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How to identify and quantify the members of the *Bacillus* genus?

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Abstract

Members of the *Bacillus* genus are widely distributed throughout natural environments and have been studied for decades among others for their physiology, genetics, ecological functions, and applications. However, despite its prevalence in nature, the characterization and classification of *Bacillus* remain challenging due to its complex and ever-evolving taxonomic framework. This review addresses the current state of the *Bacillus* taxonomic landscape and summarizes the critical points in the development of *Bacillus* phylogeny. With a clear view of *Bacillus* phylogeny as a foundation, we subsequently review the methodologies applied in identifying and quantifying *Bacillus*, while also discussing their respective advantages and disadvantages.

INTRODUCTION

The *Bacillus* genus encompasses a diverse set of species with the highly distinctive feature of forming dormant endospores that survive harsh conditions such as radiation (Setlow, 2006), drought (Vardharajula et al., 2011), or heat (Mandic-Mulec et al., 2015). Microbiologists have constantly discovered *Bacillus* and related species in diverse various natural environments like soil, air, and ocean sediments, as well as human-created niches such as clean rooms in spacecraft, or hospitals (Rüger et al., 2000; Satomi et al., 2006; Seuylemezian et al., 2020; Shivaji et al., 2006; Xu et al., 2020). Members of the *Bacillus* genus are involved in numerous ecosystem functions, reflecting the diverse environmental habitats in which they are distributed (Saxena et al., 2020).

Although the initial characterization of *Bacillus* species took place around 150 years ago, the taxonomic classification of *Bacillus* remains notoriously confusing (Zeigler & Perkins, 2021). One of the reasons is the loose criteria used in the past, whereby diverse bacteria were assigned to the *Bacillus* genus simply based on the ability to form spores aerobically (Combet-Blanc et al., 1995; Denariaz et al., 1989). The development of *Bacillus* phylogeny has been a remarkable reflection of the continuous advancements in methods deployed for bacterial characterization and identification. Rapid progress in molecular genetics led to an exponential influx of novel species in a short period. This has, on the one hand, expanded our knowledge of the diversity, distributions, and functions of the members of the *Bacillus* genus (Fortina et al., 2001; Rooney et al., 2009; Rössler et al., 1991; Wisotzkey et al., 1992). However, it has also led to an intricate genus containing hundreds of taxa grouped under the same genus name but without any well-defined characteristics that are commonly shared among and exclusive to them (Logan & De Vos, 2009). Accurate characterization of the member in the *Bacillus* genus provides information for inferring evolutionary relatedness and genetic diversity among the species, where phylogenetic analysis helps species delineation and novel strain identification, making the characterization and phylogeny of the *Bacillus* genus mutually informative and complementary.

Here, in this review, we first systematically survey the taxonomic development of the *Bacillus* genus by summarizing the emergence of novel species, re-classification, and re-description of its members at a few critical milestone time points. The comprehensive

understanding of the *Bacillus* phylogenetic framework serves as the cornerstone for the accurate characterization of species in the *Bacillus* genus. Then, we provide an overview of methodologies applied for the identification and quantification of species in the *Bacillus* genus, particularly, concerning the advancements and limitations. By synthesizing the current state of molecular methodologies, we aspire to offer suggestions for refining and advancing the identification and quantification of *Bacillus*.

A RETROSPECTIVE EXAMINATION OF *BACILLUS* PHYLOGENY DEVELOPMENT

Bacillus subtilis and *Bacillus anthracis* were the earliest species of the genus *Bacillus* that were described by Cohn and Koch in the late 1870s (Cohn, 1876; Koch, 1876). The first description of *B. subtilis* was provided by Cohn, specifically noting the formation, germination, and heat resistance of endospores (Cohn, 1876). The initial identification of *B. anthracis* was solely dependent on a series of animal inoculations from suspect cultures that were followed up upon the development of anthrax (Irenge & Gala, 2012). At that time, isolates that were unable to cause anthrax in laboratory animals were simply categorized as *B. cereus* or *B. anthracis similis* (Turnbull, 1999).

For the next 50 years, many bacteria that were rod-shaped, Gram-positive, spore-forming, and aerobic were classified as *Bacillus* or as a member of the *Bacillaceae* family. However, such a vague definition failed to fit a diverse genus as *Bacillus* which has no exclusive phenotypic characteristics (Heather & Geraldine, 2011). For instance, in a few cases, *Bacillus* isolates exhibit a Gram-variable staining response (Burke & McDonald, 1983). Certain species display round or coccoid shapes under specific growth phases or nutrient-deficient conditions (Gray et al., 2019). This genus includes aerobic, anaerobic, and facultative anaerobe species (Clements et al., 2002). The use of crude criterion resulted in an extreme polyphyly and heterogeneity of *Bacillus* species. In the past decade, to better understand the phylogenetic and evolutionary history of the *Bacillus* genus, certain species were reclassified into other genera (Dunlap et al., 2020; Gupta et al., 2020; Patel & Gupta, 2020), the remaining species have amended the description (Dunlap, 2015a; Dunlap et al., 2016; Gordon et al., 1977), and prospective species within these genera have set new criteria (Carroll et al., 2020).

The comparative phylogenomic analysis on >300 *Bacillus*/*Bacillaceae* genomes *Gupta* et al. performed is an important milestone in the development of *Bacillus* systematics, where they first identified six novel clades of *Bacillus* and transferred species from these clades into genera including *Peribacillus gen. nov.*, *Cytobacillus gen. nov.*, *Mesobacillus gen. nov.*, and so forth (Gupta et al., 2020). Moreover, they proposed that the *Bacillus* genus should be restricted to only the 'subtilis clade' and 'cereus clade' (Bhandari et al., 2013) and confirmed most of the species outside these 2 clades robustly formed 17 distinct clades and reclassified these as new genera (Patel & Gupta, 2020). As a result, 206 of the 291 known *Bacillus* species were reclassified to other genera, remaining 27 and 19 species as part of the subtilis and cereus clades. The 'subtilis clade' encompasses the type species *B. subtilis* and represents the genus *Bacillus* sensu stricto. The 'cereus clade' comprises human pathogens including *B. anthracis* and *Bacillus cereus*. Furthermore, they proposed that all novel species of the *Bacillus* genus should meet the minimal criteria that prospective species with the 'cereus clade' or 'subtilis clade' should be supported either by a 16S sequence-based phylogenetic tree or concatenated protein sequences.

Given the current classification of *Bacillus*, we outline the major changes in *Bacillus* taxonomy by reviewing the 'subtilis clade' and 'cereus clade', respectively. We hope that retrospectively examining the phylogenetic development of the genus *Bacillus* in a historical overview, will provide a clearer understanding of its intricate taxonomy and laid groundwork for its characterization.

THE *B. SUBTILIS* GROUP

Species of the *B. subtilis* group are genetically closely related and hardly distinguishable phenotypically. Most vegetative cells of these organisms are <1 μm, they are generally mesophilic and neutrophilic, although some can be tolerant to high pH levels (Oualha et al., 2020). This group is identified as prolific secondary metabolites producers with at least 4%–5% genome of the genome of a given strain in this group devoted to secondary metabolites synthesis (Caulier et al., 2019; Steinke et al., 2021). Among the wide array of secondary metabolites produced by the *B. subtilis* group, compounds such as fengycin and surfactin are involved in many biological control activities with the traits of antifungals, antibacterial, and elicitor of induced systemic resistance of plants (Kiesewalter et al., 2021; Ongena & Jacques, 2008). Therefore, marketed biofertilizers and biofungicides are mostly from this group (Dunlap, 2019b; Pérez-García et al., 2011).

The first proposed species *B. subtilis*, *Bacillus licheniformis*, *Bacillus pumilus*, and *Bacillus amyloliquefaciens* in this group were described more than 50 years ago (Gordon et al., 1973). Phylogenomics of the *B. subtilis* group underwent various changes with influxes of novel species and previous species being reclassified (Figure 1). Many studies provided reliable phylogenetic terms and molecular signatures that enabled the

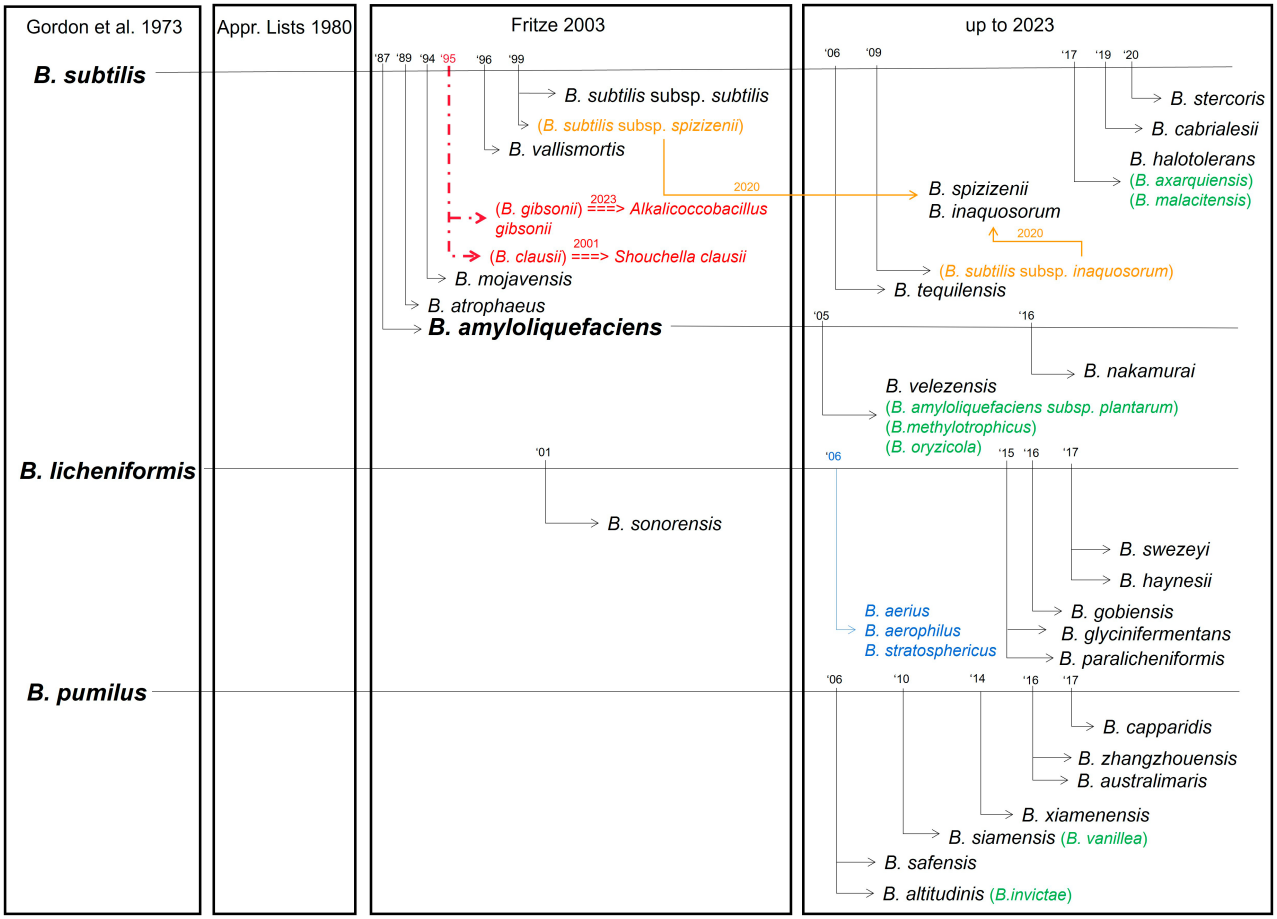

FIGURE 1 Taxonomy development of the species from the *B. subtilis* group. The species are classified following their relatedness to the closest original member of the group (bold text) and listed by the published years. Species coloured in red were re-classified and assigned as other genera. Species coloured in blue were placed on the list of rejected names as they were not available from any collection. Species coloured in green were identified as earlier or later heterotypic synonyms of the respective species on the same branch. Species coloured in orange were promoted to species status. Species coloured in black are validly published under the International Code of Nomenclature of Prokaryotes (ICNP). This figure is an updated version of the study done by Fritze (2004).

re-demarcation of several clades. For instance, *Bacillus gibsonii* and *Bacillus clausii* were assigned to other genera, and renamed *Alkalicoccobacillus gibsonii* and *Shouchella clausii*, respectively (Joshi et al., 2022; Kim et al., 2023). Meanwhile, species including *Bacillus aerius*, *Bacillus aerophilus*, and *Bacillus stratosphericus* that are no longer available from any strain collection were proposed to be listed as rejection names (Branquinho et al., 2015; Dunlap, 2015b). There are also species in this group not been validly published under the International Code of Nomenclature of Prokaryotes (ICNP) yet, such as species *B. subtilis* subsp. *natto* which has been applied in natto (fermented soybean food) production for almost 100 years (Kubo et al., 2011). The classification of certain subspecies within the *Bacillus* genus stems from distinctive genetic traits or adaptations to diverse habitats. However, subspecies *B. subtilis* subsp. *inaquosorum* and *B. subtilis* subsp. *spizizenii* were left as subspecies due to the lack of distinguishing phenotypes. They recently have been promoted to species status based on genomic comparisons, and phenotypical and chemotaxonomy determinations (Dunlap et al., 2020).

The *B. velezensis* species entails controversial taxonomy, initially proposed as a later heterotypic synonym of *B. amyloliquefaciens* but was overthrown based on comparative genomics and DNA–DNA relatedness calculations (Dunlap et al., 2016; Wang et al., 2008). Furthermore, plant-associated strain *B. amyloliquefaciens* subsp. *plantarum* FZB42^T had debates on whether it should be a later heterotypic synonym of *B. velezensis*. Dunlap et al. (2015) and Fan et al. (2017) demonstrated that the morphological, physiological, chemotaxonomic, and phylogenetics properties display only minor differences between these two taxa indicating FZB42 should be regarded as *B. velezensis*.

Currently, most registered commercialized species used as plant pathogen antagonists from the *B. subtilis* group have inconsistent names due to these convoluted taxonomic (re)classifications (13). For instance,

B. velezensis is the most commonly misidentified strain and is registered as either *B. subtilis* or *B. amyloliquefaciens*. This reminds us that there is much work to be done to set strict criteria to assign new species and to attain a coherent phylogeny for the *B. subtilis* group to benefit research and application.

THE *B. CEREUS* GROUP

The *B. cereus* group (termed *Bacillus cereus* sensu lato) is the other major group within the *Bacillus* genus with significant roles in agriculture, human health, food spoilage, and the environment. It encompasses a wide array of pathogenic strains: *B. anthracis*, the etiological agent of anthrax (Koch, 1876); *B. cereus*, the foodborne pathogen causing emetic and diarrhoea (Jovanovic et al., 2021); *Bacillus. thuringiensis*, a pathogen of invertebrate organisms applied as biopesticide control agents (Brar et al., 2006). The pathogenicity of the *B. cereus* group is mainly associated with the plasmids it encodes. *Bacillus anthracis* encompasses pXO1 and pXO2, one carrying genes coding for the anthrax toxin components and the other containing an operon for biosynthesis capsule that is important for host immune system evasion (Okinaka et al., 1999). Emetic *B. cereus* strains harbour plasmid pCER270 encoding the toxin cereulide biosynthesis gene cluster (Rasko et al., 2007). *Bacillus thuringiensis* contains plasmids that encode crystal proteins (Höfte & Whiteley, 1989). The ease of plasmid loss or transfer makes plasmid contents a simple but not completely reliable marker for the phenotypic delineation of these species (Vilas-Bôas et al., 2007).

The taxonomic development of this group had a lot of debates over decades (Figure 2). Smith and Gordon clustered highly correlated species into 'lumpers' rather than 'splitters', knowing that, the 'lumpers' would be dissected to form 'good species' when more advanced differentiation methods come (Gordon et al., 1973). Indeed, *B. anthracis*, *B. thuringiensis*, and *Bacillus mycoides* that were transferred as varieties of 'parent species' of *B. cereus* were reinstalled in 1980 on the Approved Lists of Bacterial Names with clearer descriptions (Skerman et al., 1980). After that, very few species were added to the *B. cereus* group for decades. It was not until 2013 that *Bacillus toyonensis* and *Bacillus cytotoxicus* were introduced as new species of *B. cereus*. *s.l.*, marking the first study that incorporated whole genome sequencing (WGS) data to describe unknown species. In 2017, nine novel species were proposed as additional novel species which effectively expanded the group. Nevertheless, it is still equivocal whether the announcement of these species led to further ambiguities due to the use of different genomospecies thresholds for species delineation.

Several studies aimed to standardize novel species identification and establish frameworks for taxonomic classification of *B. cereus*. *s.l.* Carroll et al. (2020) proposed a nomenclatural framework where they

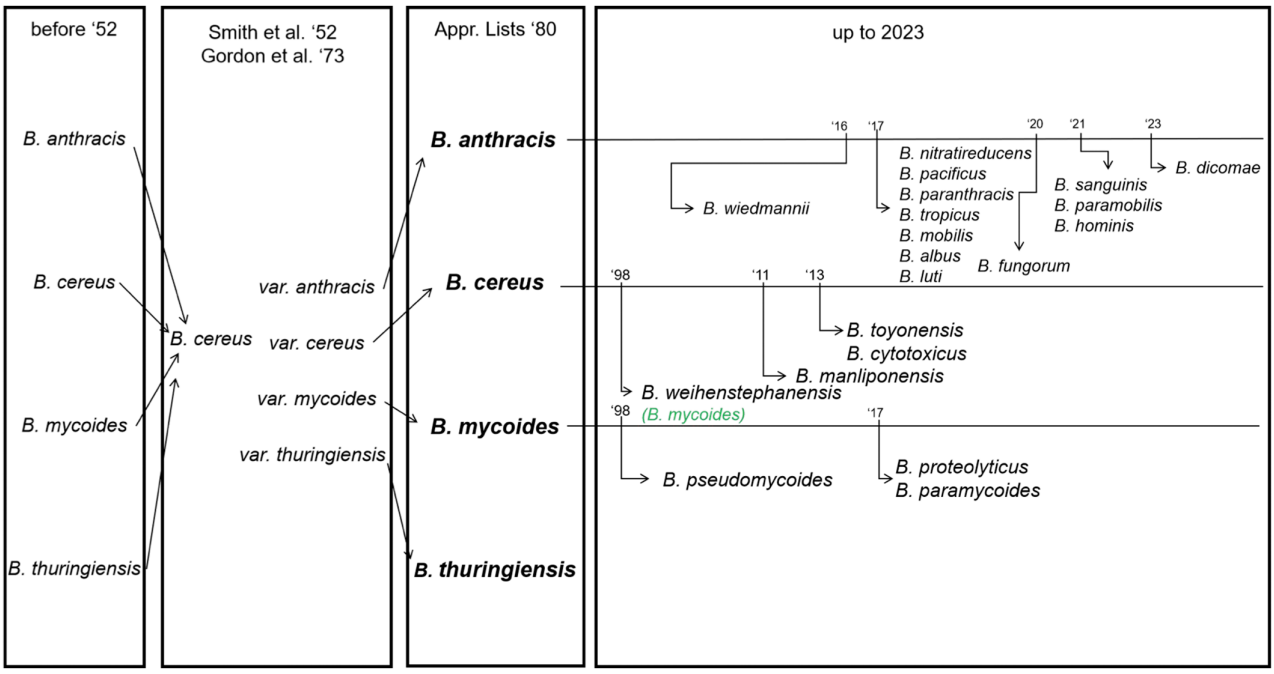

FIGURE 2 Taxonomy development of the species from the *B. cereus* group. The species are classified following their relatedness to the closest original member of the group (bold text) and listed by the published years. Species coloured in green were identified as earlier or later heterotypic synonyms of corresponding species on the same branch. Species coloured in black are validly published under the ICNP. The figure is an updated version of the study done by Fritze (2004).

reassigned the species in the *B. cereus* group and designated the medically important species into sublineages. In this case, the emetic *B. cereus* is referred to as *B. mosaicus subsp. cereus*. However, this nomenclature method has not been widely adapted to date. Despite the phylogenetic unrelatedness of *B. cereus* and *B. anthracis* to the subtilis clade, it needs to be retained within the genus *Bacillus*. This retention is attributed to the deeply ingrained terminology in publications and daily usage, coupled with the highly pathogenic traits of these species. Thus, according to Rule 56a of the code, transferring species from the *B. cereus* group is not recommended, and any nomenclatural framework should undergo rigorous tests to avoid confusion.

IDENTIFICATION AND QUANTIFICATION OF *BACILLUS*

From Gram smear to 16S rRNA sequencing, the relentless pursuit of scientists to accurately describe bacteria has yielded profound implications across domains such as health care, agriculture, biotechnology, etc. In the case of pathogenic *Bacillus* species, a swift and high-resolution identification is crucial in guiding the choice and duration of medical treatments. As a basis for agriculture, the identification and characterization of the exact *Bacillus* species hold immense significance, facilitating the discernment of potential field applications. Additionally, the multifaceted involvement of *Bacillus* in ecosystem functions such as nitrification, soil organic matter degradation, and phosphorus solubilisation necessitates species identification to comprehend their ecological roles within natural environments. Altogether, the identification and characterization of *Bacillus* holds far-reaching significance in numerous fields. Therefore, scientists have developed a range of approaches from traditional phenotypic characterization to molecular analyses. In the following sections, we critically review the methodologies applied for the identification and characterization of *Bacillus* concerning the benefits and limitations of each approach.

BACILLUS IDENTIFICATION IN THE PRE-NGS ERA: PHYSIOLOGICAL AND MORPHOLOGICAL TESTS

The advent of next-generation sequencing (NGS) enables culture-independent, large-scale, time-efficient approaches to profile microbiomes on the level of single isolate and complex communities (Knight et al., 2018). Consequently, conventional methods including biochemical and physiological tests seem to have lost their significance in defining a bacterial species. Nevertheless, for several medically important species, a simple look through the microscope or biochemical tests may still be faster than 16S rRNA gene sequencing which allows quick identification to assist clinical diagnosis (Irenge & Gala, 2012; Rao et al., 2010).

Physiological tests for the differentiation of the *B. subtilis* group are not frequently used, primarily because morphologies vary in response to environmental conditions, resulting in diverse colony patterns on solid media (Tasaki et al., 2017). Nevertheless, certain physiological tests were employed to distinguish between specific species in the *B. subtilis* group. For instance, *B. pumilus* is known as starch hydrolysis negative and hippurate-positive (Peng et al., 2013); *B. licheniformis* was reported to be distinguishable from *B. pumilus* as it is facultatively anaerobe, propionate-positive, and grows up to 55°C (Fritze & Pukall, 2011); *B. atrophaeus* and *B. subtilis* were observed distinguishable based on the formation of pigments when cultured on tyrosine medium (Burke et al., 2004). Besides, *B. subtilis* was documented as distinguishable from *B. amyloliquefaciens* based on a faster acid production from lactose, and slower gluconate utilization (Nakamura, 1987). However, caution is advised with morphology-based methods in this group, as molecular identification and evolving taxonomy reveal its inaccuracies.

Phenotypic characteristics remain as a main approach for preliminary taxonomic classification of *B. cereus s. l.* species (Carroll et al., 2022). For example, *B. anthracis* is non-haemolytic, non-motile, susceptible to lysis by γ phage, and incapable of decomposing tyrosine (Logan & De Vos, 2015; Tallent et al., 2019); *B. mycoides* and *Bacillus pseudomycoides* form rhizoid colonies on agar medium (Logan & De Vos, 2015; Nakamura & Jackson, 1995); *B. thuringiensis* forms crystals during the stationary phase that can be detected using microscopy; and *B. cereus* produces lecithinase and do not ferment mannitol on mannitol-egg yolk-polymyxin agar medium (Baldwin, 2020; Schnepf et al., 1998). Phenotypic analysis of pathogenic and harmless *B. cereus* strains remains difficult, but the tests described above are adequate for distinguishing *B. cereus* from the other members of the *B. cereus* group (Kamar et al., 2013).

CONVENTIONAL DNA SEQUENCING

Prior to the implementation of targeted sequence typing schemes, methods such as restriction fragment length polymorphism (Joung & Côté, 2001; Palmisano et al., 2001), pulsed field gel electrophoresis (Gaviria Rivera & Priest, 2003), random amplified polymorphic DNA (Rivera & Priest, 2003), multi-locus variable number tandem repeat (Dhakal et al., 2013; Durmaz et al., 2012), multi-locus enzyme electrophoresis were crucial in distinguishing members of the *Bacillus* genus

(Helgason et al., 2000; Zahner et al., 1994). Subsequently, single- and multi-locus sequence typing (SLST and MLST) approaches became, and remain instrumental methods for the identification of *Bacillus* species or subspecies.

At the flourishing time of molecular genetics, the 16S rRNA gene was a pillar of SLST approach to classifying bacteria, making it possible to reconstruct phylogeny on an unprecedented scale (Goto et al., 2000; Miranda et al., 2008; Sacchi et al., 2002). Nevertheless, it has insufficient differentiating ability of all *Bacillus* species. It has been shown that 93.93% of members of the *Bacillus* genera carry multiple copies of 16S rRNA genes and 55.32% of the 16S alleles are identical to other species (Strube, 2021). Alternative protein-coding genes such as *rpoB* (Ki et al., 2009; Mohkam et al., 2016), *gyrA* (Chun & Bae, 2000; Jongsik & Kyung, 2000; Liu et al., 2022), and *gyrB* were proposed as potential biomarkers to identify a *Bacillus* species (Bavykin et al., 2004; Chen & Tsen, 2002; La et al., 2004; Wang et al., 2007; Yamada et al., 1999). For a primary delineation, Dunlap (2019a) suggested *gyrA* or *gyrB* to be used for the *B. subtilis* group, and *pycA* for the *B. cereus* group. Recently, we thoroughly analysed primer sets frequently employed in literature and revealed that *gyrA* and *rpoB*-based primers have high intra-species specificity within the *B. subtilis* group (Xu et al., 2023). Surprisingly, *gyrB*-based degenerated primers had no amplification of certain *Bacillus* genomes which prompted the doubts about phylogenetic discrimination capacity of *gyrB*. Meanwhile, we proved elongation factor thermal unstable Tu (*tuf*) is a good phylogenetic marker that is not only specific for the *Bacillus* genus but also adequately discriminates the so far described species within the genus. These highly conserved protein-encoding genes offer a preliminary characterization of isolates from the *Bacillus* genus at either the species or subspecies level in instances where complete genomes are not accessible yet.

Other than SLST, a multitude of diverse methods was employed for the discrimination of *Bacillus* species or subspecies many of which were regarded as the 'golden standard' during different periods. The use of concatenated sets of housekeeping genes scattered along the genome, known as MLST, is one of the most powerful tools to discriminate closely related species. It has been widely applied to investigate the evolutionary history and population genetics of *B. cereus s.l.* group (Helgason et al., 2004; Hoffmaster et al., 2008), but specific approaches have also been developed for the *B. subtilis* group (Madslien et al., 2012). Despite the advantages of being unambiguous, reproducible, and easily portable between laboratories, MLST has faced the main demand of *Bacillus* discrimination less towards the genus/species level and more towards the strain level where it fails to yield clear-cut discrimination. However, the advancement of NGS has revolutionized the standards for novel strain typing and achieved strain-level discrimination. As described in the following section, NGS has opened new avenues for studying *Bacillus* populations and their diverse ecological roles.

THE NGS ERA, RESHAPING THE IDENTIFICATION OF *BACILLUS*

The advances in NGS have fostered the rapid development of microbiome research (Shendure & Ji, 2008). As one of the most frequently applied NGS approaches, WGS contributed to the explosion of novel *Bacillus* species discovery and provided tremendous quantities of genome sequences. The utilization of WGS has circumvented the low resolution achieved by conventional approaches and re-clarified the evolutionary relationships of *Bacillus* species. At the time of writing, more than a thousand complete *Bacillus* genomes are deposited in the National Center for Biotechnology Information (NCBI) and the number keeps rising. To systematically characterize novel *Bacillus* isolates, it has been recommended to perform polyphasic analyses that go beyond solely WGS regardless of the circumstances. Ideally, biochemical, phenotypic, and genotypic testing, together with full-length 16S rRNA gene analysis and comparative genome analysis subsequently to WGS should jointly provide information for *Bacillus* characterization.

EXPLORING *BACILLUS* DIVERSITY IN COMMUNITY SETTINGS USING NGS

Nowadays, there is a widespread recognition that microorganisms are not solitary players but rather intricately woven within their microbial networks. Thus, people have shifted their focus from single cultures to complex communities which more accurately reflect the natural lifestyles of microbiomes.

Advances in DNA-based, high-throughput sequencing technologies, such as amplicon sequencing (also known as metataxonomics or marker gene sequencing) and metagenome sequencing have revolutionized our ability to investigate the composition and function of natural microbial communities (Shendure & Ji, 2008). Among these technologies, 16S rRNA gene amplicon sequencing has emerged as a cost-efficient, rapid method to profile bacterial community composition and is now routinely used. However, as highlighted above, the 16S rRNA gene has an exceptionally high allele multiplicity in the species of the *Bacillus* genus and extensive species overlap, therefore the amplicons obtained on these genomes are rarely unique for the individual species (Pan et al., 2023; Strube, 2021). For

instance, routinely used 16S amplicon primers targeting the V3V4 hypervariable region show a high allele multiplicity of 63.90% and a species overlap of 74.47% for *Bacillus* (Strube, 2021). Consequently, when analysing a community containing *B. subtilis* with V3V4 metataxonomics, it will incorrectly define several unique amplicon sequence variants due to the presence of multiple alleles of *B. subtilis* resulting in overinflated richness in the sample. Moreover, in a sample containing *B. thuringiensis*, one may even incorrectly infer the presence of no <14 other species, as all these have V3V4 alleles shared with one another.

An alternative term for amplicon sequencing is marker gene sequencing, which is self-explanatory as it involves targeting a specific region of a gene of interest to profile microbial phylogenies. To further elevate the specificity for the *Bacillus* genus, several studies have adapted conserved genes that have high discrimination power that applied in SLST and developed amplicon-based approaches to investigate *Bacillus* communities. Porcellato et al. (2019) selected the three most discriminating genes of *B. cereus* group members from an MLST scheme and demonstrated *panC* gene had better discrimination power than *glpT* and *pycA*. They found psychrotrophic strains of the genus *Bacillus*, including *B. weihenstephanensis*, *B. mycoides*, and *B. thuringiensis* strains were the most abundant phylogenetic clusters in milk samples. Additionally, the housekeeping gene *gyrA*, which encodes DNA gyrase subunit A was deployed as another molecular marker to determine the diversity of *Bacillus* species (Liu et al., 2022). It demonstrated the ability to detect at least 92 *Bacillus* species and resolve 6 phylogenetic clusters out of 8 strains in a mock community but has not been tested for environmental samples.

The two aforementioned studies have certain limitations in the detection spectrum of *Bacillus* species. *panC*-based amplicon sequencing specifically targeted the *B. cereus* group while the *gyrA*-based approach focuses on the *B. subtilis* group. It is challenging to find genes that contain a highly variable region that can be used universally for *Bacillus* species or sub-species identification and flanked by highly conserved regions that can serve as binding sites for amplicon primers. Recently, a *tuf*-targeted amplicon sequencing approach was developed which exhibits the highest coverage of *Bacillus* species diversity reported to date with high specificity (Xu et al., 2023). It allows precise species resolution of the *Bacillus* community in natural soil communities and could be potentially applied to track the persistence of *Bacillus* inoculant in the field.

Metagenomics plays an irreplaceable role in resolving microbial community structure at the species or even strain level, as well as in profiling functional genes, pathways, and metabolism (Daniel, 2005). For instance, metagenome sequencing was applied to track the persistence of inoculated plant protective agent *B. amyloliquefaciens* in the field, where indigenous rhizosphere community shifts were analysed caused by the inoculant-produced secondary metabolites (Kröber et al., 2014). Similarly, Huang et al. (2022) employed metagenomics to investigate the effects of bio-inoculant *B. subtilis* subsp. H11 on the microbial community structure and the metabolic potential of aged flue-cured tobacco. Furthermore, metagenomics was also applied to profile the *Bacillus* phage abundance in naturally fermented soybean food. This highlights the potential of metagenomics to also decipher viral–bacterial interactions (Tamang et al., 2022).

Without a doubt, NGS technology allows a much deeper characterization of the role that the *Bacillus* genus plays in natural environment contexts. Nevertheless, it only semi-quantified the abundance of *Bacillus* as it assesses the relative, but not absolute abundances of individual microbes. Recently, cell-based (flow cytometry [FCM]) and molecular methods (qPCR) were integrated with NGS data to estimate the absolute microbial abundance. It remains unclear to what extent these quantification methods eliminated the bias introduced by amplicon sequencing (Tettamanti et al., 2020). Thus, in the last section, the quantification methods are reviewed specifically for *Bacillus* which holds another significant aspect of research in this field.

EMPHASIZING QUANTIFICATION: THE CRUCIAL ROLE BEYOND IDENTIFICATION IN *BACILLUS* SPECIES

Under certain circumstances, quantifying bacterial abundance is more crucial than mere detection or identification. As one of the most versatile used bacteria genera, commercial products derived from *Bacillus* sp. range from biofertilizers (Borriss, 2011; Sun et al., 2020), biofungicides (Lahlali et al., 2013), and biopesticides (Brar et al., 2006), to probiotics (Elshaghabee et al., 2017), enzymes (Contesini et al., 2018), and vitamins (Schallmey et al., 2004), where addressing critical questions regarding the efficacy, safety, and consistency of these products necessitates quantitative data. Do biocontrol agents applied in the field actively promote plant growth? Do probiotics have adequate amounts of bacteria that consistently and effectively confer benefits to the host? What are the residue levels of biopesticide throughout the food chain? Here, we highlight the studies that use quantitative data to address these real-world challenges.

Conventional culture-dependent approaches that rely on counting the total number of colony-forming units grown on solid media generally underestimate the total abundance. Culture-independent approaches such as quantitative real-time polymerase chain reaction (qPCR) and fluorescence in situ hybridization (FISH) quantify populations based on the DNA of bacterial cells without the necessity of laborious colony count tests. These DNA-based approaches have been

successfully used for the quantitative analysis of *Bacillus* and provide irreplaceable information in industrial applications.

Xie et al. (2020) developed a primer/probe set for rapid quantitative detection of *B. subtilis* populations and successfully detected the colonization dynamic of inoculants within the *Arabidopsis thaliana* rhizosphere. Their approach demonstrates significant implications in agriculture especially when multiple strains serve as biological control agents (BCAs) for pathogen suppression. In such a scenario, qPCR assay could quantitatively detect the development and population shift of BCAs in response to environmental changes and enable the selection of 'superior performers' in field trials (Lim et al., 2011; Rotolo et al., 2016). In the food industry, *Bacillus* is unwelcome due to its spoilage capability and pathogenic potential. Therefore, qPCR allows quantitative detection of foodborne pathogens and ensures the hygiene standards within the food industry (Cattani et al., 2016; Dzieciol et al., 2013; Kwon et al., 2021; Sadeghi et al., 2014).

One of the major limitations of any PCR-based molecular methods is the overestimation of cell numbers by amplification of nonviable cells. This shortcoming has been addressed by using propidium monoazide (PMA) as a nucleic acid-intercalating dye to inhibit the amplification of DNA from dead cells. Importantly, PMA-qPCR could enumerate not only vegetative cells but also the activated spores of *Bacillus*, a relevant criterion for the spore-forming *Bacillus* (Guo et al., 2022; Rawsthorne et al., 2009).

Another limitation of qPCR is its inability to provide insights into the interactions of bacteria within their environment. FISH as an alternative technique allows the identification and visualization of individual microbe cells in natural environments by utilizing a fluorescent-labelled probe that hybridizes to specific target sequences within the intact cells (Levsky & Singer, 2003). In a study conducted by Posada et al. (2016), a specific probe was designed for plant growth-promoting bacterium *B. subtilis* EA-CB0575, where it successfully hybridized with several strains of *B. subtilis* and failed to hybridize with other closely related species. To avoid indigenous bacteria autofluorescence and root structure interference, a catalysed reporter deposition-FISH (CARD-FISH) methodology targeting this strain was further developed (Posada et al., 2018). They demonstrated both FISH and CARD-FISH techniques effectively detected *B. subtilis* on plant roots growing in different culture systems. Moreover, Pasulka et al. (2021) designed FISH probes to quantitatively measure *Bacillus* abundance in direct-fed microbial products and crop microbial biostimulants to ensure these probiotics have adequate amounts of bacteria. Still, the potential of FISH to be used for enumerating *Bacillus* within products has largely been unexplored. We are convinced that with further development, the use of FISH to visualize and quantify *Bacillus* will be more prevalent to enable scientists to better study the ecological impact of *Bacillus* after application in natural systems.

Other culture-independent techniques such as FCM offer rapid determination of cell numbers, size-related scatter signals, and fluorescence (Müller & Nebe-von-Caron, 2010). This technique provides a useful and complementary approach to culture-based and other molecular methods for the study of complex environments, such as sediments, water, soil, and sludge

TABLE 1 Characteristics of methods used to quantify the *Bacillus* genus in complex environments.

Methods	Advantages	Limitations	Application to *bacillus* genus
Colony counting	• Simple • Cost-effective	• Laborious • Not applied to unculturable bacteria	(Gorsuch et al., 2019)
Quantitative real-time PCR	• High specificity and sensitivity • Unaffected by cell size	• Not discriminated between live and dead cells • Requires primer and probe optimization • Soil particles might contaminate DNA extraction and negatively affect PCR	(Cattani et al., 2016; Dzieciol et al., 2013; Fernández-No et al., 2011; Guo et al., 2022; Kwon et al., 2021; Lim et al., 2011; Rawsthorne et al., 2009; Rotolo et al., 2016; Sadeghi et al., 2014; Xie et al., 2020)
Fluorescent in situ hybridization	• In situ detection • Rapid analysis • Visualization • High specificity	• Requires confocal or epifluorescence microscope • Requires optimization of probe design and hybridization conditions	(Pasulka et al., 2021; Posada et al., 2016, 2018)
Flow cytometry/ Fluorescence-activated Cell Sorting	• High specificity • Rapid analysis • Capable of enumerate viable but non-culturable state	• Expensive • Complex data analysis • Limited applicability for sample types	(Cronin & Wilkinson, 2010; Majeed et al., 2018)

(Amalfitano & Fazi, 2008). It can efficiently separate vegetative cells or endospores of *B. cereus* from the food matrix and label them with specific fluorescent tags (Cronin & Wilkinson, 2010). A study conducted by Majeed et al. highlighted the use of FCM to enumerate the resuscitation stage of *Bacillus coagulans* (corrected name: *Heyndrickxia coagulans*) in commercial formulations like capsules and tablets (Majeed et al., 2018). The application of FCM allowed the quantification of the state in which bacteria retain characteristics of living cells but are not culturable (Davis, 2014). This capability is particularly valuable since the traditional culture-dependent method may underestimate the extent of viable but not culturable populations. The advantages and limitations of the quantification techniques are discussed in Table 1 which aims to provide comprehensive insights for researchers.

CONCLUSIONS

Smith and Gordon highlighted in their work, 'When only a few strains of a group are available, as often happens, their species descriptions must remain tentative until verified by the study of more strains' (Berkeley, 2002). With the decreasing cost and advancements in sequencing technologies, microbiologists will continue to explore *Bacillus* in diverse habitats. This will undoubtedly expand the existing genome database, enabling more comprehensive descriptions of novel species. Nevertheless, amidst these advancements, researchers should be careful with novel species proposals and classifications of new isolates. We should not solely depend on single techniques for novel isolate characterization but embrace multi-dimensional approaches. The integration of omics data, including genomics, transcriptomics, proteomics, and metabolomics will keep reshaping contemporary *Bacillus* taxonomics, providing invaluable insights into their functionalities, evolution histories, characteristics, and ecological roles they play in nature.

Nowadays, the identification of the *Bacillus* genus is shifting towards high accuracy, high throughput, and high speed on an unprecedented scale. Moreover, the focus of identifying a *Bacillus* species is transitioning to two directions, on the one hand, doing detailed molecular analyses, down to the level of strains, even clones; and on the other, moving up to the level of community, studying lifestyle in their natural habitat (e.g., soil). Although strictly speaking, the identification of bacteria relies on the given reference database used, such taxonomy assignment can be changed subsequently with the dynamic taxonomy landscapes. Thus, only through continuous collaborations among taxonomists and microbiologists, a refined genome reference database and a highly accurate identification system can be developed.

Despite the extensive application of the *Bacillus* genus in medicine, agriculture, and industry, studies on quantifying *Bacillus* numbers in complex communities are scarce. One reason is the lack of proper methods to determine their populations along with the challenges of selecting appropriate methodologies for the diverse environments. From the studies summarized here, answers to those real-world challenges call for the development of *Bacillus*-specific tools that can quantify *Bacillus* community members in situ. Together with the versatile omics approaches, studying the functionality and metabolism will move a step forward to understanding the ecology of *Bacillus*.

AUTHOR CONTRIBUTIONS

Ákos T. Kovács: Conceptualization; supervision; funding acquisition; writing—review and editing; project administration. **Xinming Xu:** Conceptualization; writing—original draft; formal analysis.

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CONFLICT OF INTEREST STATEMENT

The authors declare there is no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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