, DL models are only as good as the quantity and quality of the data they are trained upon (Robinson et al., 2021). DL algorithms also require huge amounts of data to train their models, which might be a deterring factor to its success in some occasions (Bonetta and Valentino, 2020). Another setback is their inability to predict true biological novelty, as these models are built upon preexisting knowledge (Robinson, 2022; Robinson et al., 2021).

Protein function prediction is indubitably a grand challenge of the post-genomic era. The progress made so far has been substantial, and despite the fact that year upon year new protein function prediction tools keep surfacing, with ever-improving performance and accuracy, this biological conundrum is far from being solved (Makrodimitris et al., 2020). There is tremendous potential for future innovation, and plenty of room for improvement, in order to enhance protein function prediction and genome annotation in the years to come (Bonetta and Valentino, 2020; Makrodimitris et al., 2020). We envision that new

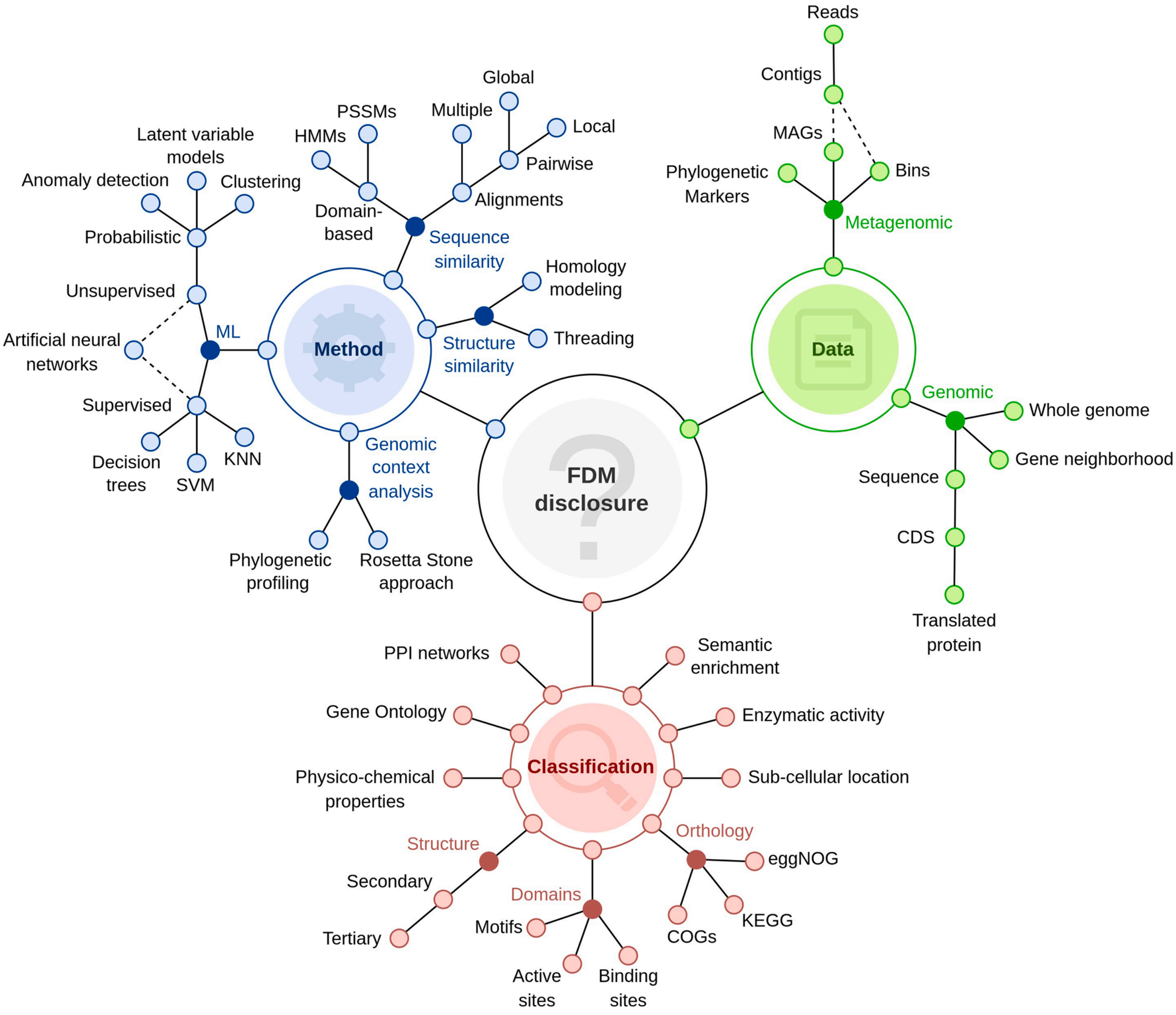


Fig. 3. Mind-map of *in silico* concepts relating to FDM disclosure. The three main hubs depict the general categories each concept belongs to. A non-exhaustive overview of some of the major concepts is shown for each hub, as well as how/whether these concepts hierarchically relate to one another. The first concept (i. e., node) that has “offspring” concepts relating to it has its respective node and text shown with a darker color. A dashed line represents a relation between concepts that are shared by more than one “parent”. CDS: coding sequence; COGs: clusters of orthologous genes; eggNOG: evolutionary genealogy of genes, non-supervised orthologous groups; FDM: functional dark matter; HMMs: hidden Markov models; KEGG: Kyoto encyclopedia of genes and genomes; KNN: k-nearest neighbors algorithm; MAGs: metagenome-assembled genomes; ML: machine-learning; PPI: protein-protein interaction; PSSMs: position-specific scoring matrices; SVM: support-vector machine.

developments in DL will continue to expedite opportunities for (meta) genomic data mining, thereby allowing to characterize the massive breadth of “known unknowns” that lie overlooked amidst sequence data repositories. An optimistic outlook from this endeavor is that, a breakthrough of new molecular functions and metabolites crucial for the biotechnology industry will be made, helping to achieve ahead of time the sustainable development goals for 2030 adopted by the United Nations. Undoubtedly, the horizon goes far and wide, while the potential treasures that can be unearthed by prospecting this information are hidden in plain sight.

CRediT authorship contribution statement

Pedro Escudeiro: Conceptualization, Writing – original draft, Writing – review & editing, Visualization, Data curation. **Christopher S. Henry:** Supervision, Funding acquisition. **Ricardo P.M. Dias:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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